frontiers | Research Topics

Insights in nutrition, psychology and brain health

Edited by
Andrew Scholey

Published in
Frontiers in Nutrition

FRONTIERS EBOOK COPYRIGHT STATEMENT

The copyright in the text of individual articles in this ebook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this ebook is the property of Frontiers.

Each article within this ebook, and the ebook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this ebook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or ebook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-83252-134-2
DOI 10.3389/978-2-83252-134-2

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Insights in nutrition, psychology and brain health

Topic editor
Andrew Scholey — Monash University, Australia

Citation
Scholey, A., ed. (2023). *Insights in nutrition, psychology and brain health*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83252-134-2

Table of contents

06 Improving Brain Creatine Uptake by Klotho Protein Stimulation: Can Diet Hit the Big Time?
Sergej M. Ostojic and Dagrun Engeset

10 Association Between Blood Biochemical Factors Contributing to Cognitive Decline and B Vitamins in Patients With Alzheimer's Disease
Ting Qian, Lei Zhao, Xiaoli Pan, Shaoming Sang, Yangqi Xu, Changpeng Wang, Chunjiu Zhong, Guoqiang Fei and Xiaoqin Cheng

19 Infant Formula Supplemented With Milk Fat Globule Membrane, Long-Chain Polyunsaturated Fatty Acids, and Synbiotics Is Associated With Neurocognitive Function and Brain Structure of Healthy Children Aged 6 Years: The COGNIS Study
Ana Nieto-Ruiz, José A. García-Santos, Juan Verdejo-Román, Estefanía Diéguez, Natalia Sepúlveda-Valbuena, Florian Herrmann, Tomás Cerdó, Roser De-Castellar, Jesús Jiménez, Mercedes G. Bermúdez, Miguel Pérez-García, M. Teresa Miranda, M. Carmen López-Sabater, Andrés Catena and Cristina Campoy

38 Vitamin A Status Is More Commonly Associated With Symptoms and Neurodevelopment in Boys With Autism Spectrum Disorders—A Multicenter Study in China
Ting Yang, Li Chen, Ying Dai, Feiyong Jia, Yan Hao, Ling Li, Jie Zhang, Lijie Wu, Xiaoyan Ke, Mingji Yi, Qi Hong, Jinjin Chen, Shuanfeng Fang, Yichao Wang, Qi Wang, Chunhua Jin, Jie Chen and Tingyu Li

47 Unhealthy Dieting During the COVID-19 Pandemic: An Opinion Regarding the Harmful Effects on Brain Health
Iván Rentería, Patricia Concepción García-Suárez, José Moncada-Jiménez, Juan Pablo Machado-Parra, Barbara Moura Antunes, Fabio Santos Lira and Alberto Jiménez-Maldonado

55 Lactate Is Answerable for Brain Function and Treating Brain Diseases: Energy Substrates and Signal Molecule
Ming Cai, Hongbiao Wang, Haihan Song, Ruoyu Yang, Liyan Wang, Xiangli Xue, Wanju Sun and Jingyun Hu

72 Higher Consumption of Fruit and Vegetables Is Associated With Lower Worries, Tension and Lack of Joy Across the Lifespan
Simone Radavelli-Bagatini, Marc Sim, Lauren C. Blekkenhorst, Nicola P. Bondonno, Catherine P. Bondonno, Richard Woodman, Joanne M. Dickson, Craig Harms, Dianna J. Magliano, Jonathan E. Shaw, Robin M. Daly, Jonathan M. Hodgson and Joshua R. Lewis

84 Segmental Bioimpedance Variables in Association With Mild Cognitive Impairment
Dieu Ni Thi Doan, Boncho Ku, Kahye Kim, Minho Jun, Kyu Yeong Choi, Kun Ho Lee and Jaeuk U. Kim

98 Association of Dietary Vitamin K Intake With Cognition in the Elderly
Anni Wang, Meng Zhao, Jia Luo, Tianhao Zhang and Dongfeng Zhang

108 Diet Quality and Cognitive Performance in Children Born Very Low Birth Weight
Julie Sato, Meghan McGee, Nicole Bando, Nicole Law, Sharon Unger and Deborah L. O'Connor

116 A Combination of Green Tea, Rhodiola, Magnesium, and B Vitamins Increases Electroencephalogram Theta Activity During Attentional Task Performance Under Conditions of Induced Social Stress
Neil Bernard Boyle, Louise Dye, Clare Louise Lawton and Jac Billington

132 Synergistic effects of epigallocatechin gallate and l-theanine in nerve repair and regeneration by anti-amyloid damage, promoting metabolism, and nourishing nerve cells
Xinya Xie, Juan Wan, Xin Zheng, Wenjing Pan, Jiayi Yuan, Baozhu Hu, Meiyan Feng, Zhonghua Liu and Shuxian Cai

152 Neural responsiveness to Chinese versus Western food images: An functional magnetic resonance imaging study of Chinese young adults
Xi Xu, Jiajia Pu, Amy Shaw and Todd Jackson

166 Nutrition and mental health: A review of current knowledge about the impact of diet on mental health
Mateusz Grajek, Karolina Krupa-Kotara, Agnieszka Białek-Dratwa, Karolina Sobczyk, Martina Grot, Oskar Kowalski and Wiktoria Staśkiewicz

180 Joint analyses of human milk fatty acids, phospholipids, and choline in association with cognition and temperament traits during the first 6 months of life
Tengfei Li, Tinu M. Samuel, Ziliang Zhu, Brittany Howell, Seoyoon Cho, Kristine Baluyot, Heather Hazlett, Jed T. Elison, Di Wu, Jonas Hauser, Norbert Sprenger, Hongtu Zhu and Weili Lin

197 Myristic acid alleviates hippocampal aging correlated with GABAergic signaling
Qi Shang, Guifeng Chen, Peng Zhang, Wenhua Zhao, Honglin Chen, Die Yu, Fuyong Yu, Huiwen Liu, Xuelai Zhang, Jiahui He, Xiang Yu, Zhida Zhang, Riwei Tan, Zixian Wu, Jingjing Tang, De Liang, Gengyang Shen, Xiaobing Jiang and Hui Ren

211 Nutritional risk screening 2002 scale and subsequent risk of stroke-associated infection in ischemic stroke: The REMISE study
Xiaoli Chen, Dongze Li, Yi Liu, Ling Zhu, Yu Jia and Yongli Gao

220 The causal association between iron status and the risk of autism: A Mendelian randomization study
Li Chen, Xingzhi Guo, Chen Hou, Peng Tang, Xin Zhang, Li Chong and Rui Li

228 Evaluation of the synergistic effects of epigallocatechin-3-gallate-loaded PEGylated-PLGA nanoparticles with nimodipine against neuronal injury after subarachnoid hemorrhage
Xianguang Yang, Mengguo Han, Xue Wang, Jian Wang, Xiaoxue Sun, Chunyan Zhang, Shuaiguo Yan, Liyong Huang and Ying Chen

frontiers in Nutrition

OPINION
published: 23 December 2021
doi: 10.3389/fnut.2021.795599

Improving Brain Creatine Uptake by Klotho Protein Stimulation: Can Diet Hit the Big Time?

Sergej M. Ostojic and Dagrun Engeset*

Department of Nutrition and Public Health, University of Agder, Kristiansand, Norway

Keywords: creatine, CT1, brain, Klotho (KL), vitamin D2, curcumin, phosphate-restricted diet, low-calorie high-protein diet

OPEN ACCESS

Edited by:
Andrew Scholey, Swinburne University of Technology, Australia

Reviewed by:
Nafisa M. Jadavji, Midwestern University, United States

****Correspondence:***
Sergej M. Ostojic
sergej.ostojic@uia.no
orcid.org/0000-0002-7270-2541

Specialty section:
This article was submitted to Nutrition and Brain Health, a section of the journal Frontiers in Nutrition

Received: *15 October 2021*
Accepted: *08 December 2021*
Published: *23 December 2021*

Citation:
Ostojic SM and Engeset D (2021) Improving Brain Creatine Uptake by Klotho Protein Stimulation: Can Diet Hit the Big Time? Front. Nutr. 8:795599. doi: 10.3389/fnut.2021.795599

INTRODUCTION

Creatine plays a pivotal role in cellular bioenergetics, acting as a temporal and spatial energy buffer in cells with high and fluctuating energy requirements (1). Jeopardizing delicate creatine homeostasis can be detrimental to many energy-demanding tissues, including the brain. For instance, cerebral creatine hypometabolism accompanies various neurological conditions, including a number of developmental disorders (2, 3), neurodegenerative and cerebrovascular diseases (4, 5), and brain cancer (6). A reduced creatine availability in the brain has been thus recognized as an apposite therapeutic target, and supplying exogenous creatine to compensate for a disease-driven shortfall emerged as a first possible approach. However, early success in animal models of neurological diseases was not corroborated in human trials, with the use of creatine supplementation proved largely disappointing in clinical studies with a number of symptomatic neurological disorders [for a detailed review, see (7)]. A meager delivery of creatine to the brain could be partly due to a low activity/density of creatine transporter (CT1 or SLC6A8), a transmembrane sodium- and chloride-dependent protein that mediates creatine uptake into the target cells (8). For that reason, the upregulation of CT1 function has been identified as an innovative course of action to facilitate creatine uptake, with several exotic agents and routes were cataloged so far, including glucocorticoid-regulated kinases, mammalian target of rapamycin, ammonia, and Klotho protein (9).

CT1 STIMULATION AND KLOTHO PROTEIN

Besides other vehicles, Klotho protein (Clotho; HFTC3) is put forward as a possible stimulator of CT1 function that can uplift creatine allocation to the target tissues. This membrane-bound pleiotropic enzyme (also exists in a circulating form) participates in many metabolic pathways, including calcium-phosphate metabolism, nutrient sensing, and remyelination (10). Klotho is highly expressed in neuronal cells of the cerebral cortex, cerebellum, and spinal cord (11). The role of Klotho in high-phosphate energy metabolism modulation was revealed a few years ago when Amilaji et al. (12) found that the co-expression of Klotho protein increases a creatine-induced current in CT1-expressing cells. The authors reported that the current through CT1 was a function of the extracellular creatine levels, with the maximal creatine-induced current was higher in cells expressing CT1 together with Klotho than in cells expressing CT1 alone (29.5 vs. 20.2 nA). This implies a Klotho-driven upregulation of creatine carriers, presumably by stabilizing the carrier protein in the cell membrane, which likely nominates Klotho protein as a therapeutic proxy to accelerate creatine uptake through CT1. Several methods have been developed lately to raise Klotho levels (13), including a transgenic insertion of the klotho gene, a recombinant Klotho protein administration, or using angiotensin-converting enzyme inhibitor and thiazolidinediones.

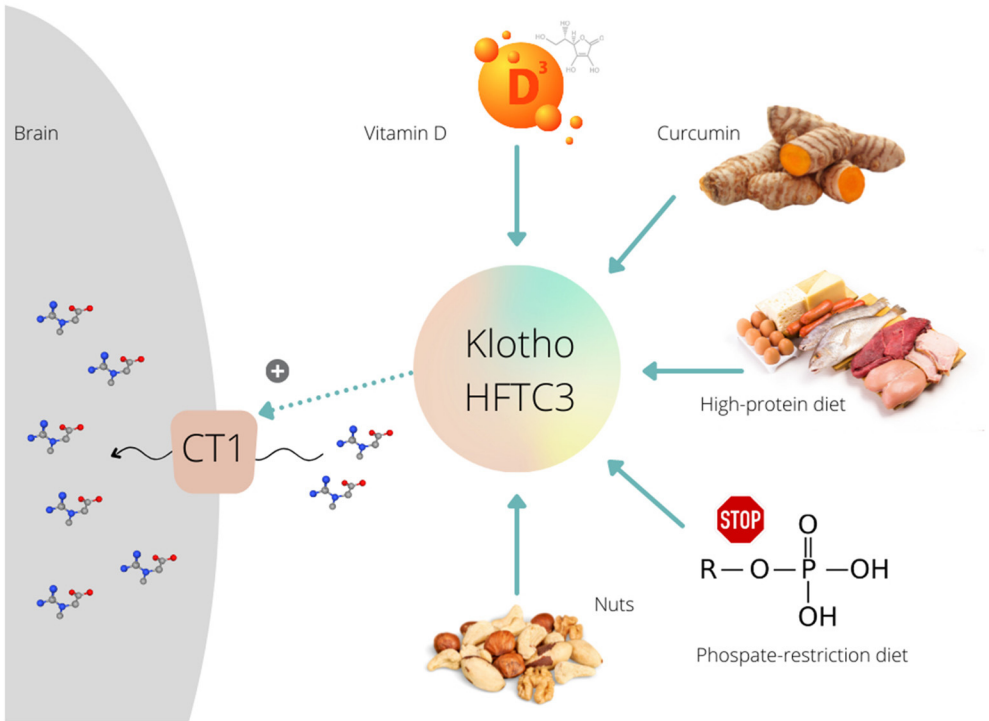


FIGURE 1 | Various dietary interventions upregulate Klotho protein expression/activity that further can stimulate (+) creatine uptake via creatine transporter (CT1) in the brain.

However, nutrition-related interventions might also be capable of enhancing endogenous Klotho production, and perhaps to unlocks CT1 for brain creatine uptake (**Figure 1**).

KLOTHO MODULATION BY DIET

Several preclinical studies demonstrated that exposure to vitamin D could modulate the activity of Klotho protein. A Japanese group (14) treated mice with a vitamin D-enriched diet and found increased expression of Klotho; the expression was upregulated and reached maximal levels 8 h after the administration of 1,25-dihydroxyvitamin D3. Vitamin D treatment induced a Klotho mRNA hyper-expression in different mouse renal cell lines (15), and in proximal kidney human cells (16). Curcumin, a polyphenol pigment and principal curcuminoid of turmeric (Curcuma longa), is another dietary agent that can upregulate Klotho protein. Hu et al. (17) demonstrated that curcumin induced Klotho expression in HK-2 human proximal tubule epithelial cell line. Recent studies found that the whole-diet interventions are also effective to intensify Klotho function, with a low-calorie high-protein diet increased Klotho levels in rats' brain (18), while a phosphate-restricted diet improved kidney klotho expression in a mouse model of polycystic kidney disease (19). Interestingly, a positive association was found between nuts portion intake and the Klotho plasma levels in middle-aged sedentary adults (20). These preliminary findings call for a set of proof-of-concept studies where supplemental creatine is co-administered with Klotho-stimulating agents while participants are monitored for changes in brain creatine levels. A recent trial confirmed favorable outcomes of a creatine-vitamin D multi-component supplement for functional performance and upregulation of energy metabolism-related kinases in elderly (21). Yet, no biomarkers of Klotho activation and/or creatine uptake by the brain have been evaluated.

CONCLUSION

Although promising, backing up the Klotho activity to improve creatine delivery to the central nervous system requires robust evidence. Basically, whether the Klotho upregulation induced by a specific dietary intervention is followed by a CT1 triggering and augmented creatine uptake in the brain currently remains unknown. Experimental transport/expression models in neurons would help with addressing CT1-Klotho activation in interventional studies, while clinical trials might include monitoring circulating Klotho as a proxy to Klotho stimulation, along with MR spectroscopy to track down changes in brain creatine levels. Future studies require a careful scrutinization of nutritional strategies utilized in terms of the length of intervention and level of exposure, and adjusted for creatine consumed from food. Additional covariates that can influence the outcome of a given trial also include concomitant pathology

(such as X-linked creatine transporter deficiency) and nutritional status of participants (e.g., vitamin D deficiency, creatine-free diet), along with other factors (including age, physical exercise, and level of education) that could play important role on CT1 and Klotho protein function and/or creatine homeostasis in the brain. For instance, animal studies suggest sex differences in Klotho protein expression, with the levels were higher in the male mice than in the female mice (22). Klotho function could also be affected by HMG-CoA reductase inhibitors (23), uremia-related compounds (24), or demethylation agents (25). Therefore, accounting for various endogenous and exogenous modulators of Klotho protein should be required for upcoming nutritional trials addressing brain creatine uptake through this pathway. The new knowledge about a possible diet-induced stimulation of Klotho protein opens an exciting opportunity for exploring various nutritional strategies aimed to potentiate cerebral uptake of creatine. This could perhaps bring forth a solution to the many who suffer from acquired or inherited creatine deficits.

AUTHOR CONTRIBUTIONS

SO designed and wrote the manuscript and has primary responsibility for the final content. All authors read, revised, and approved the final version of the manuscript.

REFERENCES

1. Wallimann T, Tokarska-Schlattner M, Schlattner U. The creatine kinase system and pleiotropic effects of creatine. *Amino Acids.* (2011) 40:1271–96. doi: 10.1007/s00726-011-0877-3
2. Fons C, Campistol J. Creatine defects and central nervous system. *Semin Pediatr Neurol.* (2016) 23:285–9. doi: 10.1016/j.spen.2016.11.003
3. Edmondson DA, Xia P, McNally Keehn R, Dydak U, Keehn B, A. magnetic resonance spectroscopy study of superior visual search abilities in children with autism spectrum disorder. *Autism Res.* (2020) 13:550–62. doi: 10.1002/aur.2258
4. van den Bogaard SJ, Dumas EM, Teeuwisse WM, Kan HE, Webb A, Roos RA, et al. Exploratory 7-Tesla magnetic resonance spectroscopy in Huntington's disease provides in vivo evidence for impaired energy metabolism. *J Neurol.* (2011) 258:2230–9. doi: 10.1007/s00415-011-6099-5
5. Lin AQ, Shou JX Li XY, Ma L, Zhu XH. Metabolic changes in acute cerebral infarction: Findings from proton magnetic resonance spectroscopic imaging. *Exp Ther Med.* (2014) 7:451–5. doi: 10.3892/etm.2013.1418
6. Kinoshita Y, Yokota A. Absolute concentrations of metabolites in human brain tumors using in vitro proton magnetic resonance spectroscopy. *NMR Biomed.* (1997) 10:2–12. doi: 10.1002/(sici)1099-1492(199701)10:1<2::aid-nbm442>3.0.co;2-n
7. Bender A, Klopstock T. Creatine for neuroprotection in neurodegenerative disease: end of story? *Amino Acids.* (2016) 48:1929–40. doi: 10.1007/s00726-015-2165-0
8. Ohtsuki S, Tachikawa M, Takanaga H, Shimizu H, Watanabe M, Hosoya K, et al. The blood-brain barrier creatine transporter is a major pathway for supplying creatine to the brain. *J Cereb Blood Flow Metab.* (2002) 22:1327–35. doi: 10.1097/01.WCB.0000033966.83623.7D
9. Ostojic SM. Modulation of CT1 function: from klotho protein to ammonia and beyond. *Front Nutr.* (2021) 8:660021. doi: 10.3389/fnut.2021.660021
10. Torbus-Paluszczak M, Bartman W, Adamczyk-Sowa M. Klotho protein in neurodegenerative disorders. *Neurol Sci.* (2018) 39:1677–82. doi: 10.1007/s10072-018-3496-x
11. Lim K, Groen A, Molostvov G, Lu T, Lilley KS, Snead D, et al. α-Klotho expression in human tissues. *J Clin Endocrinol Metab.* (2015) 100:E1308–18. doi: 10.1210/jc.2015-1800
12. Almilaji A, Sopjani M, Elvira B, Borras J, Dërmaku-Sopjani M, Munoz C, et al. Upregulation of the creatine transporter Slc6A8 by Klotho. *Kidney Blood Press Res.* (2014) 39:516–25. doi: 10.1159/000368462
13. Pastor J, Moe OW. Treating systemic Klotho deficiency. *Am J Nephrol.* (2019) 49:410–2. doi: 10.1159/000499864
14. Tsujikawa H, Kurotaki Y, Fujimori T, Fukuda K, Nabeshima Y. Klotho, a gene related to a syndrome resembling human premature aging, functions in a negative regulatory circuit of vitamin D endocrine system. *Mol Endocrinol.* (2003) 17:2393–403. doi: 10.1210/me.2003-0048
15. Barthel TK, Mathern DR, Whitfield GK, Haussler CA, Hopper HA 4th, Hsieh JC, et al. 1,25-Dihydroxyvitamin D3/VDR-mediated induction of FGF23 as well as transcriptional control of other bone anabolic and catabolic genes that orchestrate the regulation of phosphate and calcium mineral metabolism. *J Steroid Biochem Mol Biol.* (2007) 103:381–8. doi: 10.1016/j.jsbmb.2006.12.054
16. Haussler MR, Haussler CA, Whitfield GK, Hsieh JC, Thompson PD, Barthel TK, et al. The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the "Fountain of Youth" to mediate healthful aging. *J Steroid Biochem Mol Biol.* (2010) 121:88–97. doi: 10.1016/j.jsbmb.2010.03.019
17. Hu Y, Mou L, Yang F, Tu H, Lin W. Curcumin attenuates cyclosporine A-induced renal fibrosis by inhibiting hypermethylation of the klotho promoter. *Mol Med Rep.* (2016) 14:3229–36. doi: 10.3892/mmr.2016.5601
18. Shafie A, Rahimi AM, Ahmadi I, Nabavizadeh F, Ranjbaran M, Ashabi G. High-protein and low-calorie diets improved the anti-aging Klotho protein in the rats' brain: the toxic role of high-fat diet. *Nutr Metab.* (2020) 17:86. doi: 10.1186/s12986-020-00508-1
19. Omede F, Zhang S, Johnson C, Daniel E, Zhang Y, Fields TA, et al. Dietary phosphate restriction attenuates polycystic kidney disease in mice. *Am J Physiol Renal Physiol.* (2020) 318:F35–42. doi: 10.1152/ajprenal.00282.2019
20. Jurado-Fasoli L, Amaro-Gahete FJ, De-la-O A, Martinez-Tellez B, Ruiz JR, Gutiérrez Á, et al. Adherence to the Mediterranean diet, dietary factors, and S-Klotho plasma levels in sedentary middle-aged adults. *Exp Gerontol.* (2019) 119:25–32. doi: 10.1016/j.exger.2019.01.019
21. Evans M, Guthrie N, Pezzullo J, Sanli T, Fielding RA, Bellamine A. Efficacy of a novel formulation of L-Carnitine, creatine, and leucine on lean body mass and functional muscle strength in healthy older adults: a randomized, double-blind placebo-controlled study. *Nutr Metab.* (2017) 14:7. doi: 10.1186/s12986-016-0158-y
22. de Mello NP, Andreotti DZ, Orellana AM, Scavone C, Kawamoto EM. Inverse sex-based expression profiles of PTEN and Klotho in mice. *Sci Rep.* (2020) 10:20189. doi: 10.1038/s41598-020-77217-5
23. Narumiya H, Sasaki S, Kuwahara N, Irie H, Kusaba T, Kameyama H, et al. HMG-CoA reductase inhibitors up-regulate anti-aging klotho mRNA via RhoA inactivation in IMCD3 cells. *Cardiovasc Res.* (2004) 64:331–6. doi: 10.1016/j.cardiores.2004.07.011
24. Sun CY, Chang SC, Wu MS. Suppression of Klotho expression by protein-bound uremic toxins is associated with increased DNA methyltransferase expression and DNA hypermethylation. *Kidney Int.* (2012) 81:640–50. doi: 10.1038/ki.2011.445
25. Jung D, Xu Y, Sun Z. Induction of anti-aging gene klotho with a small chemical compound that demethylates CpG islands. *Oncotarget.* (2017) 8:46745–55. doi: 10.18632/oncotarget.18608

Conflict of Interest: SO serves as a member of the Scientific Advisory Board on creatine in health and medicine (AlzChem LLC.). SO owns patent "Sports Supplements Based on Liquid Creatine" at European Patent Office (WO2019150323 A1), and active patent application "Synergistic Creatine" at UK Intellectual Property Office (GB2012773.4). SO has served as a speaker at Abbott Nutrition, a consultant of Allied Beverages Adriatic and IMLEK, and has received research funding related to creatine and/or guanidinoacetic acid from the Serbian Ministry of Education, Science, and Technological Development, Provincial Secretariat for Higher Education and Scientific Research, AlzChem GmbH,

KW Pfannenschmidt GmbH, Monster Beverage Corporation, and ThermoLife International LLC. SO does not own stocks and shares in any organization.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Ostojic and Engeset. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 21 February 2022
doi: 10.3389/fnut.2022.823573

Association Between Blood Biochemical Factors Contributing to Cognitive Decline and B Vitamins in Patients With Alzheimer's Disease

Ting Qian[1,2†], *Lei Zhao*[3†], *Xiaoli Pan*[1,2†], *Shaoming Sang*[1,2], *Yangqi Xu*[1,2], *Changpeng Wang*[1,2], *Chunjiu Zhong*[1,2*], *Guoqiang Fei*[1,2*] *and Xiaoqin Cheng*[1,2*]

[1] Department of Neurology, Zhongshan Hospital (Xiamen Branch), Fudan University, Shanghai, China, [2] State Key Laboratory of Medical Neurobiology, Institutes of Brain Science and Collaborative Innovation Center for Brain Science, Fudan University, Shanghai, China, [3] Department of Neurology, Ninth People's Hospital, Shanghai Jiao Tong University, Shanghai, China

OPEN ACCESS

Edited by:
Andrew Scholey,
Swinburne University of Technology, Australia

Reviewed by:
Nafisa M. Jadavji,
Midwestern University, United States
Andrea Fuso,
Sapienza University of Rome, Italy

***Correspondence:**
Xiaoqin Cheng
cheng.xiaoqin@zs-hospital.sh.cn
Guoqiang Fei
fl0817@126.com
Chunjiu Zhong
zhongcj@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Nutrition and Brain Health, a section of the journal Frontiers in Nutrition

Received: 27 November 2021
Accepted: 27 January 2022
Published: 21 February 2022

Citation:
Qian T, Zhao L, Pan X, Sang S, Xu Y, Wang C, Zhong C, Fei G and Cheng X (2022) Association Between Blood Biochemical Factors Contributing to Cognitive Decline and B Vitamins in Patients With Alzheimer's Disease. Front. Nutr. 9:823573. doi: 10.3389/fnut.2022.823573

Background: Malnutrition, metabolism stress, inflammation, peripheral organs dysfunction, and B vitamins deficiency significantly contribute to the progression and mortality of Alzheimer's disease (AD). However, it is unclear which blood biochemical indicators are most closely related to cognitive decline and B vitamins deficiency (thiamine, folate, vitamin B12) in patients with AD.

Methods: This was a cross-sectional study of 206 AD patients recruited from six hospitals in China. Thiamine diphosphate (TDP), the bioactive form of thiamine, was measured by high-performance liquid chromatography fluoroscopy (HPLC) at a single center. Levels of biochemical indicators (except TDP) were measured by regular and standard laboratory tests in each hospital. Pearson's rank correlation analysis was used to assess relationships between B vitamins and biochemical indicators. *T*-test was used to compare the difference between ApoE ε4 and non-ApoE ε4 groups. Differences were considered statistically significant as $P < 0.05$.

Results: Among the biochemical results, in AD population, malnutrition indicators (erythrocyte, hemoglobin, serum albumin, and total protein) were most significantly associated with cognitive function, as was free triiodothyronine (FT3) levels which had been observed in previous study. Malnutrition and FT3 levels depend on age but not apolipoprotein E (ApoE) genotype. Meanwhile, Among the B vitamins, TDP was the most significantly associated with malnutrition indicators and FT3.

Conclusion: Our results indicated that TDP reduction could be a modifiable risk factor for malnutrition and FT3 that contributed to cognitive decline in AD patients. Correcting thiamine metabolism could serve as an optional therapy target for AD treatment.

Keywords: malnutrition, cognitive dysfunction, Alzheimer's disease, B vitamins, TDP

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disease clinically characterized by cognitive impairment and eventual loss of self-care ability. AD is costly to treat and places a heavy burden on society and families (1). There is also a lack of effective treatment that halt or reverse disease progression. Despite considerable research have been conducted, improved understanding of AD pathogenesis and therapeutic options are desperately needed (2).

Age and ApoE genotypes are crucial for cognitive impairment. Besides, peripheral system dysfunction (3–6) and malnutrition (7, 8) have been recognized as risk factors for cognitive impairment and loss of self-care ability in AD patients. Renal (3), thyroid (4), and liver dysfunction (5) and inflammation (9) played various roles in cognitive impairment in both healthy older people and AD patients. Metabolism stress indicated by abnormal blood lipids and glucose is also considered as a potential risk for AD progression (10). Several studies reported that anemia/abnormal hemoglobin and low serum albumin level is an independent risk factor of poor cognitive performance in the elderly and for dementia and rapid cognitive decline among the elderly (11, 12). These blood biochemical results contributing to cognitive decline were regular measured and can be collected in hospital daily. However, it has not been determined which blood biochemical indicators specific are most closely associated to cognitive decline in subjects with AD.

B vitamins deficiency—especially thiamine, folate, or B12 reduction—is a leading cause of neurological impairment and disabilities of daily living (13). Populations at risk of B vitamins deficiency including the elderly, alcoholics, obesity surgery recipients, vegetarians/vegans and AD, whom are recognized as being at high risk for cognitive impairment. Several underlying pathways were indicated to link low B vitamins status and cognitive impairment as well as neuropathological changes of AD. Folate and vitamin B12 deficiency disrupted one-carbon metabolism could lead to elevate homocysteine through lowing enzymatic activities for the remethylating or trans-sulfuration (14). Alternatively which is essential for of S-adenosyl-methionine (SAM) synthesis, and required for methylation of DNA/RNA and proteins (15), its impairment caused hypermethylated redox-related genes (NUDT15 and TXNRD1). Independently, B vitamins deficiency also induced cerebrovascular dysfunction, activation of tau kinases and enhanced Aβ metabolism contributed to AD development (16–18). David Smith and colleagues' studies also indicated a possible pathogenetic role of B vitamins (B12, Folate) in mild cognitive impairment, as B vitamins therapy could abrogate effects of iron-associated brain atrophy rate (19). Thiamine diphosphate (TDP) is the bioactive form of thiamine, which acts in the production of energy from carbohydrates in the cells and is essential in the proper functioning of the nervous system (20). As a critical cofactor of three glucose metabolism enzymes (Transketolase, Pyruvate dehyrogenase complex, α-Ketoglutarate dehydrogenase complex), TDP plays important and sometimes rate-limiting roles in the brain glucose metabolism (21), TDP reduction is closely related to glucose hypometabolism and induced oxidative stress contributed to cognitive dysfunction and AD pathologies (22, 23).

Understanding the complex relationships between the different biochemical systems regulated in the peripheral system by these vitamins may facilitate AD diagnosis and improve treatment. In AD patients, the association between B vitamins status and brain function was already observed, but whether B vitamins status linked cognitive dysfunction of AD through peripheral system biochemical risk indicators remain to be clarified.

As a continuation of our previous research (24), in this study, we performed in AD population to find out some specific biochemical indicators of peripheral blood which may aggravate cognitive decline mostly. It points out that TDP related malnutrition indicators (erythrocyte, hemoglobin, serum albumin, and total protein) and FT3 are the most significant risk factors for cognitive decline in AD patients rather than other blood biochemical indicators.

MATERIALS AND METHODS

Study Design and Subjects

From January 1, 2012 to November 30, 2020, 206 AD patients were recruited from outpatient or inpatient department of six hospitals in China (Zhongshan Hospital, Huashan Hospital, Shanghai Mental Health Center, Haiwan Hospital, Tianjin Huanhu Hospital, and Brain Hospital affiliated to Nanjing Medical University) who had no specific family history of AD. Informed consent was obtained from all participating subjects or healthcare givers. This study was approved by the Committee on Medical Ethics of hospitals.

All subjects underwent neurological examinations and neuropsychological evaluations including the Mini-Mental State Examination (MMSE), Clinical Dementia Rating (CDR), and Activity of Daily Living (ADL) scales. Their healthcare givers were also asked about the patients' abilities. All patients had cranial magnetic resonance imaging (MRI) scans within 2 weeks after questionnaires collected and were diagnosed with AD by neurologists or psychiatrists specialized in dementia (Chunjiu Zhong, Guoqiang Fei, Lei Zhao, Xiaoli Pan) according to DSM-IV criteria.

The criteria of AD patients enrollment were following: (1) primary complaint of memory decline characterized by gradual onset and continuing deterioration over 1 year; (2) MMSE score $\leq$26 (range 0–30) and at least one other cognitive deficit beyond memory impairment; (3) CDR score $\geq$0.5 (range 0–3) and impairment in performance of daily activities; (4) no diagnostic evidence of other neurological and mental disorders; and (5) cranial MRI excluded brain tumor, infarct, hematoma, and hydrocephalus. The exclusion criteria were: (1) major gastrointestinal tract disorders, (2) taking supplements containing B vitamins in the previous month, and (3) chronic alcohol abuse.

Measurement of Biochemical Factors

Blood samples were collected on the same day when questionnaires completed. Blood biochemistry factors were

measured using routine laboratory tests in respective hospital, including routine blood examination; glucose, lipid, and protein levels; renal, thyroid, liver function; and folate and vitamin B12 levels. Lipid metabolism was determined by levels of triglycerides, total cholesterol, and low-density lipoprotein (LDL). Thyroid function was assessed by measuring thyroid-stimulating hormone (TSH), free triiodothyronine (FT3), and free thyroxine (FT4). We selected alanine aminotransferase (ALT) and aspartate transaminase (AST) as liver function indicators, and urea, creatinine, and uric acid as the renal function indicators.

As the biologically active form of thiamine, TDP is abundant in erythrocytes and is a reliable reflection of the thiamine body store (25). Blood TDP levels were measured centralizedly in a single center within 21 days using HPLC fluoroscopy as previously described (26). The HPLC operators were blinded to participant information. Briefly, fresh samples of whole blood were immediately deproteinized with 7.4% perchloric acid. After centrifugation at 9,300 ×g and 4°C for 6 min, the supernatant was collected and stored at −20°C. Thiamine phosphate esters were derivatized into thiochromes using potassium ferricyanide and separated by gradient elusion with a C18 reversed-phase analytical column (250 × 4.6 mm). The derivatives were measured by HPLC fluoroscopy on an Agilent 1100 platform (Agilent Technologies, Santa Clara, CA, USA) with excitation and emission wavelengths of 367 and 435 nm, respectively.

ApoE Genotypes Analysis

A single-nucleotide polymorphism genotyping assay was used for the detection of ApoE alleles. Human genomic DNA was extracted and purified using a genome extraction kit (Tiangen Biotech, Beijing, China). We categorized all participants into two subgroups according to their ApoE ε4 status: an ApoE ε4 group, including the ε2/ε4, ε3/ε4, or ε4/ε4 genotypes, and a non-ApoE ε4 group, including the ε2/ε2, ε2/ε3, or ε3/ε3 genotypes.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences software version 18.0 (SPSS Inc., Chicago, IL, USA). Pearson's rank correlation analysis was performed to assess relationships between B vitamins and biochemical indicators. *T*-test was used to compare the difference between ApoE ε4 and non-ApoE ε4 groups. Differences were considered statistically significant as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

TABLE 1 | Baseline characteristics.

	All patients
Male/female, *n* (%)	85/121 (41/59)
Age, y	74.90 (0.73)
BMI (kg/m^2)	21.98 (0.29)
Education, y	7.23 (0.39)
MMSE	12.34 (0.56)
ADL	16.96 (0.65)
CDR	1.72 (0.07)
Nutrition factors	
Erythrocyte (10^{12}/L)	4.29 (0.04)
Hemoglobin (g/L)	130.2 (1.09)
Albumin (g/L)	40.26 (0.39)
Total protein (g/L)	68.86 (0.51)
Liver function	
AST (U/L)	22.39 (0.71)
ALT (U/L)	17.78 (0.77)
Lipid metabolism	
Triglyceride (mmol/L)	1.33 (0.04)
Cholesterol (mmol/L)	4.82 (0.09)
LDL (mmol/L)	2.76 (0.09)
Glucose metabolism	
Glucose (mmol/L)	5.68 (0.11)
Thyroid function	
FT3 (pmol/L)	4.06 (0.05)
FT4 (pmol/L)	15.7 (0.43)
TSH (mIU/L)	1.97 (0.09)
Renal function	
Urea (mmol/L)	5.73 (0.15)
Creatinine (μmol/L)	73.99 (1.82)
Uric acid (mmol/L)	294.7 (6.98)
Inflammation	
Leukocyte (10^9/L)	7.53 (1.05)
Neutrophilic granulocyte (%)	63.19 (0.73)
Thrombocyte (10^9/L)	215.80 (4.99)

Data are presented as frequency (%) for categorical and mean (SEM) for continuous variables, unless indicated otherwise.

AD, Alzheimer's disease; ADL, activities of daily living; ApoE, apolipoprotein E; BMI, body mass index; CDR, Clinical Dementia Rating; MMSE, Mini Mental State Examination. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FT3, free triiodothyronine; FT4, free thyroxine; LDL, low-density lipoprotein; ns, not significant; TSH, thyroid-stimulating hormone.

RESULTS

Malnutrition Indicators and FT3 Levels Were Most Closely Associated With AD Cognitive Decline

To assess the relationship between biochemical parameters [organ function, lipid and glucose metabolism, inflammation cells and malnutrition (erythrocyte, hemoglobin, serum albumin and total protein)] and AD, we analyzed associations between cognitive-related scores (MMSE, ADL and CDR) and biochemical indicators in 206 sporadic AD patients aged from 48 to 101 years old in China (**Table 1**).

Notably, the malnutrition indicators of erythrocyte, hemoglobin, albumin, and total protein levels were consistently and significantly associated with MMSE, ADL and CDR scores, with all levels of $P < 0.001$. In details, the *r* between erythrocyte, hemoglobin, albumin, and total protein levels and MMSE was 0.37, 0.37, 0.44, 0.27, respectively. As for ADL, the *r* was −0.35, −0.34, −0.43, −0.27, respectively. As for CDR, the *r* was −0.33, −0.35, −0.46, −0.30, respectively. Consistent with a previous study (6), we also observed that serum FT3 was most significantly associated with MMSE ($r = 0.40$, $P < 0.001$), ADL ($r = -0.36$, $P < 0.001$), and CDR ($r = -0.37$, $P < 0.001$) scores, while TSH

was less strongly but still significantly associated with MMSE ($r = 0.14$, $P < 0.05$) and CDR ($r = -0.20$, $P < 0.01$) scores. There was no significant association between serum FT4 and cognitive function (MMSE: $r = -0.03$; ADL: $r = 0.11$; CDR: $r = 0.04$. $P > 0.05$). Besides, the liver function indicator ALT, cholesterol, glucose, leukocyte, and neutrophilic granulocyte percent mildly associated with AD cognitive scores. Other indicators were not statistically associated with AD cognitive scores (all $P > 0.05$) (**Table 2**).

Association Between B Vitamins Status and Blood Biochemical Indicators

To explore the potential causes of malnutrition in AD patients, we analyzed the relationship between B vitamins status and blood biochemical indicators. The results showed that blood TDP level significantly associated with erythrocyte number ($r = 0.32$, $P < 0.001$), hemoglobin level ($r = 0.33$, $P < 0.001$), albumin level ($r = 0.30$, $P < 0.001$), and weakly associated with total protein level ($r = 0.17$, $P < 0.05$). TDP levels also significantly associated with FT3 levels ($r = 0.29$, $P < 0.001$). Liver, renal function, and lipid, glucose metabolism and inflammation indicators have no statistical association with TDP level (AST: $r = 0.09$; ALT: $r = 0.14$; Urea: $r = -0.02$; Creatinine: $r = 0.11$, Uric acid: $r = 0.10$; Triglyceride: $r = -0.04$; Cholesterol: $r = 0.00$; LDL: $r = -0.03$; Glucose: $r = 0.04$; Leukocyte: $r = -0.01$; Neutrophilic granulocyte%: $r = -0.03$; Thrombocyte: $r = 0.00$, $P > 0.05$) (**Table 3**).

Meanwhile, blood folate levels weakly associated with erythrocyte number ($r = 0.15$, $P < 0.05$) and albumin level ($r = 0.19$, $P < 0.05$) but not hemoglobin or total protein level ($r = 0.13$ and $r = 0.14$, respectively, $P > 0.05$), which was similar to FT3 ($r = 0.15$, $P < 0.05$) and neutrophilic granulocyte percent ($r = -0.17$, $P < 0.05$). There were no statistical associations between folate levels and other indicators ($P > 0.05$) (**Table 3**). B12 level only mildly associated with liver function as reflected by ALT level ($r = 0.19$, $P < 0.01$).

Malnutrition and FT3 Level Associated With Age but Not ApoE Genotypes

Aging is the greatest risk factor for AD development and progression, so we analyzed the relationship between peripheral organ dysfunction, malnutrition indicators and age. There were significantly negative associations with erythrocyte number ($r = -0.40$, $P < 0.001$), hemoglobin level ($r = -0.35$, $P < 0.001$), albumin level ($r = -0.50$, $P < 0.001$), total protein level ($r = 0.34$, $P < 0.001$), and FT3 ($r = -0.49$, $P < 0.001$, **Table 4**). Blood ALT ($r = -0.28$, $P < 0.001$), cholesterol ($r = -0.32$, $P < 0.001$), LDL ($r = -0.27$, $P < 0.01$), glucose ($r = -0.17$, $P < 0.05$), and urea ($r = 0.20$, $P < 0.01$) were also significantly associated with age (**Table 4**).

ApoE ε4 genotype is another important risk factor for AD. We detected the ApoE genotypes in a subset (86%) of AD patients in our cohort. We found no significant differences in malnutrition indicators (erythrocyte, hemoglobin, albumin, and total protein) and FT3 between non-ApoE ε4 and ApoE ε4 carrier group (**Figure 1**). Collectively, these results suggest that TDP related malnutrition and abnormal FT3 level were dependent on age but not ApoE genotypes.

TABLE 2 | Association between biochemical indicators and cognitive dysfunction in AD patients.

Blood biochemical markers		Correlation with MMSE			Correlation with ADL			Correlation with CDR		
		r	*P*	*n*	*r*	*P*	*n*	*r*	*P*	*n*
Malnutrition	Erythrocyte	0.37	***	188	−0.35	***	162	−0.33	***	187
	Hemoglobin	0.37	***	201	−0.34	***	175	−0.35	***	200
	Albumin	0.44	***	169	−0.43	***	158	−0.46	***	168
	Total protein	0.27	***	167	−0.27	***	156	−0.30	***	166
Liver function	AST	−0.02	ns	178	0.04	ns	152	−0.02	ns	177
	ALT	0.16	*	197	−0.21	**	171	−0.18	*	196
Lipid metabolism	Triglyceride	0.00	ns	161	0.00	ns	135	−0.04	ns	160
	Cholesterol	0.10	ns	159	−0.24	**	133	−0.20	*	158
	LDL	0.07	ns	122	−0.15	ns	115	−0.15	ns	121
Glucose metabolism	Glucose	0.16	*	196	−0.25	**	170	−0.22	**	195
Thyroid function	FT3	0.40	***	202	−0.36	***	176	−0.37	***	201
	FT4	−0.03	ns	202	0.11	ns	176	0.04	ns	201
	TSH	0.14	*	197	−0.14	ns	172	−0.20	**	196
Renal function	Urea	−0.11	ns	189	0.09	ns	163	0.12	ns	188
	Creatinine	0.09	ns	196	−0.02	ns	170	−0.02	ns	195
	Uric acid	0.04	ns	158	0.00	ns	148	−0.04	ns	157
Inflammation	Leukocyte	0.06	ns	191	0.18	*	165	−0.06	ns	190
	Neutrophilic granulocyte %	−0.18	*	168	0.13	ns	143	0.19	*	167
	Thrombocyte	−0.06	ns	192	0.05	ns	166	0.02	ns	191

TABLE 3 | Association between B vitamins status and biochemical indicators in AD patients.

Blood biochemical markers		Correlation with TDP			Correlation with folate			Correlation with B12		
		r	*P*	*n*	*r*	*P*	*n*	*r*	*P*	*n*
Malnutrition	Erythrocyte	0.32	***	177	0.15	*	187	0.00	ns	185
	Hemoglobin	0.33	***	190	0.13	ns	200	0.01	ns	198
	Albumin	0.30	***	162	0.19	*	168	0.05	ns	166
	Total protein	0.17	*	160	0.14	ns	166	0.04	ns	164
Liver function	AST	0.09	ns	167	−0.04	ns	177	0.07	ns	175
	ALT	0.14	ns	186	0.02	ns	196	0.19	**	194
Lipid metabolism	Triglyceride	−0.04	ns	150	0.02	ns	160	0.09	ns	158
	Cholesterol	0.00	ns	148	0.10	ns	158	0.07	ns	156
	LDL	−0.03	ns	116	0.07	ns	121	0.10	ns	119
Glucose metabolism	Glucose	0.04	ns	185	0.10	ns	195	−0.01	ns	193
Thyroid function	FT3	0.29	***	191	0.15	*	201	0.04	ns	199
	FT4	0.03	ns	191	−0.03	ns	201	0.05	ns	199
	TSH	0.01	ns	187	−0.01	ns	196	0.06	ns	194
Renal function	Urea	−0.02	ns	178	−0.06	ns	188	0.09	ns	186
	Creatinine	0.11	ns	185	−0.01	ns	195	0.07	ns	193
	Uric acid	0.10	ns	152	0.02	ns	157	0.11	ns	155
Inflammation	Leukocyte	−0.01	ns	180	0.03	ns	190	−0.02	ns	188
	Neutrophilic granulocyte %	−0.03	ns	157	−0.17	*	167	−0.14	ns	166
	Thrombocyte	0.00	ns	181	0.03	ns	191	0.06	ns	189

TABLE 4 | Peripheral organs functions and nutrition homeostasis correlated with age.

Blood biochemical markers		Correlation with ages		
		r	*P*	*n*
Malnutrition	Erythrocyte	−0.40	***	188
	Hemoglobin	−0.35	***	201
	Albumin	−0.50	***	169
	Total protein	−0.34	***	167
Liver function	AST	0.02	ns	178
	ALT	−0.28	***	197
Lipid metabolism	Triglyceride	0.10	ns	161
	Cholesterol	−0.32	***	159
	LDL	−0.27	**	122
Glucose metabolism	Glucose	−0.17	*	196
Thyroid function	FT3	−0.49	***	202
	FT4	−0.03	ns	202
	TSH	0.05	ns	197
Renal function	Urea	0.20	**	189
	Creatinine	0.12	ns	196
	Uric acid	0.13	ns	158
Inflammation	Leukocyte	0.03	ns	191
	Neutrophilic granulocyte%	0.12	ns	168
	Thrombocyte	0.05	ns	192

DISCUSSION

In this cross-sectional study, we firstly investigated most blood biomarkers to assess the association between peripheral systemic function and cognition scores in AD population. We found that malnutrition indicators consisted of erythrocyte, hemoglobin, serum albumin and total protein associated with AD cognitive scores most, as well as FT3, which is consistent with previous study (6). Among B vitamins, TDP was most significantly associated with malnutrition indicators and FT3. Malnutrition indicators and FT3 status associated with age but not ApoE genotypes.

It is widely accepted that AD is a neurodegenerative disease that involves multiple organ systems. Here we found that peripheral systemic dysfunction—especially malnutrition and FT3—significantly associated with cognitive decline in AD patients. A previous study reported that a poorer Mini-Nutritional Assessment score, lower body mass index, lower fat-free mass, and a less healthy dietary pattern were associated with a higher risk of 2-year clinical progression of AD (8). Serum FT3 levels were associated with the risk of progression to AD (6). These two risk factors both associated with TDP level significantly, indicating that they may interact with each other through some common pathways to influence the development of AD. Understand the potential mechanism may promote the diagnosis and assess the prognosis of the subsequent cognitive decline in AD.

To our knowledge, others consistently showed that low blood hemoglobin level was associated with AD cognitive impairment (12, 18). Anemia is commonly present as thiamine deficiency (27). Gene mutations related to thiamine metabolism are often found in patients with thiamine-responsive megaloblastic anemia (27). A large population study showed that a micronutrient-fortified rice diet thiamine and other nutrients significantly improved hemoglobin levels, anemia prevalence, and cognitive

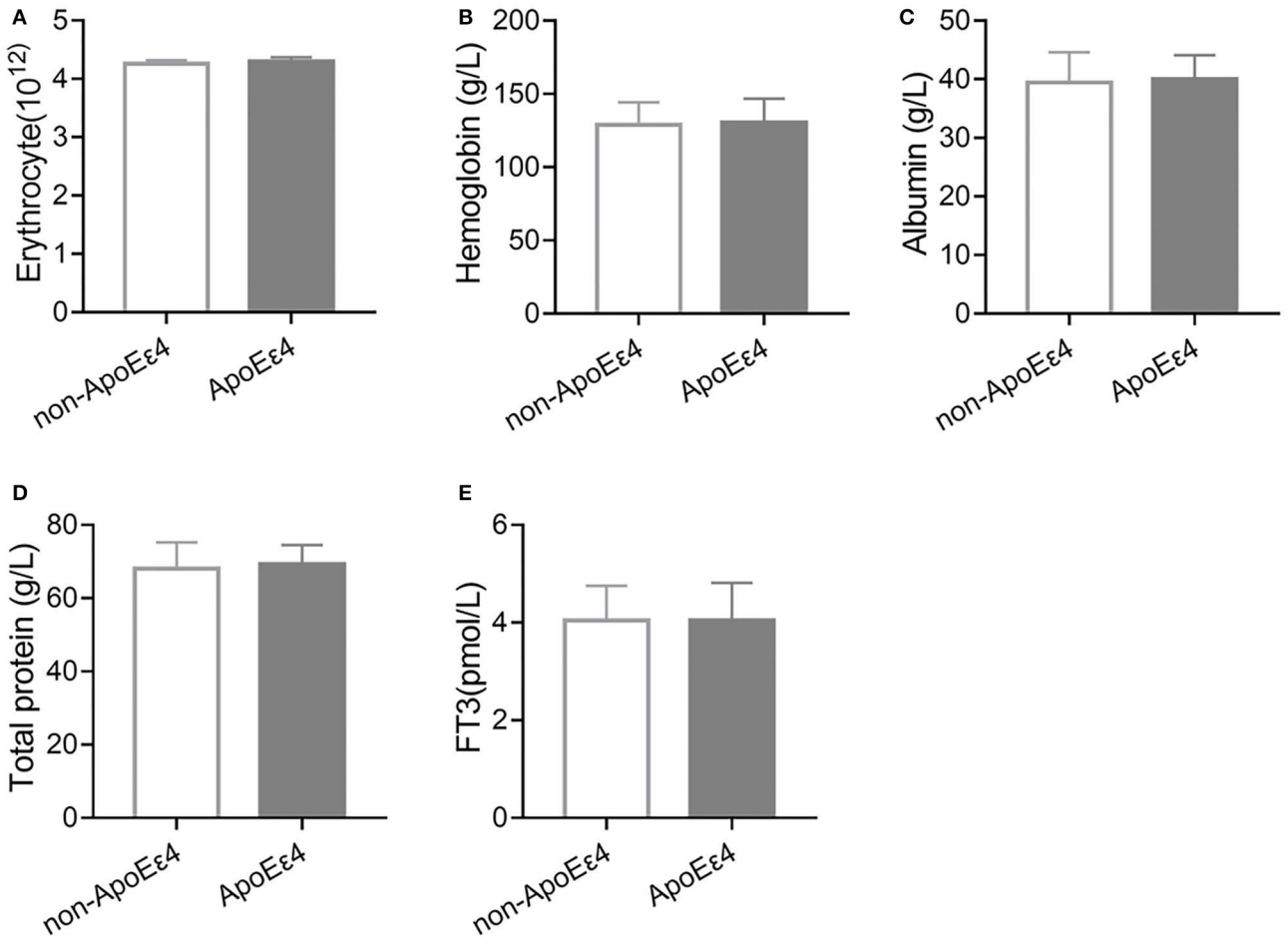


FIGURE 1 | Malnutrition indicators and FT3 levels were independent of ApoE genotype. **(A)** There was no significant change in erythrocyte number between non-ApoE ε4 ($n = 91$) and ApoE ε4 ($n = 81$, $P > 0.05$) carriers. **(B)** Hemoglobin level was similar between non-ApoE ε4 ($n = 96$) and ApoE ε4 ($n = 90$, $P > 0.05$) carriers. **(C)** There was no significant difference in albumin levels between non-ApoE ε4 ($n = 80$) and ApoE ε4 ($n = 75$, $P > 0.05$) carriers. **(D)** There was no significant difference in total protein levels between non-ApoE ε4 ($n = 79$) and ApoE ε4 ($n = 74$, $P > 0.05$) carriers. **(E)** There was no significant difference in FT3 levels between non-ApoE ε4 ($n = 95$) and ApoE ε4 ($n = 90$, $P > 0.05$) carriers.

performance among schoolchildren in India (28). At the molecular level, precursor porphyrins are the major component of hemoglobin that are synthesized in the mitochondria using the production of succinyl-coenzyme A (CoA) from the Krebs cycle (29). However, the mitochondrial pool of succinyl-CoA may limit heme biosynthesis in the setting of TDP deficiency. Decreased heme biosynthesis in erythroid cells then causes anemia (30). Previous studies also showed that thiamine deficiency induced anorexia by inhibiting the hypothalamic adenosine monophosphate-activated protein kinase signal pathway and disrupting neuroendocrine feedback control of food intake and energy metabolism, which may be related to malnutrition in AD patients (31). Although adults with vitamin B12 or folate deficiency can also present with megaloblastic anemias, our results suggest that anemia in AD patients may be caused by TDP reduction more than folate or B12 deficiency. Previous studies showed that low albumin was an independent risk marker for cognitive decline in older adults (32). Experimental and clinical studies suggested that the disturbed metabolism of thiamine could lead to tissue-specific thiamine deficiency in the kidney and increase albumin excretion and microalbuminuria in peripheral nephropathy (33). This probably occurs by impairing the activity and expression of TDP-dependent enzymes—particularly transketolase, and relating to increase of low-grade vascular inflammation and impairment of glomerular and tubular structure (34, 35). It maybe the potential pathological process for low levels of albumin or/and total protein in AD. We also observed that FT3 associated with progression of cognitive decline in AD as before (6). A possible association is that the conversion of FT3 to the acetate would proceed by the thiamine-requiring pyruvic dehydrogenase system. Thus, a thiamine deficiency could block thyroid function and consequently reduce oxidative metabolism and energy production by reducing the formation of FT3 (36). Others and our studies have reported that supplement of benfotiamine (as a synthetic thiamine precursor) is efficacious in improving cognitive function in AD patients

(37, 38). While Aisen et al. found that supplement of high-dose B vitamins (folate, vitamin B6 and B12) had no beneficial effect on the primary cognitive measure in individuals with mild to moderate AD, although total homocysteine declined (39). As a continuous study, our results also suggested that proper TDP level may play an outstanding role in the maintenance of nutrition homeostasis and cognitive function in AD patients. However, the links of thiamine deficiency with malnutrition in AD were not well-investigated.

TDP reduction was an AD-specific pathological feature that closely correlated with brain glucose metabolism (22). Many researches have demonstrated the association between the thiamine deficiency and cognition impairment in animals. Thiamine deficiency resulted in reduced activity of the crucial acetylcholine synthetic enzyme, choline acetyltransferase and neurogenesis (20). Besides, thiamine deficiency could induce excess glutamate release and selective sub-medial thalamic nucleus death involving both inflammation and oxidative stress in the brain (20). Our results indicated that low TDP may be a link between broken nutrition homeostasis in the peripheral system and brain glucose hypometabolism in AD patients. Thiamine uptake is enhanced by thiamine deficiency and reduced by thyroid hormone and diabetes (40). However, how thiamine or its derivatives protect against malnutrition and abnormal FT3 levels in older subjects or AD patients requires further investigation. According to the "homocysteine hypothesis", folate and vitamins B6 and/or B12 deficiency could contribute to cognitive decline, Aβ deposition, neurofibrillary tangles, white matter damage and brain atrophy through DNA methylation, histone modifications and microRNAs (41–44), while the researches relating epigenetics on TDP pathology in AD are still limited and need to be further explored. Although how the malnutrition and TDP reduction work in cognitive decline are still ambiguous and more efforts are needed to further explore, it is positive that correcting thiamine metabolism could be a more effective treatment strategy in patients with AD (45).

In conclusion, malnutrition and FT3 are important modifiable factors for AD progression. Thiamine has been known for protecting against the neuropathy, and TDP may be a link to explore the association among different organs metabolism function, further a potential target to understand the mechanism of cognitive decline. B vitamins supplementation, especially thiamine or its analogs, may be beneficial to prevent cognitive decline.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Committee on Medical Ethics of Zhongshan Hospital, Committee on Medical Ethics of Huashan Hospital, Committee on Medical Ethics of Shanghai Mental Health Center, Committee on Medical Ethics of Haiwan Hospital, Committee on Medical Ethics of Tianjin Huanhu Hospital, and Committee on Medical Ethics of Brain Hospital affiliated to Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XC, LZ, GF, and CZ conceived the study designed experiments. TQ, XP, SS, CW, and GF performed patients collection, thiamine metabolite detection, and ApoE genotype analysis. TQ, LZ, and XC wrote the paper. All authors read and approved the final version of the manuscript.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China (81901081, 81870822, 91332201, 82171408, 8217051360, and 8150050116), Shanghai Municipal Science and Technology Major Project (No. 2018SHZDZX01) and ZJ Lab, Science and Technology Commission of Shanghai Municipality (20ZR1411300), and the Natural Science Foundation of Fujian Province (2020CXB049).

REFERENCES

1. Alzheimer's disease facts and figures. *Alzheimers Dement.* (2021) 17:327–406. doi: 10.1002/alz.12328
2. Long JM, Holtzman DM. Alzheimer disease: an update on pathobiology and treatment strategies. *Cell.* (2019) 179:312–39. doi: 10.1016/j.cell.2019.09.001
3. Wu J, Weng S, Liang C, Lin C, Lan T, Lin S, et al. Effects of kidney function, serum albumin and hemoglobin on dementia severity in the oldest old people with newly diagnosed Alzheimer's disease in a residential aged care facility: a cross-sectional study. *Bmc Geriatr.* (2020) 20:391. doi: 10.1186/s12877-020-01789-0
4. Quinlan P, Horvath A, Eckerström C, Wallin A, Svensson J, Institutionen För Neurovetenskap Och Fysiologi SFPO, et al. Altered thyroid hormone profile in patients with Alzheimer's disease. *Psychoneuroendocrino.* (2020) 121:104844. doi: 10.1016/j.psyneuen.2020.104844
5. Nho K, Kueider-Paisley A, Ahmad S, MahmoudianDehkordi S, Arnold M, Risacher SL, et al. Association of altered liver enzymes with Alzheimer disease diagnosis, cognition, neuroimaging measures, and cerebrospinal fluid biomarkers. *JAMA Netw Open.* (2019) 2:e197978. doi: 10.1001/jamanetworkopen.2019.7978
6. Quinlan P, Horvath A, Wallin A, Svensson J. Low serum concentration of free triiodothyronine (FT3) is associated with increased risk of Alzheimer's disease. *Psychoneuroendocrino.* (2019) 99:112–9. doi: 10.1016/j.psyneuen.2018.09.002
7. Bianchi VE, Herrera PF, Laura R. Effect of nutrition on neurodegenerative diseases. A systematic review. *Nutr Neurosci.* (2021) 24:810–34. doi: 10.1080/1028415X.2019.1681088
8. Doorduijn AS, de van der Schueren M, van de Rest O, de Leeuw FA, Hendriksen H, Teunissen CE, et al. Nutritional status is associated with clinical progression in Alzheimer's disease: The NUDAD project. *J Am Med Dir Assoc.* (2020) S1525-8610(20)30895-1. doi: 10.1016/j.jamda.2020.10.020
9. Sochocka M, Zwolińska K, Leszek J. The infectious etiology of Alzheimer's disease. *Curr Neuropharmacol.* (2017) 15:996–1009. doi: 10.2174/1570159X15666170313122937

10. Huynh K, Lim W, Giles C, Jayawardana KS, Salim A, Mellett NA, et al. Concordant peripheral lipidome signatures in two large clinical studies of Alzheimer's disease. *Nat Commun.* (2020) 11:5698. doi: 10.1038/s41467-020-19473-7
11. Llewellyn DJ, Langa KM, Friedland RP, Lang IA. Serum albumin concentration and cognitive impairment. *Curr Alzheimer Res.* (2010) 7:91. doi: 10.2174/156720510790274392
12. Shah RC, Buchman AS, Wilson RS, Leurgans SE, Bennett DA. Hemoglobin level in older persons and incident Alzheimer disease: prospective cohort analysis. *Neurology.* (2011) 77:219–26. doi: 10.1212/WNL.0b013e318225aaa9
13. Blasko I, Defrancesco M, Oberacher H, Loacker L, Kemmler G, Marksteiner J, et al. Plasma phosphatidylcholines and vitamin B12/folate levels are possible prognostic biomarkers for progression of Alzheimer's disease. *Exp Gerontol.* (2021) 147:111264. doi: 10.1016/j.exger.2021.111264
14. Kennedy DO. B vitamins and the brain: mechanisms, dose and efficacy–a review. *Nutrients.* (2016) 8:68. doi: 10.3390/nu8020068
15. An Y, Feng L, Zhang X, Wang Y, Wang Y, Tao L, et al. Dietary intakes and biomarker patterns of folate, vitamin B6, and vitamin B12 can be associated with cognitive impairment by hypermethylation of redox-related genes NUDT15 and TXNRD1. *Clin Epigenet.* (2019) 11:139. doi: 10.1186/s13148-019-0741-y
16. Hoffmann J, Busse S, von Hoff F, Borucki K, Frodl T, Busse M. Association between homocysteine and vitamin levels in demented patients. *J Alzheimers Dis.* (2021) 81:1781–92. doi: 10.3233/JAD-201481
17. Hooshmand B, Refsum H, Smith AD, Kalpouzos G, Mangialasche F, von Arnim C, et al. Association of methionine to homocysteine status with brain magnetic resonance imaging measures and risk of dementia. *JAMA Psychiat.* (2019) 76:1198–205. doi: 10.1001/jamapsychiatry.2019.1694
18. Wolters FJ, Zonneveld HI, Licher S, Cremers L, Ikram MK, Koudstaal PJ, et al. Hemoglobin and anemia in relation to dementia risk and accompanying changes on brain MRI. *Neurology.* (2019) 93:e917–26. doi: 10.1212/WNL.0000000000008003
19. Jakubowski H, Zioła-Frankowska A, Frankowski M, Perła-Kaján J, Refsum H, de Jager CA, et al. B vitamins prevent iron-associated brain atrophy and domain-specific effects of iron, copper, aluminum, and silicon on cognition in mild cognitive impairment. *J Alzheimers Dis.* (2021) 84:1039–55. doi: 10.3233/JAD-215085
20. Gibson GE, Hirsch JA, Fonzetti P, Jordan BD, Cirio RT, Elder J. Vitamin B1 (thiamine) and dementia. *Ann NY Acad Sci.* (2016) 1367:21–30. doi: 10.1111/nyas.13031
21. Muller YA, Schulz GE. Structure of the thiamine- and flavin-dependent enzyme pyruvate oxidase. *Science.* (1993) 259:965–7. doi: 10.1126/science.8438155
22. Sang S, Pan X, Chen Z, Zeng F, Pan S, Liu H, et al. Thiamine diphosphate reduction strongly correlates with brain glucose hypometabolism in Alzheimer's disease, whereas amyloid deposition does not. *Alzheimers Res Ther.* (2018) 10:26. doi: 10.1186/s13195-018-0354-2
23. Chen Z, Zhong C. Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies. *Prog Neurobiol.* (2013) 108:21–43. doi: 10.1016/j.pneurobio.2013.06.004
24. Pan X, Fei G, Lu J, Jin L, Pan S, Chen Z, et al. Measurement of blood thiamine metabolites for Alzheimer's disease diagnosis. *Ebiomedicine.* (2016) 3:155–62. doi: 10.1016/j.ebiom.2015.11.039
25. Bettendorff L, Lakaye B, Kohn G, Wins P. Thiamine triphosphate: a ubiquitous molecule in search of a physiological role. *Metab Brain Dis.* (2014) 29:1069–82. doi: 10.1007/s11011-014-9509-4
26. Cheng X, Ma D, Fei G, Ma Z, Xiao F, Yu Q, et al. A single-step method for simultaneous quantification of thiamine and its phosphate esters in whole blood sample by ultra-performance liquid chromatography-mass spectrometry. *J Chromatogr B.* (2018) 1095:103–11. doi: 10.1016/j.jchromb.2018.07.030
27. Liu M, Alimov AP, Wang H, Frank JA, Katz W, Xu M, et al. Thiamine deficiency induces anorexia by inhibiting hypothalamic AMPK. *Neuroscience.* (2014) 267:102–13. doi: 10.1016/j.neuroscience.2014.02.033
28. Mahapatra S, Parker ME, Dave N, Zobrist SC, Shajie AD, King A, et al. Micronutrient-fortified rice improves haemoglobin, anaemia prevalence and cognitive performance among schoolchildren in Gujarat, India: a case-control study. *Int J Food Sci Nutr.* (2021) 72:690–703. doi: 10.1080/09637486.2020.1855126
29. Burch JS, Marcero JR, Maschek JA, Cox JE, Jackson LK, Medlock AE, et al. Glutamine via α-ketoglutarate dehydrogenase provides succinyl-CoA for heme synthesis during erythropoiesis. *Blood.* (2018) 132:987–98. doi: 10.1182/blood-2018-01-829036
30. Medeiros R, Moraes JO, Rodrigues S, Pereira LM, Aguiar H, de Cordova C, et al. Thiamine deficiency modulates p38(MAPK) and heme oxygenase-1 in mouse brain: association with early tissue and behavioral changes. *Neurochem Res.* (2020) 45:940–55. doi: 10.1007/s11064-020-02975-7
31. Marseglia A, Darin-Mattsson A, Skoog J, Rydén L, Hadarsson-Bodin T, Kern S, et al. Metabolic syndrome is associated with poor cognition: a population-based study of 70-year-old adults without dementia. *J Gerontol Ser A.* (2021) 76:2275–83. doi: 10.1093/gerona/glab195
32. Ng TP, Feng L, Niti M, Yap KB. Albumin, haemoglobin, BMI and cognitive performance in older adults. *Age Ageing.* (2008) 37:423–9. doi: 10.1093/ageing/afn102
33. Rabbani N, Thornalley PJ. Emerging role of thiamine therapy for prevention and treatment of early-stage diabetic nephropathy. *Diabetes Obesity Metab.* (2011) 13:577–83. doi: 10.1111/j.1463-1326.2011.01384.x
34. Thornalley PJ, Babaei-Jadidi R, Al Ali H, Rabbani N, Antonysunil A, Larkin J, et al. High prevalence of low plasma thiamine concentration in diabetes linked to a marker of vascular disease. *Diabetologia.* (2007) 50:2164–70. doi: 10.1007/s00125-007-0771-4
35. Ascher E, Gade PV, Hingorani A, Puthukkeril S, Kallakuri S, Scheinman M, et al. Thiamine reverses hyperglycemia-induced dysfunction in cultured endothelial cells. *Surgery.* (2001) 130:851–8. doi: 10.1067/msy.2001.117194
36. Appledorf H, Newberne PM, Tannenbaum SR. Influence of altered thyroid status on the food intake and growth of rats fed a thiamine-deficient diet. *J Nutr.* (1969) 97:271. doi: 10.1093/jn/97.3.271
37. Pan X, Chen Z, Fei G, Pan S, Bao W, Ren S, et al. Long-term cognitive improvement after benfotiamine administration in patients with Alzheimer's disease. *Neurosci Bull.* (2016) 32:591–6. doi: 10.1007/s12264-016-0067-0
38. Gibson GE, Luchsinger JA, Cirio R, Chen H, Franchino-Elder J, Hirsch JA, et al. Benfotiamine and cognitive decline in Alzheimer's disease: results of a randomized placebo-controlled Phase IIa clinical trial. *J Alzheimers Dis.* (2020) 78:989–1010. doi: 10.3233/JAD-200896
39. Aisen PS, Schneider LS, Sano M, Diaz-Arrastia R, van Dyck CH, Weiner MF, et al. High-dose B vitamin supplementation and cognitive decline in Alzheimer disease: a randomized controlled trial. *JAMA.* (2008) 300:1774–83. doi: 10.1001/jama.300.15.1774
40. Rindi G, Laforenza U. Thiamine intestinal transport and related issues: recent aspects. *Proc Soc Exp Biol Med.* (2000) 224:246–55. doi: 10.1046/j.1525-1373.2000.22428.x
41. Smith AD, Refsum H. Homocysteine, B vitamins, and cognitive impairment. *Annu Rev Nutr.* (2016) 36:211–39. doi: 10.1146/annurev-nutr-071715-050947
42. Oulhaj A, Refsum H, Beaumont H, Williams J, King E, Jacoby R, et al. Homocysteine as a predictor of cognitive decline in Alzheimer's disease. *Int J Geriatr Psych.* (2010) 25:82–90. doi: 10.1002/gps.2303
43. Athanasopoulos D, Karagiannis G, Tsolaki M. Recent findings in Alzheimer disease and nutrition focusing on epigenetics. *Adv Nutr.* (2016) 7:917–27. doi: 10.3945/an.116.012229
44. Porter K, Hoey L, Hughes C, Ward M, McNulty H. Causes, consequences and public health implications of low B-vitamin status in ageing. *Nutrients.* (2016) 8:725. doi: 10.3390/nu8110725

45. Fessel J. Supplemental thiamine as a practical, potential way to prevent Alzheimer's disease from commencing. *Alzheimers Dement.* (2021) 7:e12199. doi: 10.1002/trc2.12199

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Qian, Zhao, Pan, Sang, Xu, Wang, Zhong, Fei and Cheng. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 09 March 2022
doi: 10.3389/fnut.2022.820224

Check for updates

Infant Formula Supplemented With Milk Fat Globule Membrane, Long-Chain Polyunsaturated Fatty Acids, and Synbiotics Is Associated With Neurocognitive Function and Brain Structure of Healthy Children Aged 6 Years: The COGNIS Study

*Ana Nieto-Ruiz[1,2,3‡], José A. García-Santos[1,2,3‡], Juan Verdejo-Román[4], Estefanía Diéguez[1,2,3], Natalia Sepúlveda-Valbuena[5], Florian Herrmann[1,3], Tomás Cerdó[3,6†], Roser De-Castellar[7], Jesús Jiménez[7], Mercedes G. Bermúdez[1,2,3], Miguel Pérez-García[4,8], M. Teresa Miranda[9], M. Carmen López-Sabater[10,11,12], Andrés Catena[8,13] and Cristina Campoy[1,2,3,14]**

[1] Department of Paediatrics, School of Medicine, University of Granada, Granada, Spain, [2] Instituto de Investigación Biosanitaria (ibs.GRANADA), Health Sciences Technological Park, Granada, Spain, [3] EURISTIKOS Excellence Centre for Paediatric Research, Biomedical Research Centre, University of Granada, Granada, Spain, [4] Department of Personality, Assessment & Psychological Treatment, School of Psychology, University of Granada, Granada, Spain, [5] Nutrition and Biochemistry Department, Faculty of Sciences, Pontificia Universidad Javeriana, Bogotá, Colombia, [6] Carlos III Health Institute, Madrid, Spain, [7] Laboratorios Ordesa, Sant Boi de Llobregat, Spain, [8] Mind, Brain and Behavior Research Centre—CIMCYC, University of Granada, Granada, Spain, [9] Department of Biostatistics, School of Medicine, University of Granada, Granada, Spain, [10] Department of Nutrition, Food Sciences and Gastronomy, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain, [11] Institut de Recerca en Nutrició i Seguretat Alimentària de la UB (INSA-UB), Barcelona, Spain, [12] National Network of Research in Physiopathology of Obesity and Nutrition (CIBERobn), Institute of Health Carlos III (Barcelona's Node), Madrid, Spain, [13] Department of Experimental Psychology, School of Psychology, University of Granada, Granada, Spain, [14] National Network of Research in Epidemiology and Public Health (CIBERESP), Institute of Health Carlos III (Granada's Node), Madrid, Spain

Background: Adequate nutrient intake during the first few months of life plays a critical role on brain structure and function development.

Objectives: To analyze the long-term effects of an experimental infant formula (EF) on neurocognitive function and brain structure in healthy children aged 6 years compared to those fed with a standard infant formula or breastfed.

Methods: The current study involved 108 healthy children aged 6 years and participating in the COGNIS Study. At 0–2 months, infants were randomized to receive up to 18 months of life a standard infant formula (SF) or EF enriched with milk fat globule membrane (MFGM), long-chain polyunsaturated fatty acids (LC-PUFAs) and synbiotics. Furthermore, a reference group of breastfed (BF) infants were also recruited. Children were assessed using neurocognitive tests and structural Magnetic Resonance Imaging (MRI) at 6 years old.

OPEN ACCESS

Edited by:
David Vauzour, University of East Anglia, United Kingdom

Reviewed by:
Sylvie Vancassel, INRA Centre Bordeaux-Aquitaine, France
Rosaura Leis, University of Santiago de Compostela, Spain

***Correspondence:**
Cristina Campoy
ccampoy@ugr.es

†Present address:
Tomás Cerdó, Maimonides Institute for Research in Biomedicine of Cordoba (IMIBIC), Cordoba, Spain

‡These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Nutrition and Brain Health, a section of the journal Frontiers in Nutrition

Received: *22 November 2021*
Accepted: *01 February 2022*
Published: *09 March 2022*

Citation:
Nieto-Ruiz A, García-Santos JA, Verdejo-Román J, Diéguez E, Sepúlveda-Valbuena N, Herrmann F, Cerdó T, De-Castellar R, Jiménez J, Bermúdez MG, Pérez-García M, Miranda MT, López-Sabater MC, Catena A and Campoy C (2022) Infant Formula Supplemented With Milk Fat Globule Membrane, Long-Chain Polyunsaturated Fatty Acids, and Synbiotics Is Associated With Neurocognitive Function and Brain Structure of Healthy Children Aged 6 Years: The COGNIS Study. Front. Nutr. 9:820224. doi: 10.3389/fnut.2022.820224

Results: Experimental infant formula (EF) children showed greater volumes in the left orbital cortex, higher vocabulary scores and IQ, and better performance in an attention task than BF children. EF children also presented greater volumes in parietal regions than SF kids. Additionally, greater cortical thickness in the insular, parietal, and temporal areas were found in children from the EF group than those fed with SF or BF groups. Further correlation analyses suggest that higher volumes and cortical thickness of different parietal and frontal regions are associated with better cognitive development in terms of language (verbal comprehension) and executive function (working memory). Finally, arachidonic acid (ARA), adrenic acid (AdA), docosahexaenoic acid (DHA) levels in cheek cell glycerophospholipids, ARA/DHA ratio, and protein, fatty acid, and mineral intake during the first 18 months of life seem to be associated with changes in the brain structures at 6 years old.

Conclusions: Supplemented infant formula with MFGM components, LC-PUFAs, and synbiotics seems to be associated to long-term effects on neurocognitive development and brain structure in children at 6 years old.

Clinical Trial Registration: https://www.clinicaltrials.gov/, identifier: NCT02094547.

Keywords: neuroimaging, early nutrition, infant formula, breastfeeding, MFGM, LC-PUFAs, synbiotics, cognition

INTRODUCTION

Proteins, long–chain polyunsaturated fatty acids (LC-PUFAs), iron, zinc, iodine, and B vitamins play a critical role on brain function and structure development (1–3). Nevertheless, given that the infant brain evolves from early childhood to adolescence, early-life nutritional deficiencies may have negative long-lasting or permanent effects on later cognitive function (4), including impairment on visuo-perceptual functions (5), working memory (6, 7), language (8), and executive functions (9).

It is well-established that breastfeeding is related to optimal brain maturation (10, 11) and neurodevelopment (12) in life. When breastfeeding is not possible, infant formulas are available to satisfy infant's nutritional and energy requirements. However, their functional and nutritional properties vary considerably from breast milk, and new bioactive compounds are being added to narrow the functional and nutritional gap with breast milk (13). In this regard, supplementation of infant formula with LC-PUFAs, mainly with docosahexaenoic and arachidonic acids (DHA and ARA, respectively), seems to be related with short and long-term beneficial effects on cognitive and visual development (14), learning, vocabulary, intelligence (15), processing information (16), and attention and inhibition systems (17). Other studies, however, have shown no long-term effects of LC-PUFA-enriched infant formula on neurodevelopmental and psychomotor outcomes (18, 19). On the other hand, infant formula supplementation with milk fat globule membrane (MFGM) has also gained interest due to its content in bioactive components and complex polar lipids that might have a potential role on infant neurodevelopment (20–22). Furthermore, it is important to notice that synbiotics (pre-plus probiotics) are also being added to infant formulas to ensure a healthy establishment of gut microbiota and communication along the microbiota-gut-brain axis, thus modulating neurodevelopment, brain function, and behavior throughout the life span (23, 24). However, the combined long-term effects of all these bioactive components are not well-established, and further studies are still needed to clarify the role of early nutrition based on bioactive compound-enriched infant formula on brain structure and cognitive function later in life.

Having in mind these considerations, the aim of this study was to analyze the long-term effects of an infant formula supplemented with MFGM, LC-PUFAs, and synbiotics on neurocognitive function and brain structure in healthy children aged 6 years compared to those who received a standard infant formula or breastfeeding.

Abbreviations: ARA, Arachidonic acid; AdA, Adrenic acid; BENCI, Computerized battery for neuropsychological evaluation of children; BF, Breastfed infants; BMI, Body Mass Index; DHA, Docosahexaenoic acid; DPA, docosapentaenoic acid; EF, Experimental infant formula; EFSA, European Food Safety Authority; EPA, Eicosapentaenoic acid; FAs, Fatty acids; GWG, Gestational Weight Gain; IQ, Intelligent quotient; K-BIT, Kaufman brief intelligence test; LC-PUFAs, Long-chain polyunsaturated fatty acids; LNA, α-linolenic acid; MFGM, Milk fat globule membrane; MRI, Magnetic resonance imaging; PLON-R, Oral language test of Navarra revised.

MATERIALS AND METHODS

Ethics, Informed Consent, and Permissions

The COGNIS study was conducted according to the updated principles of the Declaration of Helsinki II (25, 26), and was approved by the Research Bioethical Committee from the University of Granada (Spain), the Bioethical Committees for Clinical Research from San Cecilio University Clinical Hospital, and University Mother-Infant Hospital of Granada (Spain). Prior to involving each child in the study, families were informed about

study procedures and a signed written informed consent was obtained from each parent, legal guardian, or caregiver.

Study Design and Subjects

The current analysis included 108 children aged 6 years and participating in the COGNIS Project, a prospective, randomized, and double-blind study consisting in a nutritional intervention based on an infant formula supplemented with bioactive nutrients (registered at https://clinicaltrials.gov/ct2/show/NCT02094547?term=NCT02094547&draw=2&rank=1, Identifier: NCT02094547). Detailed information on this project, including study design, subject recruitment, and population characteristics, have been previously described (27, 28). Originally, a total of 220 full-term healthy Spanish infants were included in the study. One hundred seventy infants, aged between 0 and 2 months old, were randomized (ratio 1:1) to receive, during their first 18 months of life, either a standard infant formula (SF: $n = 85$) or an experimental infant formula (EF: $n = 85$) enriched with MFGM components [10% of total protein content (wt:wt)], synbiotics [Fructooligosaccharides (FOS): Inulin proportion 1:1; *Bifidobacterium longum* subsp *infantis* CECT7210 (*Bifidobacterium infantis* IM1) and *Lactobacillus rhamnosus* LCS-74], LC-PUFAs (including DHA and ARA), gangliosides, nucleotides, and sialic acid. A full description of the nutritional composition of both infant formulas has been previously reported (28). Infants received initiation formula up to 6 months of age and the corresponding follow-on formula between 6 and 18 months of age. Additionally, 50 exclusively breastfed infants (BF) for at least 2 months were enrolled between 0 and 6 months old as a reference group.

A detailed participant's flowchart from the baseline visit to 6 years old is shown in **Figure 1**. Up to 18 months of life, a total of 40 infants had to be excluded in the formula groups as follows: 24 were excluded in the SF group (1 infant due to perinatal hypoxia, 1 infant had formula non-related growth deficiency, 15 infants did not take the infant formula, 2 had infantile colic, 3 were excluded due to lactose intolerance, 1 infant due to digestive surgical intervention, and 1 infant suffered hydrocephalus); 16 infants were excluded in the EF group (2 infants presented formula non-related growth deficiency, 2 infants lactose intolerance, 11 infants did not take the infant formula, and 1 was excluded due to epileptic seizure). Furthermore, one infant from the BF group was excluded because he was not exclusively breastfed for at least 2 months. During the follow-up visits, some participants decided to drop out of the study. Afterwards, 108 children attended the follow up call at 6 years old, and their neurocognitive performance was evaluated using the Kaufman Brief Intelligence Test (K-BIT) (29), the Oral Language Test of Navarra Revised (PLON-R) (30), and the Computerized Battery for Neuropsychological Evaluation of Children (BENCI) (31) (SF: $n = 37$; EF: $n = 39$; BF: $n = 32$). All participants were also invited to participate in a Magnetic Resonance Imaging (MRI) session. However, some data from participants ($n = 30$) were taken out of current analysis due to poor image quality caused by the excessive motion of children during MRI acquisition. Therefore, neuroimage data were finally obtained from 78 children aged 6 years (SF: $n = 30$; EF: $n = 27$; BF: $n = 21$). Furthermore, nutritional information from participants in the COGNIS study was also collected, including fatty acid (FA) status up to 18 months of age (SF: 33; EF: 34; BF: 31) and complete dietary intake up to 6 years old (SF: 35; EF: 35; BF: 30).

Data Collection and Assessment

Baseline information regarding parents was obtained upon study entry, including age, pre-conceptional maternal body mass index (BMI), gestational weight gain (GWG), smoking during pregnancy, educational level, place of residence, employment, and socioeconomic status. Postpartum depression was evaluated with the Spanish version of the Edinburgh Postnatal Depression Scale (32). Parents' intelligence quotient (IQ) was assessed using the G factor of the Cattel Intelligence test (33, 34).

Baseline characteristics of infants (gestational age, type of delivery, sex, siblings, and timing of breastfeeding) were collected using questionnaires and medical records. In addition, child anthropometric parameters such as BMI, head circumference, and waist circumference were also registered at 6 years of age.

Fatty Acid Status

Analysis of FA status was performed from cheek cell samples collected at 3, 6, 12, and 18 months of life, as previously reported (35). This method is a valid index of essential fatty acid status as it can be monitored frequently and is reported to be associated with functional parameters in infants (36). In addition to the easy and non-invasive nature of this technique, cheek cell fatty acids may serve as a marker of the essential fatty acid content, especially of DHA and ARA, in plasma, tissue concentrations (red blood cells), and the diet (37). Briefly, samples from the inside of the infants' cheeks were collected 1 h after feeding using a Rovers® EndoCervex-Brush® supplied by Deltalab (Barcelona, Spain), and cell pellets were obtained by centrifugation and stored at −80°C until further analysis. Cheek cell glycerophospholipid fraction was isolated using methanol with butylated hydroxytoluene (BHT), while FA methyl esters were obtained using sodium methylate in methanol (25 wt% in methanol) and boron trifluoride methanol solution (14% v/v). Rapid gas chromatography was used to separate FAs in cheek cell samples (35, 38), and quantification was done by normalization. Lastly, the results were expressed in relative amounts (percentage).

Dietary Intake

Participants' dietary intake was evaluated at 6, 12, and 18 months and 2.5, 4, and 6 years old by a quantitative 3-day dietary record based on the methods for food monitoring and nutrient intake indicated by Food and Agriculture Organization of the United Nations (FAO) (39). DIAL software (Alce Ingeniería, Madrid, Spain) (40) was used to analyze dietary records, which converts food consumption data into nutrient intake (macro- and micronutrients, including FAs profile) in accordance to a previously described methodology (41).

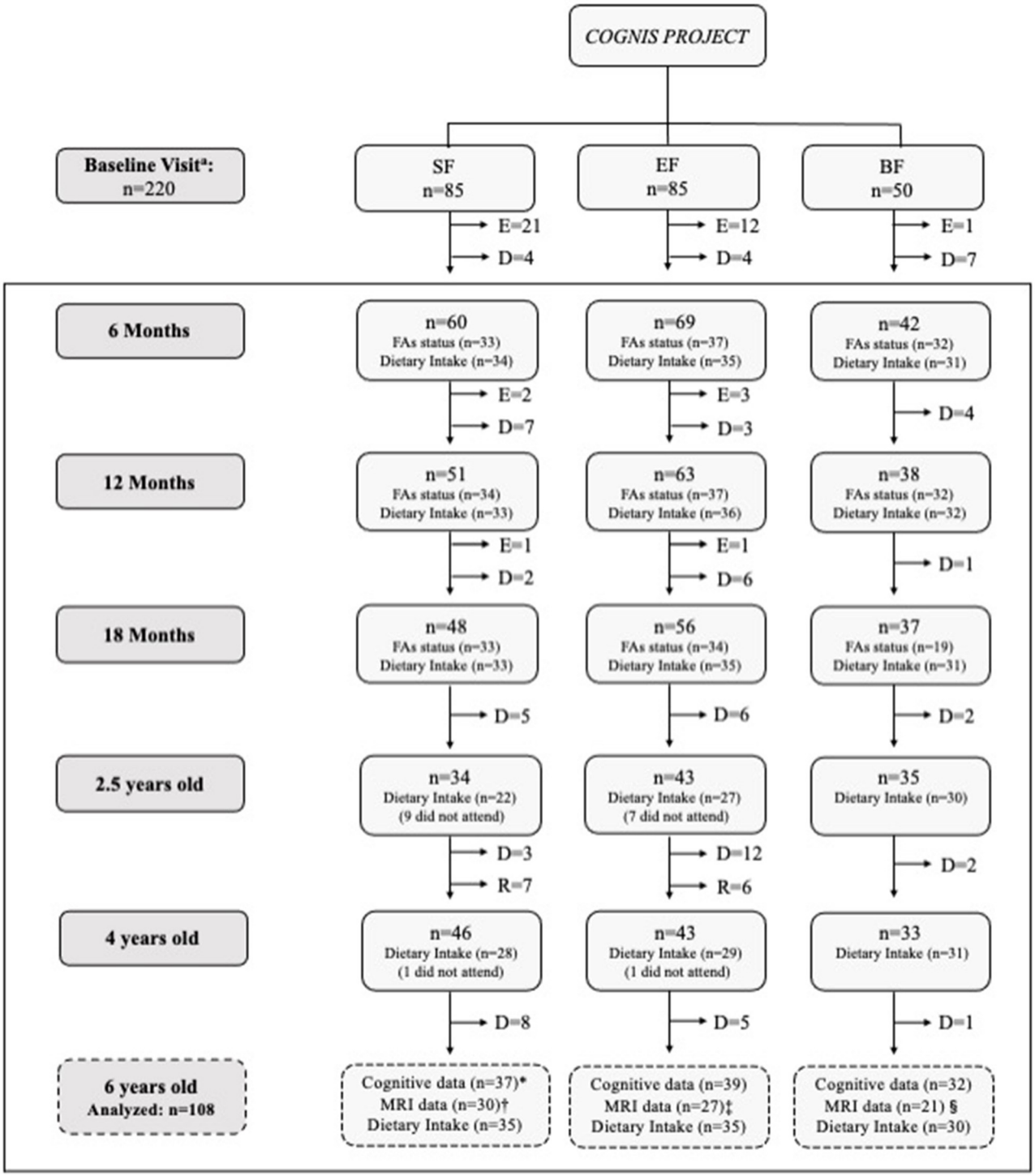


FIGURE 1 | Participant flow-chart from baseline visit to 6 years old. BF, Breastfed infants; D, Drop-outs; E, Exclusions; EF, Experimental infant formula; MRI, Magnetic Resonance Imaging; *n*, Sample size; R, Recovered; SF, Standard infant formula. Fatty acid (FA) status was analyzed from available cheek cell samples of children who attended to 6 years follow-up visit. Incomplete dietary intake data were excluded from the current analysis. At 6 years old: *2 children did not attend to cognitive assessment. Some MRI data from participants ($n = 30$) were eliminated because the quality of the brain images was not adequate to be analyzed due to excessive movement of children inside the scanner: †Unable to analyze the data from 7 children; ‡Unable to analyze the data from 14 children; §Unable to analyze the data from 11 children. [a]BF infants were randomized between 0–6 months of age.

Neurocognitive Evaluation

Kaufman Brief Intelligence Test

The Spanish version of K-BIT (29) was used to evaluate verbal and non-verbal intelligence through two subtests: *vocabulary and matrices*. In the *vocabulary* subtest, children observed a series of pictures and named the object presented on them. *Matrices* subtest is a measure of abstract reasoning. In this case, the child selected a picture or abstract design that best completes a visual pattern following a visual analogy. In both subtests, the dependent variable was the number of correct responses. Furthermore, the K-BIT test also provides a general IQ based on the sum of scores obtained in the *vocabulary* and *matrices* subtests. According to K-BIT test standards, the normal range of typical scores is between 85 and 115 points (29, 42).

Oral Language Test of Navarra Revised (PLON-R): Language Assessment

Oral Language Test of Navarra Revised (PLON-R) is a standardized test that allows an early detection or screening of the oral language development in children aged between 3 and 6 years old. This test is not only focused on the language dimensions (form, content, and use) with specific activities for each dimension, but also provides a total punctuation on language development. The scores of each one of the dimensions are transformed into typical scores. The normal score, according to PLON-R test standardized by age, is as follows: form $= 65$; content ≥ 62; use ≥ 52; total ≥ 51 (30).

Computerized Battery for Neuropsychological Evaluation of Children

Computerized Battery for Neuropsychological Evaluation of Children (BENCI) is a computerized tool that evaluates neuropsychological functions and neurodevelopmental domains in children, such as *immediate and delayed memory, attention, visual motor coordination, verbal fluency and comprehension, processing speed, and executive functions* (31, 43). All these tests were developed using valid neuropsychological procedures assumed from the literature of neuropsychological assessment, and were principally based on the NUTRIMENTHE Neuropsychological Battery (43, 44). In the current study, neurocognitive function was assessed using the following tasks (45):

- *Verbal Comprehension (images) (language)*. A combination of images of a given category (animals) was shown, and the children received instructions (auditory) through which they should select a given image that fulfills the indicated conditions (type of animal, position, type of activity that one can carry out, and/or color: e.g., *"Touch the frog that is next to the dog"*).
- *Continuous performance (sustained attention).* Various blocks in a series of letters (100 essays) appeared on the screen and the child should touch the tablet screen each time the correct stimulus appears (an A after an X). The rest of the letters were used as distractor elements.
- *Semantic Fluency (executive function)*. This test indicates a category (for example, animals) and the child should respond, as fast as he/she can, with all the words he/she knows within the same category in 60 s.
- *Working Memory (executive function).* The children listened to 8 series of number and color sequences. After each sequence, the child should separately repeat the numbers and the colors that he/she had heard in the same order.
- *Verbal Memory (short and long-term memory).* At the beginning of the task, the child listened to 6 series of words, and should memorized as many as possible. After each sequence, the child should repeat all the words that he/she could remember. After 20 min, in the delayed recall essay, the child should repeat out loud all those words he/she could remember from the previous list. Finally, in the recognition essay, the child listened to a series of words, half of which were in the list above. The child answered *"yes"* or *"no"* to whether each word was in the list.
- *Simple Reaction Time Test (processing speed).* This test required that the child to press any key as fast as possible every time a cross (+) appeared on the screen (50 essays).
- *Go/no-Go task (executive function: inhibition).* During this test, two alternating elements (bear and dolphin) kept appearing on the screen. In the first phase of the test, the child should state the distinctive element of the two (bear) and touch the tablet screen when it appeared. After listening to a sound that represents the phase change, the distinctive element appeared to be the other (dolphin), to which the child should press the tablet screen when it appeared. The dependent measure was the total number of correct answers.

Magnetic Resonance Imaging Procedure

Imaging Data Acquisition

Prior to the neuroimaging session, children were familiarized with scanner's sounds and MRI environment using a mock MRI scanner. Brain data were acquired using a 3T MRI scanner equipped with a 32-channel phased-array head coil for reception (Magnetom Trio Siemens Medical System, ERLANGEN, Germany) located at Mind, Brain, and Behavior Research Center (CIMCYC, University of Granada, Spain). A high resolution T1-weighted 3D magnetization-prepared rapid gradient-echo (MPRAGE) sequence was acquired for each participant with the following parameters: Repetition Time (TR) $= 2.3$ ms, Echo Time (TE) $= 3.1$ ms, flip angle $= 9°$, Field of View (FOV) $= 256 \times 256$ mm, matrix size=320 $\times$ 320, and number of slices=208, resulting in an isotropic resolution of 0.8 $\times$ 0.8 $\times$ 0.8 mm. Total acquisition time for the T1 sequence was 6 min and 35 s. Head movements were minimized using a foam system around the participant's head. Furthermore, a cartoon film was projected to reassure the child during the MRI scanning.

Neuroimage Processing

All images were visually inspected for major artifacts and realigned to the anterior commissure-posterior commissure (AC-PC) line. Image processing was performed using the automated processing *"recon-all"* pipeline in FreeSurfer software (version 6.0, http://surfer.nmr.mgh.harvard.edu/) on the Alhambra Cluster of the University of Granada (Spain). Preprocessing steps involved intensity normalization, registration to Talairach

space, skull stripping, segmentation of white matter (WM), tessellation of the WM boundary, and automatic correction of topological defects. After that, cerebral cortex was parceled into regions of interest (ROIs) based on gyral and sulcal structures from the Destrieux atlas (46–49). FreeSurfer outputs were also visually inspected to check for correct segmentation and parcellation. Volumes and cortical thicknesses of brain parcellation based on the Destrieux atlas were extracted along with brain subcortical volumes.

Statistical Analysis

All statistical analyses were performed using IBM® SPSS Statistics® program, version 22.0 (SPSS Inc. Chicago, IL, USA). Normally distributed variables were presented as mean and standard deviation (SD), and non-normal variables as median and interquartile range (IQR). Categorical variables were shown as frequencies and percentages. ANOVA or Welch test were performed for normally distributed variables, Kruskal Wallis test was performed for non-normal continuous variables, and Chi-square or Fisher test was performed for categorical variables. Normal distribution was tested using Kolmogorov-Smirnov and/or Shapiro Wilk test. We also used one-way ANOVAs to examine differences between SF, EF, and BF groups in neurocognitive tests scores, brain volumes, and cortical thicknesses. Total intracranial volume was included as covariate in all brain volume analyses. Moreover, to discard the differences that could be driven by confounders *(maternal age, familiar socioeconomic status, smoking during pregnancy, age, and sex*) (50–52), we performed additional analyses of group comparisons using a one-way analysis of covariance (ANCOVA) that included these confounders. In the event of significant group differences, Bonferroni-corrected *post-hoc* comparisons were used to identify significant pair-wise group differences. To explore whether the brain differences could be driven by the FA concentrations and dietary intake, we performed stepwise linear regression analyses, using one brain region as dependent variable and FA concentration, dietary intake, and the aforementioned confounders as independent variables. Finally, Pearson correlation analyses were performed to estimate the relationship between child neurocognitive performance and brain volumes and thickness of those regions that were statistically different between study groups. $p < 0.05$ were considered statistically significant.

RESULTS

Parental and Child Characteristics of the Study Participants at 6 Years Old

A comparative analysis between study groups of parents' and children's baseline characteristics participating at 6 years old in the COGNIS follow-up study is shown in **Table 1**. Significant differences between study groups were found in parents' age, educational level, and in their socioeconomic status. In fact, BF mothers were older and showed higher educational level than mothers of SF and EF infants ($p = 0.013$ and $p = 0.001$, respectively). No significant differences were found between study groups in other maternal baseline characteristics related to child neurocognitive function and brain structure, including pre-conceptional BMI (pBMI), GWG, and smoking during pregnancy. **Table 1** also shows that fathers from the BF group were older compared to fathers of SF infants ($p = 0.033$) and presented higher educational level than EF-fed infants' fathers ($p = 0.005$). Concerning socioeconomic status, those parents whose children were breastfed had higher status compared to both SF- and EF-fed infants' parents ($p = 0.002$).

All infants participating in the COGNIS study were more frequently born by vaginal delivery, and no significant differences were found for characteristics at birth among study groups. All children were born at term and with birth weight adequate for gestational age. However, as expected, due to the COGNIS study design, days of breastfeeding significantly differed between formula (SF and EF) and BF groups ($p < 0.001$). Finally, at 6 years old, children from the three study groups did not differ in their anthropometric characteristics, including BMI and head and waist circumferences.

Type of Early Feeding and Neurocognitive Function in Children Aged 6 Years

First, we analyze the potential long-term effects of the type of early feeding received during the first 18 months of life (EF vs. SF vs. BF) on later neurocognitive function at 6 years, comparing the K-BIT, PLON-R, and BENCI scores between the three COGNIS groups (**Table 2**). In the adjusted model, controlling for *maternal age, smoking during pregnancy, familiar socioeconomic status, and sex,* EF children showed higher IQ and vocabulary standard score in the K-BIT test than BF children ($p_{adj} = 0.031$, and $p_{adj} = 0.022$, respectively). Moreover, both SF and EF children presented less errors of commission in continuous performance task from BENCI battery ($p_{adj} = 0.001$) in comparison to BF children (**Table 2**). No significant differences in child language dimensions (PLON-R Test) at 6 years old were found between the three groups (**Table 2**).

Analysis of Brain Volume and Cortical Thickness in Children Aged 6 Years and Effects of Early Nutrition

Next, we tested whether the type of early nutrition during the first 18 months of life *(SF vs. EF vs. breastfeeding)* had any effects on later brain structure, brain volume, and cortical thickness. Significant results, adjusted by *smoking during pregnancy, maternal age, familiar socioeconomic status, age, and sex (plus total brain volume for volume brain analysis),* are presented in **Table 3**. Children fed with EF presented higher volumes in parietal regions than the SF children ($p_{adj} = 0.002$), particularly, in the right postcentral gyrus ($p_{adj} = 0.015$) and in the right precuneus ($p_{adj} = 0.009$). EF children also showed higher left orbital volume than BF children ($p_{adj} = 0.012$). Regarding cortical thickness, adjusted analyses showed that EF children presented higher thicknesses in the left inferior circular insular sulcus compared to SF and BF children ($p_{adj} = 0.012$). Moreover, children fed with EF presented increased cortical thickness in the left occipito-temporal sulcus compared to the SF group ($p_{adj} = 0.027$), and in the right postcentral sulcus compared to the

TABLE 1 | Baseline characteristics of parents and children participating in the COGNIS study depending on their type of feeding during infancy[1].

		SF ($n = 37$)	EF ($n = 39$)	BF ($n = 32$)	p^2
Mother					
Age (years)		31.5 (10.8)[a]	30.0 (5.0)[a]	34.0 (8.5)[b]	**0.013**
pBMI (kg/m^2)		24.5 ± 4.2	25.6 ± 4.3	24.6 ± 2.8	0.468
GWG (kg)		5.8 ± 5.2	6.7 ± 4.8	6.4 ± 3.4	0.723
Type of delivery	Vaginal	28 (75.7)	28 (71.8)	24 (75.0)	0.919
	Cesarean section	9 (24.3)	11 (28.2)	8 (25.0)	
IQ (points)		104.0 (16.5)	100.0 (19.0)	111.0 (21.0)	0.105
Educational level	NS/Primary	4 (10.8)[a,b]	12 (30.9)[a]	2 (6.3)[b]	**0.001**
	Secondary	10 (27.0)	10 (25.6)	2 (6.3)	
	VT	14 (37.8)	10 (25.6)	9 (28.1)	
	University/Ph.D	9 (24.4)[a]	7 (17.9)[a]	19 (59.4)[b]	
Postpartum depression	No	29 (78.4)	32 (84.2)	27 (84.4)	0.749
Smoking during pregnancy	No	30 (81.1)	33 (84.6)	30 (93.8)	0.299
Employment status	Unemployed	9 (24.3)	5 (12.8)	5 (15.6)	0.525
	Domestic work	1 (2.7)	3 (7.7)	1 (3.1)	
	Temporary contract	2 (5.4)	7 (17.9)	4 (12.5)	
	Stable employment	25 (67.6)	24 (61.5)	22 (68.8)	
Familiar					
Socioeconomic status	Low	7 (18.9)	8 (20.5)	1 (3.1)	**0.002**
	Middle-Low	18 (48.6)	19 (48.7)	8 (25.0)	
	Middle-High	10 (27.0)	10 (25.6)	13 (40.6)	
	High	2 (5.4)[a]	2 (5.1)[a]	10 (31.3)[b]	
Place of residence	Urban	14 (37.8)	11 (28.2)	6 (18.8)	0.216
	Rural	23 (62.2)	28 (71.8)	26 (81.3)	
Siblings	0	8 (21.6)	11 (28.2)	5 (15.6)	0.445
	≥1	29 (78.4)	28 (71.8)	27 (84.4)	
Father					
Age (years)		32.3 ± 7.0[a]	33.2 ± 6.1[a,b]	36.2 ± 4.5[b]	**0.033**
Educational level	NS/Primary	8 (21.6)[a,b]	17 (43.6)[a]	5 (15.6)[b]	**0.005**
	Secondary	18 (48.6)	10 (25.6)	7 (21.9)	
	VT	5 (13.5)	8 (20.5)	8 (25.0)	
	University/Ph.D	6 (16.3)[a,b]	4 (10.3)[a]	12 (37.5)[b]	
IQ (points)		106.8 ± 13.9	104.7 ± 16.2	106.5 ± 13.0	0.822
Employment status	Unemployed	5 (13.5)	4 (11.1)	1 (3.1)	0.302
	Domestic work	0 (0.0)	0 (0.0)	0 (0.0)	
	Temporary contract	6 (16.2)	2 (5.6)	3 (9.4)	
	Stable employment	26 (70.3)	30 (83.3)	28 (87.5)	
Neonate					
Gestational age (weeks)		40.0 (2.0)	40.0 (2.0)	40.0 (2.8)	0.826
Breastfeeding lactation (days)		13 (21.0)[a]	12 (31.0)[a]	450 (240.0)[b]	**<0.001**
Sex	Boy	24 (64.9)	25 (64.1)	13 (40.6)	0.073
	Girl	13 (35.1)	14 (35.9)	19 (59.4)	
Children					
Age (days)		2225 (38)	2222 (42)	2221.5 (26)	0.565
BMI (kg/m^2)		16.1 (2.4)	16.6 (2.6)	15.8 (2.1)	0.308
Head circumference (cm)		51.5 ± 1.8	51.8 ± 1.6	51.6 ± 1.1	0.797
Waist circumference (cm)		53.9 (4.7)	55.2 (5.9)	53.9 (6.5)	0.356

[1]*Data are presented as means ± SD for parametrically distributed data, n (%) for categorical data, and medians (IQRs) for non- parametrically distributed data.* [2]*P-values for overall differences between COGNIS-groups. ANOVA test for normally distributed variables, Kruskal–Wallis test for non-normal continuous variables, and Chi-square or Fisher test for categorical variables. Values not sharing the same suffix (a,b) were significantly different in the Bonferroni post-hoc test. P < 0.05 are highlighted in bold. SF, Standard infant formula; EF, Experimental infant formula; BF, Breastfed infants; pBMI, pre-conceptional body mass index; GWG, Gestational Weight Gain; NS, No schooling; IQ, Intelligence quotient; VT, Vocational training; BMI, Body mass index.*

TABLE 2 | Children's neurocognitive function at 6 years old depending on the type of feeding during infancy[1].

	SF ($n = 37$)	EF ($n = 39$)	BF ($n = 32$)	p^2	p_{adj}
Kaufman brief intelligence test (K-BIT)					
Vocabulary (typical score)	107.91 ± 13.44[a,b]	113.16 ± 13.31[a]	103.73 ± 14.26[b]	0.177	**0.022**
Matrices (typical score)	111.51 ± 12.07	113.21 ± 12.00	111.78 ± 11.07	0.798	0.113
IQ (typical score)	109.64 ± 11.67[a,b]	113.46 ± 11.50[a]	105.54 ± 12.40[b]	0.302	**0.031**
Oral language test of Navarra-Revised (PLON-R)					
Form (typical score)	46.92 ± 17.30	47.46 ± 14.89	49.84 ± 12.61	0.702	0.991
Content (typical score)	54.95 ± 24.65	55.38 ± 15.23	60.34 ± 19.87	0.468	0.886
Use (typical score)	52.65 ± 23.01	53.95 ± 21.38	61.00 ± 16.38	0.207	0.896
Total score PLON-R (typical score)	55.78 ± 27.34	55.31 ± 20.33	63.34 ± 19.42	0.201	0.889
Computerized battery for neuropsychological evaluation of children (BENCI)					
Verbal comprehension (successes)	10.65 ± 2.06	11.16 ± 1.55	11.26 ± 1.86	0.327	0.237
Continuous performance (successes)	53.92 ± 4.72	51.17 ± 10.00	50.06 ± 8.51	0.055	0.115
Continuous performance (errors of commission)	8.14 ± 7.87[a]	9.44 ± 12.02[a]	18.35 ± 17.43[b]	**0.003**	**0.001**
Semantic fluency (successes)	7.65 ± 2.70	7.39 ± 2.11	8.06 ± 2.39	0.518	0.927
Working memory (successes)	4.00 ± 1.43	4.05 ± 1.39	4.00 ± 1.55	0.984	0.256
Verbal short-term memory (successes)	4.39 ± 1.10[a,b]	4.19 ± 0.95[a]	4.89 ± 0.73[b]	**0.010**	0.238
Verbal long-term memory (successes)	4.41 ± 1.76	3.95 ± 2.10	4.68 ± 1.58	0.251	0.263
Verbal long-term memory (recognition successes)	10.57 ± 1.88	10.97 ± 2.02	11.13 ± 1.12	0.387	0.525
Reaction time (ms)	542.92 ± 54.25	557.55 ± 64.87	553.42 ± 151.25	0.569	0.828
Go/no-Go task (successes)	46.57 ± 4.50	48.28 ± 6.80	48.13 ± 5.73	0.308	0.335
Go/no-Go task (errors of commission)	3.24 ± 2.50	3.03 ± 2.45	3.94 ± 2.32	0.283	0.071

[1]*Data are presented as means ± SD.*

[2]*P-values for differences between COGNIS-groups. ANOVA test for normally distributed variables.*

P_{adj} are univariate analysis of covariance (ANCOVA) adjusted by maternal age, smoking during pregnancy, familiar socioeconomic status and sex of the children. Values not sharing the same suffix (a,b) were significantly different in the Bonferroni post-hoc test. $P < 0.05$ are highlighted in bold.

SF, Standard infant formula; EF, Experimental infant formula; BF, Breastfed infants; ms, milliseconds.

BF group (p_{adj} =0.017). Taken together, these results suggest a greater volume of parietal regions and higher cortical thickness in EF children aged 6 years than SF children (**Figure 2**, light blue), BF children (**Figure 2**, dark blue), or both (**Figure 2**, purple).

Associations Between Brain Structure and Neurocognitive Function in Children Aged 6 Years

Afterwards, we inquired whether there might be potential associations between child neurocognitive function at 6 years and brain structure (volume brain and cortical thicknesses), particularly in those regions in which significant differences in previous adjusted model analyses were found. In this regard, Pearson correlation analyses revealed that greater volume in the right parietal and right precuneus was positively associated with better verbal comprehension ($r = 0.267$, $p = 0.019$ and $r = 0.278$, $p = 0.014$, respectively), while better working memory was only positively related to higher right parietal volume ($r = 0.257$, $p = 0.024$; **Table 4**). In this line, our previous analysis showed that children fed with EF or BF presented greater volume in these regions (**Table 3**) and more successes, but without statistically significant differences in verbal comprehension and working memory in the BENCI Neuropsychological battery (**Table 2**). Furthermore, cortical thickness in the left occipito-temporal sulcus was positively correlated to better working memory ($r = 0.245$, $p = 0.032$; **Table 4**). It is important to note that children who were fed with EF showed significantly greater cortical thickness in this region (**Table 3**) and better scores in the abovementioned neuropsychological test (**Table 2**), although no statistically significant differences were found.

Infant's LC-PUFAs Status During the First 18 Months

To gain additional insight into the effects of early nutrition on brain structure, we analyzed FA levels of buccal cheek cell phospholipids in the COGNIS children at 6, 12, and 18 months of life, depending on their type of early feeding (**Table 5**). At 6 months of life, BF infants presented higher concentrations of ARA ($p < 0.001$), adrenic acid (AdA) ($p < 0.001$), and DHA ($p < 0.001$) in comparison to formula-fed infants. Moreover, as expected, both ARA and DHA concentrations were also increased in EF infants compared to SF infants. Nevertheless, we found higher concentrations of eicosapentaenoic acid (EPA) in EF infants than in SF infants ($p < 0.001$). AdA concentrations were also higher in the BF group compared to the EF infants ($p = 0.002$) at 12 months of life, while both BF and EF infants showed higher DHA levels than SF infants ($p < 0.001$). At 18 months of life, formula-fed infants presented higher ARA and AdA levels than BF infants ($p = 0.002$ and $p < 0.001$, respectively), and DHA

TABLE 3 | Differences in brain volume and thickness between 6 years old children fed with standard infant formula (SF), experimental infant formula (EF), or breastfed (BF) during their first 18 months of life[1].

Brain region	SF ($n = 30$)	EF ($n = 27$)	BF ($n = 21$)	P^2	p_{adj}
Volume (mm^3)					
Right parietal	71,562.67 ± 7,999.80[a]	77,167.15 ± 8,496.22[b]	73,602.10 ± 7,684.33[b]	**<0.001**	**0.002**
Right postcentral gyrus	4,258.53 ± 633.89[a]	4,885.78 ± 911.37[b]	4,429.24 ± 797.78[a,b]	**0.008**	**0.015**
Right precuneus	7,144.43 ± 1,201.38[a]	7,856.89 ± 1,164.59[b]	7,283.62 ± 974.12[a,b]	**0.029**	**0.009**
Left orbital	3,071.83 ± 503.06[a,b]	3,098.48 ± 535.85[a]	2,628.38 ± 554.63[b]	**0.006**	**0.012**
Cortical thickness (mm)					
Left inferior circular insular sulcus	3.08 ± 0.17[b]	3.21 ± 0.20[a]	3.069 ± 0.17[b]	**0.010**	**0.012**
Left occipito-temporal sulcus	2.69 ± 0.15[a]	2.78 ± 0.12[b]	2.734 ± 0.15[a,b]	**0.047**	**0.027**
Right postcentral sulcus	2.46 ± 0.13[a]	2.53 ± 0.13[b]	2.423 ± 0.12[a,b]	**0.013**	**0.017**

[1]*Data are means ± SD.*
[2]*P-values for differences between the COGNIS-groups. ANOVA test for normally distributed variables. Volume analysis is corrected by total brain volume.*
P_{adj} are univariate analysis of covariance (ANCOVA) adjusted by smoking during pregnancy, maternal age, familiar socioeconomic status, age and sex of the children. Values not sharing the same suffix (a,b) were significantly different in the Bonferroni post-hoc test. $P < 0.05$ are highlighted in bold.
SF, Standard infant formula; EF, Experimental infant formula; BF, Breastfed infants.

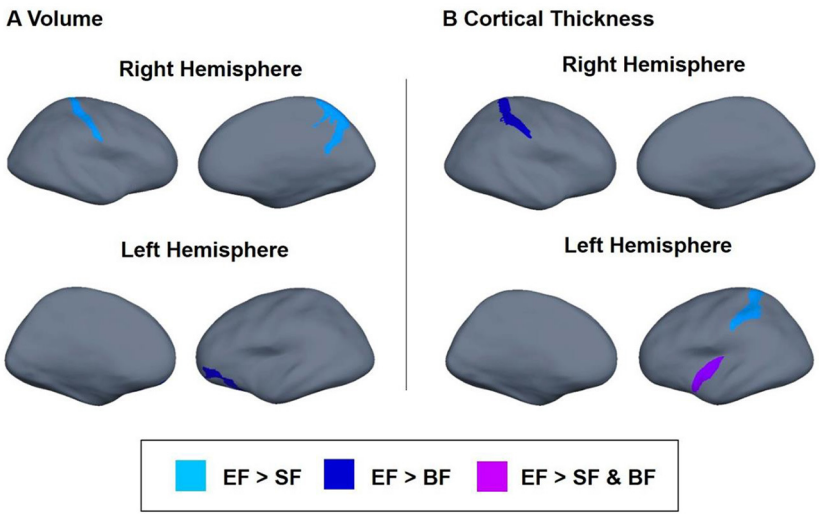


FIGURE 2 | Differences in brain volume **(A)** and cortical thickness **(B)** between children participating in the COGNIS study at 6 years of life. Analysis adjusted by smoking during pregnancy, maternal age, familiar socioeconomic status, age, and sex of the children. Brain volume analysis was also corrected for total brain volume. Experimental infant formula (EF) > Standard infant formula (SF) (light blue); EF > BF (dark blue); and EF > SF and EF > BF (purple). SF, Standard infant formula; EF, Experimental infant formula; BF, breastfed infants.

concentrations were higher in the EF group compared to the SF group ($p = 0.001$). Finally, ARA/DHA index, which reflects both endogenous synthesis and exogenous supply, were lower in BF and EF infants compared to SF infants at 6, 12, and 18 months of life ($p < 0.001$; **Table 5**).

Differences in Dietary Intake Between the Three Study Groups

Afterwards, dietary intake was analyzed during the follow-up period (up to 6 years old). Regarding the age of complementary feeding introduction, it was significantly earlier in infant formula groups (SF: 17.06 ± 2.50 weeks; EF: 17.37 ± 2.64 weeks) than in the BF group (22.75 ± 4.91 weeks) ($p < 0.001$). As shown in **Supplementary Table 1**, significant differences in dietary intake between the three groups were mainly found during the first 18 months of life. In fact, formula-fed infants at 6 months of life showed higher proteins, carbohydrates (CHO), and n-3-PUFA intake in terms of g/day, and calcium, iron, and zinc in terms of mg/day compared to BF infants (all $p < 0.001$). However, daily intake of total lipids ($p < 0.001$) and various LC-PUFAs, including α-linolenic acid (LNA), EPA, and docosapentaenoic acid (DPA) ($p < 0.001$, $p = 0.024$, and $p < 0.001$, respectively), were higher in the BF group compared to SF and EF infants. It is also important to note that ARA and DHA daily intake were different between the three groups, being higher in those infants who were breastfed and lower in those who received SF (both $p < 0.001$).

TABLE 4 | Relationship between brain structure and neurocognitive developmental scores in children aged 6 years ($n = 78$)[a].

Brain regions		Neurocognitive tasks	
		Verbal comprehension (successes)	Working memory (successes)
Volume (cm^3)			
Right parietal	*r*	0.267	0.257
	P^1	**0.019**	**0.024**
Right precuneus	*r*	0.278	0.125
	P^1	**0.014**	0.277
Cortical thickness (mm)			
Left occipito-temporal sulcus	*r*	0.048	0.245
	P^1	0.680	**0.032**

[a]*P-values for correlations between brain structure and neurocognitive development. P < 0.05 are highlighted in bold.*
r, Pearson correlation coefficient.

TABLE 5 | Influence of type of early feeding on long-chain polyunsaturated fatty acids (LC-PUFAs) concentration in infants at 6, 12, and 18 months of life[1].

FAs concentrations (%)	SF	EF	BF	p^2
6 months	**(*n* = 33)**	**(*n* = 37)**	**(*n* = 32)**	
ARA	1.75 ± 0.56^a	2.16 ± 0.63^b	2.80 ± 0.59^c	**<0.001**
AdA	0.22 ± 0.08^a	0.21 ± 0.08^a	0.29 ± 0.08^b	**<0.001**
EPA	0.10 ± 0.05^a	0.13 ± 0.06^b	$0.12 \pm 0.05^{a,b}$	**0.027**
DHA	0.35 ± 0.19^a	0.83 ± 0.31^b	1.10 ± 0.33^c	**<0.001**
ARA/DHA	5.52 ± 1.52^a	2.79 ± 0.73^b	2.72 ± 0.78^b	**<0.001**
12 months	**(*n* = 34)**	**(*n* = 37)**	**(*n* = 32)**	
ARA	2.08 ± 0.56	2.13 ± 0.56	2.36 ± 0.59	0.101
AdA	$0.24 \pm 0.05^{a,b}$	0.21 ± 0.05^a	0.27 ± 0.07^b	**0.002**
EPA	0.12 ± 0.06	0.14 ± 0.06	0.12 ± 0.05	0.144
DHA	0.48 ± 0.18^a	0.77 ± 0.25^b	0.87 ± 0.30^b	**<0.001**
ARA/DHA	4.59 ± 1.29^a	2.90 ± 0.60^b	2.86 ± 0.72^b	**<0.001**
18 months	**(*n* = 33)**	**(*n* = 34)**	**(*n* = 19)**	
ARA	2.21 ± 0.55^a	2.33 ± 0.56^a	1.79 ± 0.46^b	**0.002**
AdA	0.28 ± 0.07^a	0.27 ± 0.06^a	0.21 ± 0.05^b	**<0.001**
EPA	0.13 ± 0.06	0.16 ± 0.07	0.15 ± 0.10	0.172
DHA	0.59 ± 0.20^a	0.84 ± 0.29^b	$0.75 \pm 0.33^{a,b}$	**0.001**
ARA/DHA	4.05 ± 1.18^a	2.96 ± 0.82^b	2.70 ± 0.95^b	**<0.001**

[1]*Data are means ± SD for parametrically distributed data.* [2]*P-values for overall differences between COGNIS-groups in the ANOVA test (normally distributed variables). Values not sharing the same suffix (a,b,c) were significantly different in the Bonferroni post-hoc test. P < 0.05 are highlighted in bold. SF, Standard infant formula; EF, Experimental infant formula; BF, Breastfed infants; FAs, Fatty acids; ARA, Arachidonic acid; EPA, Eicosapentaenoic acid; AdA, Adrenic acid; DHA, Docosahexaenoic acid; ARA/DHA, Arachidonic acid/Docosahexaenoic acid ratio.*

At 12 months of life, BF infants presented higher total lipids and LNA intake than SF infants ($p = 0.001$ and $p = 0.022$, respectively). Furthermore, higher linoleic acid (LA) and $n-6$-PUFA intake were found in infants fed with EF compared to BF infants ($p = 0.024$ and $p = 0.004$, respectively). Interestingly, EF and BF infants presented similar ARA and DHA daily intake, but had higher intake than SF infants (both $p < 0.001$). Conversely, formula-fed infants showed lower daily intakes of EPA and DPA but higher n-3 PUFAs intakes compared to those who were breastfed (all $p < 0.001$). Regarding minerals, EF infants presented higher calcium daily intake than BF ones ($p = 0.016$), and both formula-fed groups showed higher intake of iron and zinc compared to BF group (all $p < 0.001$; **Supplementary Table 1**).

Our analysis also showed that participants significantly differed in daily intake of essential macronutrients at 18 months of life. In this regard, daily energy intake was higher in EF-fed infants compared with BF-fed infants ($p = 0.047$). In addition, their protein intake was higher compared to both SF and BF groups ($p = 0.001$). Regarding total lipid intake, the analysis revealed that SF infants showed lower intake than EF and BF infants ($p = 0.046$). However, carbohydrate (CHO) intake was significantly higher in both formula groups in comparison with the BF group ($p = 0.002$). Significant differences were also found for the daily intake of specific PUFAs. Overall, $n-3$-PUFA intake was higher in infants fed with EF compared with BF infants ($p = 0.021$), without differences in n-6 PUFAs between study groups. Particularly, both ARA and DHA intake remained higher in EF and BF infants compared to SF infants (both $p < 0.001$). Daily EPA intake was lower in formula-fed infants in contrast to the BF group ($p < 0.001$), and DPA intake was lower in the SF group with respect to the BF group ($p = 0.007$). Furthermore, higher daily intake of iron and zinc were found in EF infants in comparison with BF infants ($p = 0.003$ and $p = 0.001$, respectively) (**Supplementary Table 1**).

It is important to note that at 2.5 and 6 years old, no differences were found regarding dietary intake between the three groups. Nevertheless, at 4 years old, we only found differences in total energy (kcal/day) and n-3-PUFA intake being higher in the SF children than in the BF ones ($p = 0.043$ and $p = 0.022$, respectively) (**Supplementary Table 1**).

Relationships Between Fatty Acid Status During the First 18 Months of Life and Brain Structure at 6 Years Old

Using stepwise linear regression analysis, we next evaluated potential long-term influence of FA concentrations during the first 18 months of life on later brain structure (brain volume and cortical thickness) at 6 years (**Table 6**). In this regard, we found that both DHA levels and ARA/DHA index during early life were strongly correlated to later brain volume, mainly in the right parietal and right precuneus regions. In fact, our analysis showed a positive association between the right parietal volume at 6 years old and DHA concentrations presented in infants at 6, 12, and 18 months of age, while ARA/DHA index at these ages were negatively related to right parietal volume later in life (all

TABLE 6 | Potential long-term effects of fatty acids status during the first 18 months of life on children's brain structure at 6 years old[a].

	Fatty acids							
Brain regions	**6 months**				**12 months**		**18 months**	
Volume (cm^3)	**ARA**	**EPA**	**DHA**	**ARA/DHA**	**DHA**	**ARA/DHA**	**DHA**	**ARA/DHA**
Right parietal	$\beta = 0.176$ $P = \mathbf{0.021}$	N/S	$\beta = 0.330$ $P < \mathbf{0.001}$	$\beta = -0.374$ $P < \mathbf{0.001}$	$\beta = 0.341$ $P < \mathbf{0.001}$	$\beta = -0.291$ $P < \mathbf{0.001}$	$\beta = 0.295$ $P < \mathbf{0.010}$	$\beta = -0.313$ $P < \mathbf{0.001}$
Left orbital	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Right postcentral gyrus	N/S	N/S	$\beta = 0.261$ $P = \mathbf{0.021}$	$\beta = -0.344$ $P = \mathbf{0.002}$	N/S	N/S	N/S	N/S
Right precuneus	$\beta = 0.203$ $P = \mathbf{0.039}$	N/S	$\beta = 0.340$ $P = \mathbf{0.001}$	$\beta = -0.385$ $P < \mathbf{0.001}$	$\beta = 0.350$ $P = \mathbf{0.001}$	$\beta = -0.249$ $P = \mathbf{0.013}$	N/S	$\beta = -0.350$ $P = \mathbf{0.001}$
Cortical thickness (mm)								
Left circular insular sulcus	N/S	$\beta = 0.315$ $P = \mathbf{0.012}$	N/S	N/S	N/S	N/S	N/S	N/S
Left occipito–temporal sulcus	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Right postcentral sulcus	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S

[a] *P-values for linear regression analysis. P-values < 0.05 are highlighted in bold.*

ARA, Arachidonic acid; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; ARA/DHA, Arachidonic acid/Docosahexaenoic acid ratio; β, Beta coefficient; N/S, Not significance.

$p < 0.001$). A positive association was also found between later right parietal volume and ARA levels at 6 months ($p = 0.021$).

Similar associations were also found between later right precuneus volume and FA concentrations analyzed in early life. Thus, volume in this brain region exhibited a positive association with ARA levels at 6 months ($p = 0.039$) and DHA levels at 6 and 12 months (both $p = 0.001$), while ARA/DHA index at 6, 12, and 18 months showed negative association with the volume of right precuneus at 6 years old ($p < 0.001$, $p = 0.013$, and $p = 0.001$, respectively). Finally, right postcentral gyrus volume in children aged 6 years was positively related to DHA levels ($p = 0.021$), but negatively associated with ARA/DHA index ($p = 0.002$) at 6 months.

Regarding cortical thickness, our analysis showed a positive association between thickness of the left circular insula and EPA levels in cheek cell glycerophospholipids at 6 months of age ($p = 0.012$).

Long-Term Influences of Early Nutrient Intake on Children's Brain Structure at 6 Years Old

To further explore the long-term effects of early nutrition on brain structure, we also performed stepwise linear regression analyses that included brain regions which were different between study groups as dependent variable, both nutrients with which daily intake was significantly different, and several confounder variables *(maternal age, familiar socioeconomic status, smoking during pregnancy, age, and sex of the children)* as predictors. The significant associations obtained are shown in **Table 7**.

Our results showed that daily intake of certain macro- and micronutrients during early life was positively related to right brain region volume. In this line, we found that right parietal volume showed a positive association with ARA and DHA daily intake at 6 months of life ($p = 0.018$ and $p = 0.040$, respectively), and left orbital volume presented a positive association with iron intake at 6 months of life ($p = 0.007$). Likewise, right postcentral gyrus volume displayed a positive association with lipid intake, LNA, ARA, DHA, n-3-PUFAs, and calcium at 12 months of life ($p = 0.009$, $p = 0.014$, $p = 0.019$, $p = 0.015$, $p = 0.033$, and $p = 0.028$, respectively), and with protein daily intake at 18 months of life ($p = 0.013$). Furthermore, right precuneus volume was positively associated with ARA intake at 6 months of life ($p = 0.032$), DHA intake at both 12 and 18 months of life ($p = 0.018$ and $p = 0.036$, respectively), and EPA intake at 18 months of life ($p = 0.027$).

On the other hand, negative associations were found between the left orbital volume and lipids, ARA, DPA, and DHA daily intake at 6 ($p = 0.006$, $p = 0.015$, $p = 0.001$, and $p = 0.003$, respectively), and 12 months ($p = 0.001$, $p = 0.005$, $p = 0.001$, and $p = 0.012$, respectively). Daily LNA and EPA intake at 12 months of life were also negatively associated with left orbital volume ($p < 0.001$ and $p = 0.002$, respectively).

Regarding cortical thickness, our analysis revealed a positive association between left circular insular sulcus and LA and n-6-PUFA intake at 12 months of life ($p = 0.029$ and $p = 0.033$, respectively). Finally, protein and zinc intake at 18 months were positively related to cortical thickness of the right postcentral sulcus ($p = 0.011$ and $p = 0.040$).

DISCUSSION

To our knowledge, this is the first study that evaluates long-term impact of an early nutritional intervention based on an infant formula supplemented with several bioactive compounds during the first 18 months of life, on later brain structures, and related neurocognitive function in healthy children aged 6 years. Having in mind that both neurocognitive outcomes and brain structures obtained in all participants are within the normal range, our results seem to show a slightly better neurocognitive performance, particularly in terms of IQ, vocabulary (K-BIT test), and attention (BENCI test) in children fed with the supplemented infant formula compared to those who were breastfed. While long-term effects of early nutrition on neurocognitive development resulted to be lesser than expected, major changes were observed on the brain structure at 6 years old, mainly in children fed with the supplemented infant formula compared to those who received the standard one, and similar to those found in breastfed children. In fact, EF children seem to have greater volumes in the parietal and frontal regions and higher cortical thickness in the insular, parietal, and temporal regions with respect to the SF group (**Figure 2**). Interestingly, both volumes and cortical thickness in the parietal region were similar among EF and BF groups. Further correlation analyses suggested that EF-related changes in brain structure were positively associated with cognitive performance. In addition to the type of milk feeding received during the first 18 months of life, more detailed nutritional evaluation suggests that proteins, minerals and, fatty acid intake during the first 18 months of life, along with FAs concentrations in cheek cell glycerophospholipids, seem to influence later brain structure. Overall, results obtained here suggest that the intake of an infant formula supplemented with MFGM, LC-PUFAs, synbiotics (nutritional composition closer to breast milk), and key nutrients (FAs, proteins, calcium, iron, and zinc) during the first 18 months of life and the FA status in childhood may influence neurocognitive development and brain structure at least up to 6 years of age.

It is well-established that early nutrition plays a key role on optimal brain structure and function, particularly regarding optimal intake of certain nutrients (proteins, LC-PUFAs, iron, folate, among others) during sensitive periods of brain growth and development (53, 54). Although breastfeeding is the gold standard for infant nutrition during early postnatal life, infant formula intake is currently increasing in low- and high-income countries (55, 56). Consequently, great efforts have been made to enrich or supplement infant formulas with bioactive nutrients found in breast milk to narrow nutritional and functional gaps between both types of infant feeding (13). We have previously reported both short- and long-term positive effects of this infant formula supplemented with bioactive nutrients, including MFGM, LC-PUFAs, and synbiotics, on brain maturation and function assessed as visual function and language development

TABLE 7 | Long-Term influence of early dietary intake on brain structure in children aged 6 years[a].

	Brain regions					
	Volume (cm^3)				Cortical Thickness (mm)	
Macronutrients	**Right parietal**	**Left orbital**	**Right post-central gyrus**	**Right precuneus**	**Left circular Insular sulcus**	**Right post-central sulcus**
6 months						
Lipids (g/day)	N/S	β = −0.237	N/S	N/S	N/S	N/S
		***P* = 0.006**				
ARA (g/day)	β = 0.182	β = −0.222	N/S	β = 0.210	N/S	N/S
	***P* = 0.018**	***P* = 0.015**		***P* = 0.032**		
DPA (g/day)	N/S	β = −0.297	N/S	N/S	N/S	N/S
		***P* = 0.001**				
DHA (g/day)	β = 0.162	β = −0.273	N/S	N/S	N/S	N/S
	***P* = 0.040**	***P* = 0.003**				
Iron (mg/day)	N/S	β = 0.249	N/S	N/S	N/S	N/S
		***P* = 0.007**				
12 months						
Lipids (g/day)	N/S	β = −0.294	β = 0.281	N/S	N/S	N/S
		***P* = 0.001**	***P* = 0.009**			
LA (g/day)	N/S	N/S	N/S	N/S	β = 0.242	N/S
					***P* = 0.029**	
LNA (g/day)	N/S	β = −0.322	β = 0.264	N/S	N/S	N/S
		***P*<0.001**	***P* = 0.014**			
ARA (g/day)	N/S	β = −0.264	β = 0.257	N/S	N/S	N/S
		***P* = 0.005**	***P* = 0.019**			
EPA (g/day)	N/S	β = −0.303	N/S	N/S	N/S	N/S
		***P* = 0.002**				
DPA (g/day)	N/S	β = −0.300	N/S	N/S	N/S	N/S
		***P* = 0.001**				
DHA (g/day)	N/S	β = −0.242	β = 0.272	β = 0.237	N/S	N/S
		***P* = 0.012**	***P* = 0.015**	***P* = 0.018**		
n−6-PUFAs (g/day)	N/S	N/S	N/S	N/S	β = 0.236	N/S
					***P* = 0.033**	
n−3-PUFAs (g/day)	N/S	N/S	β = 0.230	N/S	N/S	N/S
			***P* = 0.033**			
Calcium (mg/day)	N/S	N/S	β = 0.236	N/S	N/S	N/S
			***P* = 0.028**			
18 months						
Protein (g/day)	N/S	N/S	β = 0.275	N/S	N/S	β = 0.330
			***P* = 0.013**			***P* = 0.011**
EPA (g/day)	N/S	N/S	N/S	β = 0.218	N/S	N/S
				***P* = 0.027**		
DHA (g/day)	N/S	N/S	N/S	β = 0.208	N/S	N/S
				***P* = 0.036**		
Zinc (mg/day)	N/S	N/S	N/S	N/S	N/S	β = 0.267
						***P* = 0.040**

[a] *P-values for linear regression analysis. P-values < 0.05 are highlighted in bold.*

CHO, Carbohydrates; LA, Linoleic acid; LNA, α-Linolenic acid; ARA, Arachidonic acid; EPA, Eicosapentaenoic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid; PUFAs, Polyunsaturated fatty acids; n−6, Omega-6; n−3, Omega-3; β*, Beta coefficient; N/S, Not significance.*

at 18 months of life and 4 years, respectively (27, 57). In addition to these benefits, the current study also suggests that supplemented infant formula seems to have a beneficial impact on child neurocognitive development at 6 years old. In fact, children who received this type of infant formula during their first 18 months of life showed higher scores in IQ, vocabulary, and attention than those who were breastfed, although values obtained were within the normal range in both cases (29, 42). This finding should be doubly discussed. First, better neurocognitive scores obtained in EF children might be partially due to the supplementation of infant formula with bioactive compounds. In this regard, according to the European Food Safety Authority (EFSA) recommendations, our experimental infant formula was supplemented with DHA and ARA (58), which are not only essential for neurogenesis, neuronal migration, and synaptogenesis processes (59), but also have a promising beneficial role on cognitive function and visual acuity (60–63), particularly with higher proportion of DHA and ARA, as well as longer supplementation duration (64). Furthermore, recent studies support the need to supplement infant formulas with DHA together with ARA to achieve optimal plasma concentrations of both fatty acids (14, 65). Moreover, infant formula tested in the COGNIS study was also supplemented with other bioactive nutrients which have separately shown positive impact on neurodevelopment and cognitive function, including MFGM (20–22, 66) and synbiotics (23, 24, 57). However, keeping in mind that each stage of brain development can be affected by different nutrients (67), we cannot determine whether the effects on brain and cognitive function reported here are only related with MFGM, LC-PUFAs, synbiotics or other bioactive compounds supplementation, or, more likely, to their synergistic action.

Secondly, it is important to highlight that the mentioned bioactive compounds are also present in breast milk. Hence, the results obtained here should be considered under controversial relationship between breastfeeding and neurocognitive development at later ages. In fact, although several studies have reported positive effects on neurocognitive and brain development (68–71), others failed to demonstrate long-term effects of breastfeeding on neurocognitive functions (72). This is particularly relevant for child IQ and other cognitive/neurological soft outcomes later in life, on which breastfeeding has little or no positive impact after adjustment for confounding factors including maternal IQ, socioeconomic characteristics, environmental factors (school or parental stimulation), genetic background, and nutritional constituents of breastfeeding (73–77). In the current study, although important potential confounder factors have been considered, we have not been able to control all related factors of social stimulation or nutritional status of the exclusively breastfed infants, including maternal nutritional status, maternal-infant interactions or other physiologic, genetic, or environmental factors that may influence bioavailability, along with status and capacity of LC-PUFAs and other bioactive components that are transferred via breast milk (78–80). Consequently, further studies should be carried out to clarify the role of breastfeeding and the new infant formulas on later cognitive performance, while always keeping in mind that child cognitive development is influenced by a complex mix of genetic and environmental factors, and, probably, by gut microbiota composition and function, as is being recently communicated by different studies (81, 82). However, despite the ideas mentioned above, our findings should not detract from current breastfeeding recommendations (83) because long-term benefits of breastfeeding for both mother and child integral development remain unmatched.

More advanced MRI techniques may offer greater insights about the role of early nutrition on later cognitive performance, identifying both those brain regions involved in neurocognitive development (84) and structural and functional effects of certain nutrients on brain development. In fact, rather than sole nutrient composition, better brain development seems to be associated with whole formula composition, achieving better results in those supplemented or enriched with different bioactive and/or essential nutrients such as LC-PUFA, iron, choline, sphingomyelin, and folic acid (85). In this line, also in accordance with above mentioned neurocognitive effects, our results suggest that early intake of MFGM, LC-PUFAs, and synbiotics through a supplemented infant formula seems to be related to in-depth changes in brain structure development compared to children fed with standard infant formula. Thus, having in mind once more that values obtained are within normal range, EF children showed higher volumes than SF children in different brain regions localized in the parietal lobe which is closely related to attentional and perceptual processes and linguistic functions (86, 87). Additionally, significant differences were also observed in cortical thickness between both formula groups, obtaining higher values in EF children in the inferior circular insula (associated with socio-emotional development) (88, 89), in the occipito-temporal sulcus (related to lexically capacities) (90, 91), and in the postcentral sulcus (related to sensorimotor functional organization) (92). Interestingly, minor differences in both brain volume and cortical thickness were found between EF and BF children, although the latter presented lower volume in the left orbital region which is associated with emotion, attention, inhibition, and memory processes (93–96). Further analysis of our data seems to suggest that aforementioned brain structural differences are related to child neurodevelopment. As a matter of fact, greater brain volumes were correlated to better brain function in terms of executive function and language development. According to Pietschnig et al. larger brain volume has been linked to better cognitive performance across different ages (97). This brain growth has been attributed to axonal density, myelination, and/or increases of fiber diameter (98). In this line, our findings might suggest an accelerated brain development in children fed with the new EF compared to those fed a SF, although further long-term studies are needed to confirm these results.

In addition, to better understand the neurobiological basis of child neurodevelopment, findings obtained so far also seem to support the potential impact of early and later nutrition on neurodevelopment. Overall, after adjusting for confounder variables, our results showed that brain structure later in life might be associated with FA status and protein, mineral, and fatty acid intake during the first 18 months of life. Regarding

FAs, among other nutrients, it is well-established that brain myelination process requires both optimal LC-PUFA intake and tissue concentrations (99). In addition, infant brain and subsequent cognitive development is partly affected by the myelination of neural networks (100, 101). In order to determine this interaction, both FA intake analysis and FA profile present in cheek cells were performed. In this line, different studies have observed that dietary fat intake is reflected in the fatty acid composition of the brain and some region-specific differences (102). Furthermore, previous studies have found cognitive differences in children with LC-PUFA supplementation and pointed out a long-term beneficial effect of early life DHA in equilibrium with ARA supplementation, specifically in the attention and inhibition systems and structural, functional, and neurochemical neuroimaging 8 years after supplementation ended (17). Furthermore, during their first 18 months of life, EF infants presented higher DHA, AdA, and ARA concentrations in cheek cell glycerophospholipids and FA intake than SF infants, but no significant differences with respect to the BF group were found. Although it is known that fat quality, rather than its total amount, plays a key role on infants' long-term health outcomes (80), findings obtained in the present study reflect negative associations between the left orbital region volume and FA intake. In fact, BF infants showed higher FA intake and lower volume in this region, along with worse execution in the attention task (more errors of commission in a performance continuous attention task). This association should be interpreted with caution. Since BF is the gold standard, the smaller left orbital region may indicate a different growth pattern of the brain (slower growth) in these children. However, this finding might also suggest potential influence of other factors on brain structure and function, apart from other bioactive components present only in human milk, including physical activity, sleep patterns, or scholar and family routines, that are worthy of further study (103). Unfortunately, we cannot confirm a long-term positive role of BF in the present study. Nevertheless, it seems that BF promotes a better LC-PUFA status and is able to determine a specific pattern of brain development different from that found in formula-fed children, even in those EF with similar LC-PUFA status.

Dietary intake analysis also showed that BF infants presented lower mineral intake (calcium, iron, and zinc) up to 18 months of life compared to formula-fed infants. Likewise, we found positive associations between the abovementioned mineral intake and the left orbital and right postcentral volumes and right postcentral sulcus cortical thickness. Due to their key role on brain development (104), we hypothesized that neurocognitive and brain structure outcomes obtained in BF children aged 6 years might be partly influenced by lower intakes of iron, calcium, and zinc. In fact, iron is essential for neurogenesis processes, neurotransmitter synthesis, brain growth, and dendrite density. Consequently, its deficiency during childhood has been associated with short- and long-term deficiencies in cognitive, motor, socio-emotional, and behavioral development (105, 106). Iron is present in small amounts in human milk (0.03 mg/100 ml) (107) although it has greater bioavailability in relation to infant formulas (108). However, exclusive breastfeeding maintained after 6 months of age is associated with increased risk of iron deficiency. If complementary feeding does not meet this need, iron supplementation is required in some cases (105, 109). Regarding infant formulas, the amount of iron contained in them is sufficient to meet the requirements (58, 110). Additionally, calcium participates in the production of neurons and glial cells (111). Its content in human milk is 32 mg/100 ml, while its content in infant formulas varies between 33.5 and 93.8 mg/100 ml (112), which could explain the lower content in breastfed infants compared to formula-fed ones. Finally, zinc is essential for child growth and development (113). Its supplementation improves motor development and cognitive performance, especially reasoning capacity (114, 115). Its content in human milk is 0.17 mg/100 ml, while its content in infant formulas ranges from 0.34 to 1.01 mg/100 ml (112).

The main strength of this study is its design as a prospective, randomized, double-blind longitudinal study. The COGNIS study is the second study in the world in healthy term infants that includes a long-term follow-up (until 6 years of age) and neuroimaging examination. In the current study, we aimed to demonstrate the long-term effects of early nutrition on brain structure and its consequences on neurodevelopment. To achieve this aim, cognitive function was evaluated using wide range of valid, reliable, and age-appropriate tests focused on diverse child brain domains (43, 44) in contrast with other studies based on evaluation of a single brain domain. Interestingly, our nutritional intervention was performed from 0 to 2 months and prolonged up to 18 months of life, which yields results with added value with respect to other studies with a shorter time of intervention. The experimental infant formula was supplemented with a set of functional nutrients (including MFGM, LC-PUFAs, and synbiotics), thereby providing added value compared to other studies aiming to demonstrate the effect of a single bioactive nutrient. Moreover, it is well-known that neurodevelopment and brain structure in children are influenced by several environmental factors such as nutrition, gender, maternal education, or socioeconomic status, among others (116–119). Thereby, although it is difficult to control all factors involved in child neurodevelopment, several confounding factors previously pointed out (50–52) were taken into account in the statistical analysis performed in the present study in order to obtain consistent results and conclusions.

Nonetheless, the current study has a series of limitations that must be considered. At the beginning of the study, there were no differences between infant formula groups regarding baseline characteristics (27). However, there were differences in parents' age, educational level, and socioeconomic status in the BF group with respect to infant formula groups. The present study has taken those differences into account to carry out the statistical analysis, given that it is well-known that those confounding factors may influence neurodevelopment. On the other side, our study groups are relatively small, not only because of the drop-outs along the long-term follow-up, but also because MRI scanning requires children to remain still. Thus, these results should be interpreted with caution. Moreover, in our study, an individual analysis of each infant's breast milk composition

during follow-up has not been performed, and the nutrient intake in the BF infants was estimated based on a complete mature human milk composition reported in the United States Department of Agriculture (USDA) National Nutrient Database for standard reference (107). Furthermore, $p < 0.05$ was used as the statistical threshold, but in the structural analysis, corrections for multiple comparisons were not applied to multiple tests. The current work is an exploratory study, and hence, future studies will be necessary to confirm or refute these results using larger sample size.

In conclusion, our findings suggest that MFGM component-, LC-PUFA-, and synbiotics-supplemented infant formula might be associated to beneficial long-term effects on neurocognitive development and brain structure in terms of brain volumes and cortical thickness in children aged 6 years. These results bring us closer to understand the effects of an adequate nutrition during the first years of life on later brain development and its neuropsychological effects. Analysis of brain structure could provide new knowledge about neural structure underlying neurocognitive function and origins and progression of brain and mental disorders. Therefore, the present study would open future opportunities to develop prevention strategies against brain and mental disorders based on ensuring adequate and individualized nutrition during the first 18 months of life.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Bioethical Committee from the University of Granada (Spain), as well as the Bioethical Committees for Clinical Research from San Cecilio University Clinical Hospital and University Mother-Infant Hospital of Granada (Spain). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CC is the principal investigator, responsible for the design and coordination of the research, obtained the funding, the guarantor of this work and as such, had full access to all the data in the study and take responsibility for the integrity of the data, accuracy of the data analysis, and the final content of the manuscript. AN-R, ED, and NS-V recruited children and collected clinical and nutritional data. JV-R, AC, MM, and AN-R conducted the statistical analyses and discussed the results. AN-R and JG-S wrote the first draft of the manuscript. All authors edited, reviewed, and approved the manuscript.

FUNDING

This project has been funded by Laboratorios Ordesa, S.L. Contract University of Granada General Foundation, No. 3349 and SMARTFOODS (CIEN) Contract University of Granada General Foundation, No. 4003, Spanish Ministry of Economy, Industry and Competitiveness. Furthermore, the project has been partially funded by HORIZON 2020 EU DynaHEALTH Project (GA No. 633595).

ACKNOWLEDGMENTS

The authors want to acknowledge the parents and children who participated in the study, the pediatricians and technicians of the EURISTIKOS team at the Department of Pediatrics, School of Medicine, University of Granada, Spain, the technicians of the RMN, Jose and Félix, at the CIMCYC, University of Granada, Spain, and the Laboratorios Ordesa, S.L. Barcelona, Spain for providing the infant formulas.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.820224/full#supplementary-material

REFERENCES

1. Knickmeyer RC, Gouttard S, Kang C, Evans D, Wilber K, Smith JK, et al. A structural MRI study of human brain development from birth to 2 years. *J Neurosci.* (2008) 28:12176–82. doi: 10.1523/JNEUROSCI.3479-08.2008
2. Prado EL, Dewey KG. Nutrition and brain development in early life. *Nutr Rev.* (2014) 72:267–84. doi: 10.1111/nure.12102
3. Lin W, Baluyot KR, Yao M, Yan J, Wang L, Li G, et al. Early-Life nutrition and cognitive development: imaging approaches. *Nestle Nutr Inst Workshop Ser.* (2019) 90:121–35. doi: 10.1159/000490300
4. Chen Y, Baram TZ. Toward understanding how early-life stress reprograms cognitive and emotional brain networks. *Neuropsychopharmacology.* (2016) 41:197–206. doi: 10.1038/npp.2015.181
5. Agyei SB, van der Weel FRR, van der Meer ALH. Development of visual motion perception for prospective control: brain and behavioral studies in infants. *Front Psychol.* (2016) 7:100. doi: 10.3389/fpsyg.2016.00100
6. Rosales FJ, Reznick JS, Zeisel SH. Understanding the role of nutrition in the brain and behavioral development of toddlers and preschool children: identifying and addressing methodological barriers. *Nutr Neurosci.* (2009) 12:190–202. doi: 10.1179/147683009X423454
7. Belfort MB, Anderson PJ, Nowak VA, Lee KJ, Molesworth C, Thompson DK, et al. Breast Milk feeding, brain development, and neurocognitive outcomes: a 7-year longitudinal study in infants born at less than 30 weeks' gestation. *J Pediatr.* (2016) 177:133–9.e1. doi: 10.1016/j.jpeds.2016.06.045
8. Fedorenko E. The role of domain-general cognitive control in language comprehension. *Front Psychol.* (2014) 5:335. doi: 10.3389/fpsyg.2014.00335
9. Rochat TJ, Houle B, Stein A, Coovadia H, Coutsoudis A, Desmond C, et al. Exclusive breastfeeding and cognition, executive function, and behavioural disorders in primary school-aged children in rural South Africa: a cohort analysis. *PLoS Med.* (2016) 13:e1002044. doi: 10.1371/journal.pmed.1002044
10. Ou X, Andres A, Pivik RT, Cleves MA, Snow JH, Ding Z, et al. Voxel-Based morphometry and fMRI revealed differences in brain gray matter in

breastfed and milk formula-fed children. *AJNR Am J Neuroradiol.* (2016) 37:713–719. doi: 10.3174/ajnr.A4593
11. Bauer CE, Lewis JW, Brefczynski-Lewis J, Frum C, Schade MM, Haut MW, et al. Breastfeeding duration is associated with regional, but not global, differences in white matter tracts. *Brain Sci.* (2019) 10:19. doi: 10.3390/brainsci10010019
12. Bardanzellu F, Peroni DG, Fanos V. human breast milk: bioactive components, from stem cells to health outcomes. *Curr Nutr Rep*. (2020) 9:1–13. doi: 10.1007/s13668-020-00303-7
13. Kaneko KJ, Fasano J, Choudhuri S. Bioactive ingredients in infant formula: the value for a robust interdisciplinary discussion on safety assessment paradigm. *J Pediatr.* (2020) 216:250–1. doi: 10.1016/j.jpeds.2019.08.035
14. Koletzko B, Bergmann K, Brenna JT, Calder PC, Campoy C, Clandinin MT, et al. Should formula for infants provide arachidonic acid along with DHA? *A* position paper of the European academy of paediatrics and the child health foundation. *Am J Clin Nutr.* (2020) 111:10–6. doi: 10.1093/ajcn/nqz252
15. Colombo J, Carlson SE, Cheatham CL, Shaddy DJ, Kerling EH, Thodosoff JM, et al. Long-term effects of LCPUFA supplementation on childhood cognitive outcomes. *Am J Clin Nutr.* (2013) 98:403–12. doi: 10.3945/ajcn.112.040766
16. Willatts P, Forsyth S, Agostoni C, Casaer P, Riva E, Boehm G. Effects of long-chain PUFA supplementation in infant formula on cognitive function in later childhood. *Am J Clin Nutr.* (2013) 98:536S−42S. doi: 10.3945/ajcn.112.038612
17. Lepping RJ, Honea RA, Martin LE, Liao K, Choi I-Y, Lee P, et al. Long-chain polyunsaturated fatty acid supplementation in the first year of life affects brain function, structure, and metabolism at age nine years. *Dev Psychobiol.* (2019) 61:5–16. doi: 10.1002/dev.21780
18. Ryan AS, Astwood JD, Gautier S, Kuratko CN, Nelson EB, Salem N. Effects of long-chain polyunsaturated fatty acid supplementation on neurodevelopment in childhood: a review of human studies. *Prostaglandins Leukot Essent Fatty Acids.* (2010) 82:305–14. doi: 10.1016/j.plefa.2010.02.007
19. Rosenfeld E, Beyerlein A, Hadders-Algra M, Kennedy K, Singhal A, Fewtrell M, et al. IPD meta-analysis shows no effect of LC-PUFA supplementation on infant growth at 18 months. *Acta Paediatr.* (2009) 98:91–7. doi: 10.1111/j.1651-2227.2008.00988.x
20. Hernell O, Domellof M, Grip T, Lonnerdal B, Timby N. Physiological effects of feeding infants and young children formula supplemented with milk fat globule membranes. *Nestle Nutr Inst Workshop Ser.* (2019) 90:35–42. doi: 10.1159/000490291
21. Timby N, Domellöf E, Hernell O, Lönnerdal B, Domellöf M. Neurodevelopment, nutrition, and growth until 12 mo of age in infants fed a low-energy, low-protein formula supplemented with bovine milk fat globule membranes: a randomized controlled trial. *Am J Clin Nutr.* (2014) 99:860–8. doi: 10.3945/ajcn.113.064295
22. Timby N, Domellof M, Lonnerdal B, Hernell O. Supplementation of infant formula with bovine milk fat globule membranes. *Adv Nutr.* (2017) 8:351–5. doi: 10.3945/an.116.014142
23. Tognini P. Gut microbiota: a potential regulator of neurodevelopment. *Front Cell Neurosci.* (2017) 11:25. doi: 10.3389/fncel.2017.00025
24. Carlson AL, Xia K, Azcarate-Peril MA, Goldman BD, Ahn M, Styner MA, et al. Infant gut microbiome associated with cognitive development. *Biol Psychiatry.* (2018) 83:148–59. doi: 10.1016/j.biopsych.2017.06.021
25. The World Medical Association Inc. Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. *Ferney-Voltaire.* (2008) 1–5. Available online at: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/
26. Association WM. World medical association declaration of helsinki: ethical principles for medical research involving human subjects. *JAMA.* (2013) 310:2191–4. doi: 10.1001/jama.2013.281053
27. Nieto-Ruiz A, García-Santos JA, Bermúdez MG, Herrmann F, Diéguez E, Sepúlveda-Valbuena N, et al. Cortical visual evoked potentials and growth in infants fed with bioactive compounds-enriched infant formula: results from COGNIS randomized clinical trial. *Nutrients.* (2019) 11:2456. doi: 10.3390/nu11102456
28. Nieto-Ruiz A, Diéguez E, Sepúlveda-Valbuena N, Herrmann F, Cerdó T, López-Torrecillas F, et al. The effects of an infant formula enriched with milk fat globule membrane, long-chain polyunsaturated fatty acids and synbiotics on child behavior up to 2.5 years old: the COGNIS study. *Nutrition.* (2020) 12:3825. doi: 10.3390/nu12123825
29. Kaufman AS, Kaufman NL. *Kauman Brief Intelligence Test [Test breve de Inteligencia de Kaufman]* (2000).
30. Aguinaga G, Armentia ML, Fraile A, Olangua, P, Uriz N. *PLON-R : Prueba de Lenguaje Oral Navarra - Revisada : Manual. 2nd ed*. Madrid: TEA Ediciones (2005).
31. Cruz-Quintana F, Pérez-García M, Roldán-vilchez LM, Fernández-López A, Marfil MNP. *BENCI: Batería de Evaluación Neuropsicológica Infantil*. Siger (2013).
32. Garcia-Esteve L, Ascaso C, Ojuel J, Navarro P. Validation of the edinburgh postnatal depression scale (EPDS) in Spanish mothers. *J Affect Disord.* (2003) 75:71–6. doi: 10.1016/S0165-0327(02)00020-4
33. Cattell RB. Theory of fluid and crystallized intelligence: a critical experiment. *J Educ Psychol.* (1963) 54:1–22. doi: 10.1037/h0046743
34. Cattell RB, Cattell AKS. *g Factor Test. Scale 2. Form A* (1994).
35. Salas Lorenzo I, Chisaguano Tonato MA, de la Garza Puentes A, Nieto A, Herrmann F, Dieguez E, et al. The effect of an infant formula supplemented with aa and dha on fatty acid levels of infants with different FADs genotypes: the COGNIS Study. *Nutrition.* (2019) 11:602. doi: 10.3390/nu11030602
36. Hoffman DR, Birch EE, Birch DG, Uauy R. Fatty acid profile of buccal cheek cell phospholipids as an index for dietary intake of docosahexaenoic acid in preterm infants. *Lipids.* (1999) 34:337–42. doi: 10.1007/s11745-999-0371-y
37. Connor SL, Zhu N, Anderson GJ, Hamill D, Jaffe E, Carlson J, et al. Cheek cell phospholipids in human infants: a marker of docosahexaenoic and arachidonic acids in the diet, plasma, and red blood cells. *Am J Clin Nutr.* (2000) 71:21–7. doi: 10.1093/ajcn/71.1.21
38. De La Garza Puentes A, Montes Goyanes R, Chisaguano Tonato AM, Castellote AI, Moreno-Torres R, Campoy Folgoso C, et al. Evaluation of less invasive methods to assess fatty acids from phospholipid fraction: cheek cell and capillary blood sampling. *Int J Food Sci Nutr.* (2015) 66:936–42. doi: 10.3109/09637486.2015.1102872
39. Worls Health Organization. *Preparation and Use of Food-Based Dietary Guidelines*. WHO (1998).
40. Alce Ingenieria. *Rograma Dial De Cálculos Nutricionales*. Available online at: http://www.alceingenieria.net/nutricion.htm (accessed January 9, 2018).
41. Sepúlveda-Valbuena N, Nieto-Ruiz A, Diéguez E, Herrmann F, Escudero-Marín M, De-Castellar R, et al. Growth patterns and breast milk/infant formula energetic efficiency in healthy infants up to 18 months of life: the COGNIS study. *Br J Nutr.* (2021) 126:1809–22. doi: 10.1017/S000711452100057X
42. Kaufman A. Manual for the kaufman brief test of intelligence. *Circ Pines Am Guid Serv*. (2004)
43. Escribano J, Luque V, Canals-Sans J, Ferré N, Koletzko B, Grote V, et al. Mental performance in 8-year-old children fed reduced protein content formula during the 1st year of life: safety analysis of a randomised clinical trial. *Br J Nutr.* (2016) 122:S22–30. doi: 10.1017/S0007114515000768
44. Pérez-García M, Luna JD, Torres-Espínola FJ, Martínez-Zaldívar C, Anjos T, Steenweg-de Graaff J, et al. Cultural effects on neurodevelopmental testing in children from six European countries: an analysis of NUTRIMENTHE global database. *Br J Nutr.* (2019) 122:S59–67. doi: 10.1017/S0007114517000824
45. Fasfous A, Peralta-Ramírez M, Pérez-Marfil MN, Cruz-Quintana F, Catena A, Perez-Garcia M. Reliability and validity of the Arabic version of the computerized battery for neuropsychological evaluation of children (BENCI). *Child Neuropsychol.* (2014) 21:210–24. doi: 10.1080/09297049.2014.896330
46. Dale AM, Fischl B, Sereno MI. Cortical surface-based analysis. I. Segmentation and surface reconstruction. *Neuroimage.* (1999) 9:179–94. doi: 10.1006/nimg.1998.0395
47. Destrieux C, Fischl B, Dale A, Halgren E. Automatic parcellation of human cortical gyri and sulci using standard anatomical nomenclature. *Neuroimage.* (2010) 53:1–15. doi: 10.1016/j.neuroimage.2010.06.010
48. Fischl B, Sereno MI, Dale AM. Cortical surface-based analysis. II: Inflation, flattening, and a surface-based coordinate system. *Neuroimage.* (1999) 9:195–207. doi: 10.1006/nimg.1998.0396

49. Fischl B, Dale AM. Measuring the thickness of the human cerebral cortex from magnetic resonance images. *Proc Natl Acad Sci USA*. (2000) 97:11050–5. doi: 10.1073/pnas.200033797
50. Verdejo-Román J, Björnholm L, Muetzel RL, Torres-Espínola FJ, Lieslehto J, Jaddoe V, et al. Maternal prepregnancy body mass index and offspring white matter microstructure: results from three birth cohorts. *Int J Obes.* (2019) 43:1995–2006. doi: 10.1038/s41366-018-0268-x
51. Gaillard R, Welten M, Oddy WH, Beilin LJ, Mori TA, Jaddoe VW V, et al. Associations of maternal prepregnancy body mass index and gestational weight gain with cardio-metabolic risk factors in adolescent offspring: a prospective cohort study. *BJOG.* (2016) 123:207–16. doi: 10.1111/1471-0528.13700
52. Jharap V V, Santos S, Steegers EAP, Jaddoe VW V, Gaillard R. Associations of maternal obesity and excessive weight gain during pregnancy with subcutaneous fat mass in infancy. *Early Hum Dev.* (2017) 108:23–8. doi: 10.1016/j.earlhumdev.2017.03.006
53. Mattei D, Pietrobelli A. Micronutrients and brain development. *Curr Nutr Rep.* (2019) 8:99–107. doi: 10.1007/s13668-019-0268-z
54. Georgieff MK, Ramel SE, Cusick SE. Nutritional influences on brain development. *Acta Paediatr.* (2018) 107:1310–21. doi: 10.1111/apa.14287
55. Bass JL, Gartley T, Kleinman R. Outcomes from the centers for disease control and prevention 2018 breastfeeding report card: public policy implications. *J Pediatr.* (2020) 218:16–21.e1. doi: 10.1016/j.jpeds.2019.08.059
56. Victora CG, Bahl R, Barros AJD, França GVA, Horton S, Krasevec J, et al. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. *Lancet.* (2016) 387:475–90. doi: 10.1016/S0140-6736(15)01024-7
57. Nieto-Ruiz A, Diéguez E, Sepúlveda-Valbuena N, Catena E, Jiménez J, Rodríguez-Palmero M, et al. Influence of a functional nutrients-enriched infant formula on language development in healthy children at four years old. *Nutrients.* (2020) 12:535. doi: 10.3390/nu12020535
58. EFSA Panel on Dietetic Products, Nutrition and Allergies. Scientific opinion on the essential composition of infant and follow-on formulae. *EFSA J.* (2014) 12:3760. doi: 10.2903/j.efsa.2014.3760
59. Schipper L, van Dijk G, van der Beek EM. Milk lipid composition and structure: the relevance for infant brain development. *OCL.* (2020) 27. doi: 10.1051/ocl/2020001
60. Qawasmi A, Landeros-Weisenberger A, Bloch MH. Meta-analysis of LCPUFA supplementation of infant formula and visual acuity. *Pediatrics.* (2013) 131:e262–72. doi: 10.1542/peds.2012-0517
61. Lien EL, Richard C, Hoffman DR. DHA and ARA addition to infant formula: current status and future research directions. *Prostagland Leukot Essent Fatty Acids.* (2018) 128:26–40. doi: 10.1016/j.plefa.2017.09.005
62. Birch EE, Carlson SE, Hoffman DR, Fitzgerald-Gustafson KM, Fu VLN, Drover JR, et al. The DIAMOND (DHA intake and measurement of neural development) study: a double-masked, randomized controlled clinical trial of the maturation of infant visual acuity as a function of the dietary level of docosahexaenoic acid. *Am J Clin Nutr.* (2010) 91:848–59. doi: 10.3945/ajcn.2009.28557
63. Drover JR, Hoffman DR, Castaneda YS, Morale SE, Garfield S, Wheaton DH, et al. Cognitive function in 18-month-old term infants of the DIAMOND study: a randomized, controlled clinical trial with multiple dietary levels of docosahexaenoic acid. *Early Hum Dev.* (2011) 87:223–30. doi: 10.1016/j.earlhumdev.2010.12.047
64. Koletzko B, Boey CCM, Campoy C, Carlson SE, Chang N, Guillermo-Tuazon MA, et al. Current information and Asian perspectives on long-chain polyunsaturated fatty acids in pregnancy, lactation, and infancy: systematic review and practice recommendations from an early nutrition academy workshop. *Ann Nutr Metab.* (2014) 65:49–80. doi: 10.1159/000365767
65. Miklavcic JJ, Larsen BMK, Mazurak VC, Scalabrin DMF, MacDonald IM, Shoemaker GK, et al. Reduction of arachidonate is associated with increase in B-cell activation marker in infants: a randomized trial. *J Pediatr Gastroenterol Nutr.* (2017) 64:446–53. doi: 10.1097/MPG.0000000000001283
66. Hernell O, Timby N, Domellöf M, Lönnerdal B. Clinical benefits of milk fat globule membranes for infants and children. *J Pediatr.* (2016) 173 (Suppl):S60–5. doi: 10.1016/j.jpeds.2016.02.077
67. Cusick SE, Georgieff MK. The role of nutrition in brain development: the golden opportunity of the "first 1000 days". *J Pediatr.* (2016) 175:16–21. doi: 10.1016/j.jpeds.2016.05.013
68. Isaacs EB, Fischl BR, Quinn BT, Chong WK, Gadian DG, Lucas A. Impact of breast milk on intelligence quotient, brain size, and white matter development. *Pediatr Res.* (2010) 67:357–62. doi: 10.1203/PDR.0b013e3181d026da
69. Horta BL, de Sousa BA, de Mola CL. Breastfeeding and neurodevelopmental outcomes. *Curr Opin Clin Nutr Metab Care.* (2018) 21:174–8. doi: 10.1097/MCO.0000000000000453
70. Kar P, Reynolds JE, Grohs MN, Bell RC, Jarman M, Dewey D, et al. Association between breastfeeding during infancy and white matter microstructure in early childhood. *Neuroimage.* (2021) 236:118084. doi: 10.1016/j.neuroimage.2021.118084
71. Ottolini KM, Andescavage N, Keller S, Limperopoulos C. Nutrition and the developing brain: the road to optimizing early neurodevelopment: a systematic review. *Pediatr Res.* (2020) 87:194–201. doi: 10.1038/s41390-019-0508-3
72. Girard L-C, Doyle O, Tremblay RE. Breastfeeding, cognitive and noncognitive development in early childhood: a population study. *Pediatrics.* (2017) 139:e20161848. doi: 10.1542/peds.2016-1848
73. Holme A, MacArthur C, Lancashire R. The effects of breastfeeding on cognitive and neurological development of children at 9 years. *Child Care Health Dev.* (2010) 36:583–590. doi: 10.1111/j.1365-2214.2009.01068.x
74. Der G, Batty GD, Deary IJ. Effect of breast feeding on intelligence in children: prospective study, sibling pairs analysis, and meta-analysis. *BMJ.* (2006) 333:945. doi: 10.1136/bmj.38978.699583.55
75. McCann JC, Ames BN. Is docosahexaenoic acid, an n−3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals. *Am J Clin Nutr.* (2005) 82:281–95. doi: 10.1093/ajcn/82.2.281
76. Michaelsen KF, Lauritzen L, Mortensen EL. Effects of breast-feeding on cognitive function. *Adv Exp Med Biol.* (2009) 639:199–215. doi: 10.1007/978-1-4020-8749-3_15
77. Yang S, Martin RM, Oken E, Hameza M, Doniger G, Amit S, et al. Breastfeeding during infancy and neurocognitive function in adolescence: 16-year follow-up of the PROBIT cluster-randomized trial. *PLoS Med.* (2018) 15:e1002554. doi: 10.1371/journal.pmed.1002554
78. Dror DK, Allen LH. Overview of nutrients in human milk. *Adv Nutr.* (2018) 9:278S−94S. doi: 10.1093/advances/nmy022
79. Miliku K, Duan QL, Moraes TJ, Becker AB, Mandhane PJ, Turvey SE, et al. Human milk fatty acid composition is associated with dietary, genetic, sociodemographic, and environmental factors in the CHILD cohort study. *Am J Clin Nutr.* (2019) 110:1370–83. doi: 10.1093/ajcn/nqz229
80. Verduci E, Giannì ML, Vizzari G, Vizzuso S, Cerasani J, Mosca F, et al. The triad mother-breast milk-infant as predictor of future health: a narrative review. *Nutr.* (2021) 13:486. doi: 10.3390/nu13020486
81. Cowan CSM, Cryan JF. The microbiome-gut-brain axis in neurocognitive development and decline. *Mod Trends Psychiatry.* (2021) 32:12–25. doi: 10.1159/000510414
82. Cowan CSM, Dinan TG, Cryan JF. Annual research review: critical windows – the microbiota–gut–brain axis in neurocognitive development. *J Child Psychol Psychiatry.* (2020) 61:353–71. doi: 10.1111/jcpp.13156
83. WHO. *Exclusive Breastfeeding*. WHO (2017). Available online at: http://www.who.int/nutrition/topics/exclusive_breastfeeding/en/
84. Morita T, Asada M, Naito E. Contribution of neuroimaging studies to understanding development of human cognitive brain functions. *Front Hum Neurosci.* (2016) 10:464. doi: 10.3389/fnhum.2016.00464
85. Deoni S, Dean D 3rd, Joelson S, O'Regan J, Schneider N. Early nutrition influences developmental myelination and cognition in infants and young children. *Neuroimage.* (2018) 178:649–59. doi: 10.1016/j.neuroimage.2017.12.056
86. Behrmann M, Geng JJ, Shomstein S. Parietal cortex and attention. *Curr Opin Neurobiol.* (2004) 14:212–7. doi: 10.1016/j.conb.2004.03.012

87. Smirnov D, Glerean E, Lahnakoski JM, Salmi J, Jaaskelainen IP, Sams M, et al. Fronto-parietal network supports context-dependent speech comprehension. *Neuropsychologia.* (2014) 63:293–303. doi: 10.1016/j.neuropsychologia.2014.09.007
88. Uddin LQ, Nomi JS, Hébert-Seropian B, Ghaziri J, Boucher O. Structure and function of the human insula. *J Clin Neurophysiol.* (2017) 34:300–6. doi: 10.1097/WNP.0000000000000377
89. Gasquoine PG. Contributions of the insula to cognition and emotion. *Neuropsychol Rev.* (2014) 24:77–87. doi: 10.1007/s11065-014-9246-9
90. Sebastian R, Gomez Y, Leigh R, Davis C, Newhart M, Hillis AE. The roles of occipitotemporal cortex in reading, spelling, and naming. *Cogn Neuropsychol.* (2014) 31:511–28. doi: 10.1080/02643294.2014.884060
91. Mano QR, Humphries C, Desai RH, Seidenberg MS, Osmon DC, Stengel BC, et al. The role of left occipitotemporal cortex in reading: reconciling stimulus, task, and lexicality effects. *Cereb Cortex.* (2013) 23:988–1001. doi: 10.1093/cercor/bhs093
92. Zlatkina V, Amiez C, Petrides M. The postcentral sulcal complex and the transverse postcentral sulcus and their relation to sensorimotor functional organization. *Eur J Neurosci.* (2016) 43:1268–83. doi: 10.1111/ejn.13049
93. Bechara A, Damasio H, Damasio AR. Emotion, decision making and the orbitofrontal cortex. *Cereb Cortex.* (2000) 10:295–307. doi: 10.1093/cercor/10.3.295
94. Hesslinger B, Van Elst LT, Thiel T, Haegele K, Hennig J, Ebert D. Frontoorbital volume reductions in adult patients with attention deficit hyperactivity disorder. *Neurosci Lett.* (2002) 328:319–21. doi: 10.1016/S0304-3940(02)00554-2
95. Hartikainen KM, Ogawa KH, Knight RT. Orbitofrontal cortex biases attention to emotional events. *J Clin Exp Neuropsychol.* (2012) 34:588–97. doi: 10.1080/13803395.2012.666231
96. Kuusinen V, Cesnaite E, Peräkylä J, Ogawa KH, Hartikainen KM. Orbitofrontal lesion alters brain dynamics of emotion-attention and emotion-cognitive control interaction in humans. *Front Hum Neurosci.* (2018) 12:437. doi: 10.3389/fnhum.2018.00437
97. Pietschnig J, Penke L, Wicherts JM, Zeiler M, Voracek M. Meta-analysis of associations between human brain volume and intelligence differences: how strong are they and what do they mean? *Neurosci Biobehav Rev.* (2015) 57:411–2. doi: 10.1016/j.neubiorev.2015.09.017
98. Lebel C, Walker L, Leemans A, Phillips L, Beaulieu C. Microstructural maturation of the human brain from childhood to adulthood. *Neuroimage.* (2008) 40:1044–55. doi: 10.1016/j.neuroimage.2007.12.053
99. Paz Soldán MM, Pirko I. Biogenesis and significance of central nervous system myelin. *Semin Neurol.* (2012) 32:9–14. doi: 10.1055/s-0032-1306381
100. Dean DC 3rd, O'Muircheartaigh J, Dirks H, Travers BG, Adluru N, Alexander AL, et al. Mapping an index of the myelin g-ratio in infants using magnetic resonance imaging. *Neuroimage.* (2016) 132:225–37. doi: 10.1016/j.neuroimage.2016.02.040
101. Dai X, Hadjipantelis P, Wang J-L, Deoni SCL, Müller H-G. Longitudinal associations between white matter maturation and cognitive development across early childhood. *Hum Brain Mapp.* (2019) 40:4130–45. doi: 10.1002/hbm.24690
102. Fernandes MF, Mutch DM, Leri F. The relationship between fatty acids and different depression-related brain regions, and their potential role as biomarkers of response to antidepressants. *Nutrients.* (2017) 9:298. doi: 10.3390/nu9030298
103. Jirout J, LoCasale-Crouch J, Turnbull K, Gu Y, Cubides M, Garzione S, et al. How lifestyle factors affect cognitive and executive function and the ability to learn in children. *Nutrition.* (2019) 11:1953. doi: 10.3390/nu11081953
104. Georgieff MK. Nutrition and the developing brain: nutrient priorities and measurement. *Am J Clin Nutr.* (2007) 85:614S–620S. doi: 10.1093/ajcn/85.2.614S
105. Radlowski EC, Johnson RW. Perinatal iron deficiency and neurocognitive development. *Front Hum Neurosci.* (2013) 7:585. doi: 10.3389/fnhum.2013.00585
106. Lozoff B. Iron deficiency and child development. *Food Nutr Bull.* (2007) 28:S560–71. doi: 10.1177/15648265070284S409
107. USDA. *FoodData Central.* USDA. Available online at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/171279/nutrients
108. Lönnerdal B. Excess iron intake as a factor in growth, infections, and development of infants and young children. *Am J Clin Nutr.* (2017) 106:1681S–7S. doi: 10.3945/ajcn.117.156042
109. Baker RD, Greer FR. Diagnosis and prevention of iron deficiency and iron-deficiency anemia in infants and young children (0-3 years of age). *Pediatrics.* (2010) 126:1040–50. doi: 10.1542/peds.2010-2576
110. Hernell O, Fewtrell MS, Georgieff MK, Krebs NF, Lönnerdal B. Summary of current recommendations on iron provision and monitoring of iron status for breastfed and formula-fed infants in resource-rich and resource-constrained countries. *J Pediatr.* (2015) 167:S40–7. doi: 10.1016/j.jpeds.2015.07.020
111. Toth AB, Shum AK, Prakriya M. Regulation of neurogenesis by calcium signaling. *Cell Calcium.* (2016) 59:124–34. doi: 10.1016/j.ceca.2016.02.011
112. Koletzko B, Baker S, Cleghorn G, Neto UF, Gopalan S, Hernell O, et al. Global standard for the composition of infant formula: recommendations of an ESPGHAN coordinated international expert group. *J Pediatr Gastroenterol Nutr.* (2005) 41:584–99. doi: 10.1097/01.mpg.0000187817.38836.42
113. Anjos T, Altmäe S, Emmett P, Tiemeier H, Closa-Monasterolo R, Luque V, et al. Nutrition and neurodevelopment in children: focus on NUTRIMENTHE project. *Eur J Nutr.* (2013) 52:1825–42. doi: 10.1007/s00394-013-0560-4
114. Bhatnagar S, Taneja S. Zinc and cognitive development. *Br J Nutr.* (2001) 85:S139–45. doi: 10.1079/BJN2000306
115. Black MM. The evidence linking zinc deficiency with children's cognitive and motor functioning. *J Nutr.* (2003) 133:1473S–6S. doi: 10.1093/jn/133.5.1473S
116. Duncan AF, Matthews MA. Neurodevelopmental outcomes in early childhood. *Clin Perinatol.* (2018) 45:377–92. doi: 10.1016/j.clp.2018.05.001
117. Jednoróg K, Altarelli I, Monzalvo K, Fluss J, Dubois J, Billard C, et al. The influence of socioeconomic status on children's brain structure. *PLoS ONE.* (2012) 7:e42486. doi: 10.1371/journal.pone.0042486
118. Johnson SB, Riis JL, Noble KG. State of the art review: poverty and the developing brain. *Pediatrics.* (2016) 137:e20153075. doi: 10.1542/peds.2015-3075
119. McDermott CL, Seidlitz J, Nadig A, Liu S, Clasen LS, Blumenthal JD, et al. Longitudinally mapping childhood socioeconomic status associations with cortical and subcortical morphology. *J Neurosci.* (2019) 39:1365–73. doi: 10.1523/JNEUROSCI.1808-18.2018

Conflict of Interest: RD-C and JJ are employees of Ordesa Laboratories S.L., company that have funded in part the COGNIS project.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Nieto-Ruiz, García-Santos, Verdejo-Román, Diéguez, Sepúlveda-Valbuena, Herrmann, Cerdó, De-Castellar, Jiménez, Bermúdez, Pérez-García, Miranda, López-Sabater, Catena and Campoy. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 05 April 2022
doi: 10.3389/fnut.2022.851980

Vitamin A Status Is More Commonly Associated With Symptoms and Neurodevelopment in Boys With Autism Spectrum Disorders—A Multicenter Study in China

Ting Yang[1], Li Chen[1], Ying Dai[1], Feiyong Jia[2], Yan Hao[3], Ling Li[4], Jie Zhang[5], Lijie Wu[6], Xiaoyan Ke[7], Mingji Yi[8], Qi Hong[9], Jinjin Chen[10], Shuanfeng Fang[11], Yichao Wang[12], Qi Wang[13], Chunhua Jin[14], Jie Chen[1] and Tingyu Li[1]**

[1] Chongqing Key Laboratory of Childhood Nutrition and Health, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, National Clinical Research Center for Child Health and Disorders, Chongqing, China, [2] Department of Developmental and Behavioral Pediatrics, The First Hospital of Jilin University, Changchun, China, [3] Department of Pediatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [4] Department of Children Rehabilitation, Hainan Women and Children's Medical Center, Haikou, China, [5] Children Health Care Center, Xi'an Children's Hospital, Xi'an, China, [6] Department of Children's and Adolescent Health, Public Health College of Harbin Medical University, Harbin, China, [7] Child Mental Health Research Center of Nanjing Brain Hospital, Nanjing, China, [8] Department of Child Health Care, The Affiliated Hospital of Qingdao University, Qingdao, China, [9] Maternal and Child Health Hospital of Baoan, Shenzhen, China, [10] Department of Child Healthcare, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai, China, [11] Children's Hospital Affiliated of Zhengzhou University, Zhengzhou, China, [12] NHC Key Laboratory of Birth Defect for Research and Prevention, Hunan Provincial Maternal and Child Health Care Hospital, Changsha, China, [13] Deyang Maternity and Child Healthcare Hospital, Deyang, Sichuan, China, [14] Department of Children Health Care, Capital Institute of Pediatrics, Beijing, China

OPEN ACCESS

Edited by:
Andrew Scholey,
Swinburne University of Technology, Australia

Reviewed by:
Jed William Fahey,
Johns Hopkins Medicine, United States
Mostafa Waly,
Sultan Qaboos University, Oman
Nikolaj Travica,
Deakin University, Australia

***Correspondence:**
Jie Chen
jchen010@hospital.cqmu.edu.cn
Tingyu Li
tyli@vip.sina.com

Specialty section:
This article was submitted to Nutrition and Brain Health, a section of the journal Frontiers in Nutrition

Received: 10 January 2022
Accepted: 14 March 2022
Published: 05 April 2022

Citation:
Yang T, Chen L, Dai Y, Jia F, Hao Y, Li L, Zhang J, Wu LJ, Ke XY, Yi MJ, Hong Q, Chen JJ, Fang SF, Wang YC, Wang Q, Jin CH, Chen J and Li TY (2022) Vitamin A Status Is More Commonly Associated With Symptoms and Neurodevelopment in Boys With Autism Spectrum Disorders—A Multicenter Study in China. Front. Nutr. 9:851980. doi: 10.3389/fnut.2022.851980

Background: Autism spectrum disorder (ASD) is a neurodevelopmental disorder, and show a striking male bias in prevalence. Vitamin A (VA) is essential for brain development, and abnormalities in its metabolite retinoic acid are associated with the pathophysiology of ASD. This national multicenter study was conducted to investigate the relationship between serum VA level and core symptoms in ASD children and whether there are still sex differences.

Method: A total of 1,300 children with ASD and 1,252 typically-developing (TD) controls aged 2–7 years old from 13 cities in China were enrolled in this study. The symptoms of children with ASD were evaluated by the Autism Behavior Checklist (ABC), Social Responsiveness Scale (SRS), and Childhood autism rating scale (CARS). The neurodevelopmental level of the children was evaluated with the revised Children Neuropsychological and Behavior Scale (CNBS-R2016). The serum level of VA was measured by high-performance liquid chromatography (HPLC).

Results: The serum VA level in children with ASD was significantly lower than that in TD children, especially in boys with ASD. Furthermore, VA levels in male children with ASD were lower than those in female children with ASD. In addition, we found that serum VA level was negatively correlated the SRS, CARS and communication warming behavior of CBNS-R2016 scores in boys with ASD. In terms of developmental quotients, serum VA

level was positively associated with the general quotient, language quotient, gross motor quotient and personal-social quotient of boys with ASD, but no difference was found in girls with ASD.

Conclusions: ASD children, especially boys, have lower serum VA levels than TD children. Moreover, serum VA status is more commonly associated with clinical symptoms and neurodevelopment in boys with ASD.

Keywords: autism spectrum disorder, vitamin A, neurodevelopment, clinical symptoms, sex differences

INTRODUCTION

Autism spectrum disorder (ASD) is a developmental disability characterized by persistent impairments in social interaction and the presence of restricted, repetitive patterns of behaviors, interests, or activities (1). The overall prevalence of ASD in 8-year-old children is 23.0/1,000 (one in 44) in the United States (2), and in China it has been estimated to be 7.0/1,000 among 6–12-year-old children (3), with an increasing trend over time. As ASD is a lifelong disability, such increasing trend has brought an enormous burden on family and society and increased health care costs. There is a sex-bias in ASD children, ASD diagnosis is approximately 4.2 times more common among boys than girls (2). Accumulating evidence suggests that the clinical core symptoms of ASD children are inconsistent between different sexes. Boys with ASD were found to exhibit higher levels of repetitive and stereotyped behaviors than girls (4–7). More recently, studies have paid attention to the differences in blood biomarkers and metabolites between ASD children of different sexes (8, 9).

Vitamin A (VA) is essential for brain development and is transported in the blood as retinol and functions in various processes as its metabolite retinoic acid (RA) in tissues (10). RA plays prominent roles in mediating neuron differentiation, synaptic plasticity and tissue formation by activating gene transcription as a ligand for the transcription factor RA receptors (RARs) (11). Genetic deletions in mice revealed that RA signaling *via* the retinoic acid receptors RXRG and RARB and CYP26B1-dependent catabolism is associated with appropriate molecular patterns in the prefrontal and motor areas, and the development of the prefrontal cortex-medial dorsal thalamus connection, which are thought to be altered in ASD (12). Moreover, other studies suggest that VA can increase oxytocin (OXT) levels in ASD patients through RA-RAR signaling and the CD38-OXT signaling pathway, and brain activity and social skills in autistic patients may be significantly increased through OXT (13–15). Our previous clinical study showed that VA supplementation for 6 months effectively increased serum VA concentration and the expression levels of proteins involved in the RARβ-CD38-OXT axis, and reducing social dysfunction in children with ASD (16, 17).

Vitamin A deficiency (VAD) is still considered public health problem, particularly in some developing countries, with an estimated 19 million pregnant women and 140 million children suffering from VAD worldwide (18). VA is essential for brain and neural system developments begin at the embryonic stage. Our previous study found that the serum VA levels in ASD children were significantly lower than that in control children. Interestingly, we further observed a positive association between maternal micronutrients supplementation and serum VA levels in ASD children, low serum VA levels in ASD children were associated with significantly lower rates of multivitamin supplementation during pregnancy (19). We hypothesize that VAD in ASD children may begin at the embryonic stage. Accumulating evidence, including the sex-specific effect of biochemical parameters, indicates that the ASD-specific behavioral alterations are differentially regulated in boys and girls. However, no studies have been conducted to date to investigate the differences in serum VA levels between children of different sexes and their association with the core symptoms of ASD. Accordingly, the present study used a nationwide multicenter cross-sectional survey to determine the differences in serum VA levels between ASD children of different sexes, as well as the association between the VA status and core symptoms of ASD in children.

METHODS

Study Design and Ethical Aspects

This is a national cross-sectional clinical study of 2–7-year-old children in China conducted from May 2018 to December 2019. The study was prospectively sponsored by the Subspecialty Group of Developmental and Behavioral Pediatrics, the Society of Pediatrics, and the Chinese Medical Association, with the participation of 13 members of the China Autism Clinical Research Alliance. The study was approved by the ethics committee of the Children's Hospital of Chongqing Medical University, Approval Number: (2018) IRB (STUDY) NO.121, and registered in the Chinese Clinical Trial Registry (Registration number: ChiCTR2000031194). Participation in this research was voluntary, and parents provided informed consent forms for all participants.

Study Participants

The study population was recruited from 13 locations in five geographical regions of China, namely the North (Harbin, Qingdao, and Changchun), East (Shanghai and Nanjing), West (Chongqing, Deyang, and Xi'an), South (Shenzhen, Haikou, and Changsha), and Middle (Wuhan and Zhengzhou). The study profile is illustrated in **Figure 1**. A total of 2,552

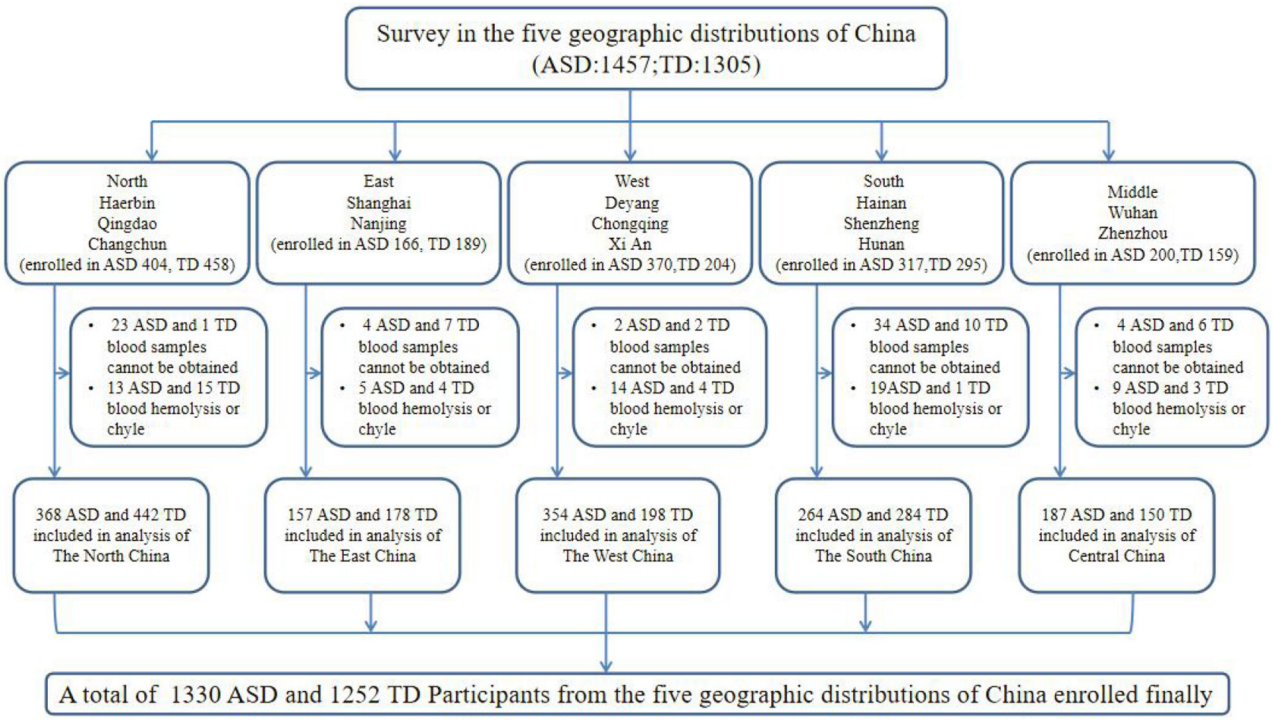


FIGURE 1 | Flow diagram illustrating the survey participants: a multi-center study in China. ASD, autism spectrum disorder; TD, typically developing.

participants (1,300 2∼7-year-old children with ASD and 1,252 typically-developing (TD) control children) were enrolled in this study. ASD children from hospital rehabilitation department or developmental behavioral pediatrics and rehabilitation training institutions were included in this study. The inclusion criteria for participants in the ASD group were the following: 1) 2–7 years old at enrollment and 2) have a diagnosis of ASD documented in their medical history [The diagnoses were conducted by an experienced psychologist or an experienced developmental pediatrician at the Children's Hospital, based on the criteria for autism defined in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)]. We also recruited participants for the TD group from online volunteers and local preschools who met the following inclusion criteria: (1) absence of ASD diagnosis and (2) no family history of ASD in first- and second-degree relatives. The exclusion criteria for participants in the ASD group were the following: (1) presence of a genetic or neurological disorder of known etiology (*e.g.*, Fragile X, Rett's syndrome), (2) significant sensory or motor impairment, (3) major physical/medical problems, (4) seizures, (5) history of a serious head injury, and (6) an acute or chronic infection in the previous 3 months. The exclusion criteria for participants in the TD group were the following: (1) significant medical or neurological conditions affecting growth, development or cognition (*e.g.*, CNS infection, seizure disorder, diabetes, tuberous sclerosis) or sensory impairments, such as significant vision or hearing loss and (2) had a history of language impairments, or social developmental disorders.

Clinical Measures of ASD Children

Information on demographics (*e.g.*, name, age, and sex), parent education level and family income were collected through face-to-face interviews with parents of the ASD and control children conducted by investigators who had received comprehensive professional training. The symptoms of the ASD children were assessed with two parent-report instruments, namely the Aberrant Behavior Checklist (ABC; in which the score of TD children should be <53) (20), the Social Responsiveness Scale (SRS; in which the score of TD children should be <65) (21), and one developmental pediatrician observation instruments, namely the Childhood Autism Rating Scale (CARS; in which the score of TD children should be <30) (22). The neurodevelopmental level of the children was evaluated with the revised Children Neuropsychological and Behavior Scale (CNBS-R2016), which has been revised and widely used in China. CNBS-R2016 includes five separate sub-scales, namely Gross Motor, Personal-Social, Language, Fine Motor, Adaptive Behavior, and an independent sub-scale (Communication Warning Behavior) was added to assess autism symptoms. A general quotient or subscale developmental quotient <70 points [<2 standard deviations (SDs)] indicates developmental delay (DD). For the sub scale of communication warning behavior, a score of <7 points indicates typical development. Follow up is required between 7 and 12 points; scores from 12 to 30 points indicate the risk of communication and interaction barriers; a score >30 indicates high suspicion of ASD. As Li et al. reported, the effectiveness of developmental assessment in children with ASD

by the CNBS-R2016 was consistent with that of the Griffiths Mental Development Scales for China (GDS-C) (23). In this study, the valid evaluation sample sizes of ABC, SRS and CARS were 1,100, 1,110, and 1,119, respectively. The valid evaluation sample size of CNBS-R2016 was 957.

Laboratory Measurements

A 5 ml aliquot of venous blood was collected from the participants by venipuncture, which was immediately centrifuged at 3,000 rpm at room temperature for 10 min to obtain the serum. The serum was then used for detecting the level of VA (retinol) by high performance liquid chromatography (HPLC) according to previously described methods (24). Briefly, serum (200 ul) was deproteinated with 200 ul dehydrated alcohol. The VA was extracted from the serum by hexane (1,000 ul). Then, 500 ul of supernatant was extracted and the hexane in it was evaporated with nitrogen gas. A mobile phase mixture (methanol: water = 97:3) was used to dissolve the VA residue. Then, the prepared sample was detected by HPLC on a Shimadzu DGU-20 As Prominence HPLC System (Shimadzu Corporation, Kyoto, Japan) at 315 nm, using a C18 column. The entire procedure was performed by the same operator in a dark room to protect the serum from light. Serum VA concentrations of >0.3, 0.2–0.3, and <0.2 mg/L were defined as VA normal (VAN), marginal VAD (MVAD), and VAD, respectively (25).

Statistical Analyses

The Kolmogorov-Smirnov goodness-of-fit test was used to test the distribution of each data set for normality before analysis. Data were analyzed using the SPSS software version 22.0 (IBM Corporation, Armonk, NY, USA). The mean and standard deviation (SD) for normal continuous variables and the frequency and percentage per category for categorical variables were used to analyze the demographic characteristics of children in each group (the TD group *vs*. ASD group). Group differences were compared by independent *t*-test of continuous variables and chi square test or Fisher's exact test of categorical variables. Univariate and multivariate (adjusted for age) logistic regression models were used to compare VA levels between the TD children and the ASD children, as well as the VA levels of TD children and ASD children of different sexes and the VA levels of ASD children of different sexes. We further compared the effect of VA on the total and subscale scores in ASD groups using the Multivariate Linear Regression Models for boys and girls separately, with age as a covariate. Odds ratio (OR) and 95% confidence interval (CI) were evaluated to determine associations. A two-sided *p*-value <0.05 is regarded as statistically significant.

RESULTS

Comparison of Demographic Characteristics Between TD and ASD Groups

As shown in **Table 1**, a total of 1,252 TD children and 1,300 children with ASD were enrolled in this study. The median interquartile range (IQR) age of the TD group was 4.39 (3.36–5.34) years, including 822 boys and 430 girls. The ASD children median IQR age was 3.97 (3.15–4.90) years, with 1,088 boys and 242 girls. There were statistically significant differences in age, sex and residence between the two groups ($P < 0.001$). The parents' educational levels in the TD group were higher than that in the ASD group ($P < 0.001$). All the Z-scores (Z_{HA}, Z_{WA}, Z_{BMIA}) showed no significant differences between TD children and ASD children.

Serum VA Levels in TD and ASD Group

To determine whether there are differences in serum VA levels in children with ASD and TD, we measured the levels of serum VA in the two groups. The results of the univariate and multivariate binary logistic regression (adjusting for age) analysis are shown in **Table 2**. The univariate binary logistic regression analysis showed that the serum VA levels in ASD children were significantly lower than that in TD children ($OR = 0.315$, 95% CI: 0.111 to 0.897, $P < 0.05$). Taking age as a covariate, the multivariate binary logistic regression analysis showed that there was still significant difference in VA level between the two groups ($OR = 0.275$, 95% CI: 0.096 to 0.789, $P < 0.05$).

Comparison of VA Levels by Group for Children of Different Sexes

We further analyzed differences in serum VA levels between the TD and ASD children in boys and girls groups using the binary logistics model. The univariate and multivariate (adjusting for age) binary logistic regression analysis showed that the VA levels in ASD children were significantly lower than that in TD children in boys (OR = 0.207, 95% CI: 0.062 to 0.693, $P < 0.05$; OR = 0.190, 95% CI:0.056 to 0.639, $P < 0.05$, respectively), but there was no difference between the two groups in girls. Additionally, VA levels of ASD children in boys were lower than those in girls (OR = 17.214, 95% CI: 2.591 to 114.375, $P < 0.05$; OR = 16.559, 95% CI: 2.471 to 110.959, $P < 0.05$, respectively) (**Table 3**).

Effect of the Serum VA Level on the Scores of ABC, SRS, CARS and Communication Warning Behavior of CBNS-R2016 in the ASD Children in Different Sex Groups

We used a linear regression model to analyze the effect of the serum VA level on the scores of ABC, SRS, CARS, and communication warning behavior of CBNS-R2016 scales, with age as a covariant. The results revealed that VA level was negatively associated with the social awareness subscale scores of SRS ($\beta = -0.065$, 95% CI: −5.478 to −0.243, $P < 0.05$), the social cognition subscale scores of SRS ($\beta = -0.066$, 95% CI: −7.356 to −0.411, $P < 0.05$), the social communication subscale scores of SRS ($\beta = -0.107$, 95% CI: −19.274 to −5.611, $P < 0.05$), the SRS total score ($\beta = -0.090$, 95% CI: −43.795 to −9.113, $P < 0.05$), the CARS total scores ($\beta = -0.126$, 95% CI: −17.000 to −6.182, $P < 0.05$), and the scores of communication warming behavior of CBNS-R2016 ($\beta = -0.080$, 95% CI: −41.721 to −5.012, $P < 0.05$). We further performed stratified analysis by sex and found that the serum VA level was negatively correlated with the SRS scores, the CARS scores and the communication warming behavior of CBNS-R2016 scores in boys, while the serum VA

TABLE 1 | Demographic characteristics of the participants in TD and ASD groups.

Variable	TD (N = 1,252)	ASD (N = 1,330)	Z/T/chi-square	P
Gender, *n* (%)				
Boys	822 (65.655)	1,088 (81.805)	$\chi^2 = 87.364$	<0.001
Girls	430 (34.345)	242 (18.195)		
Age (years), Median (IQR)	4.390 (3.360–5.340)	3.970 (3.145–4.900)	$Z = -5.789$	<0.001
Father's educational levels, *n* (%)				
Middle school or below	143 (12.181)	259 (20.394)	$\chi^2 = 45.991$	<0.001
High school	204 (17.376)	276 (21.732)		
College or above	827 (70.443)	735 (57.874)		
Mother's educational levels, *n* (%)				
Middle school or below	142 (12.014)	301 (23.516)	$\chi^2 = 106.904$	<0.001
High school	185 (15.651)	309 (24.141)		
College or above	855 (72.335)	670 (52.344)		
Residential place, *n* (%)				
Urban	1,043 (89.145)	964 (75.549)	$\chi^2 = 76.621$	<0.001
Rural	127 (10.855)	312 (24.451)		
Boys	$N = 797$	$N = 904$		
Z_{HA}, mean ± SD	0.120 ± 1.130	0.223 ± 1.167	$T = -1.844$	0.065
Z_{WA}, mean ± SD	0.322 ± 1.121	0.393 ± 1.156	$T = -1.287$	0.198
Z_{BMIA}, mean ± SD	0.365 ± 1.173	0.370 ± 1.374	$T = -0.083$	0.934
Girls	$N = 413$	$N = 198$		
Z_{HA}, mean ± SD	0.051 ± 1.148	0.228 ± 1.104	$T = -1.802$	0.072
Z_{WA}, mean ± SD	0.199 ± 1.038	0.249 ± 1.151	$T = -0.536$	0.592
Z_{BMIA}, mean ± SD	0.243 ± 1.128	0.141 ± 1.323	$T = 0.987$	0.324

ASD, autism spectrum disorder; TD, typically developing; IQR, inter-quartile range.
Z_{HA}, Z-scores for height; Z_{WA}, Z-scores for weight; Z_{BMIA}, Z-scores for BMI.
Data was shown as Median (IQR) or number (percentage). Chi-square test and Mann-Whitney U test were used in the analysis.

TABLE 2 | Comparison of the levels of serum VA between children with ASD and TD.

Group	VA Mean (SD)	Unadjusted	P	Adjusted	P
		OR (95% CI)		OR (95% CI)	
TD (N = 1,252)	0.353 (0.074)	reference	0.03	reference	0.016
ASD (N = 1,330)	0.347 (0.073)	0.315 (0.111 to 0.897)		0.275 (0.096 to 0.789)	

TD, typically developing; ASD, autism spectrum disorder; OR, odds ratio; CI, confidence interval.
Uni-variate Logistics Regression was used for unadjusted model and Binary Logistics Regression was used for adjusted model (adjusted for age).

level was only negatively associated with CARS score in girls. The differences in each subscale for both sex groups are detailed in **Table 4**.

Associations Between VA and Neurodevelopmental Levels of Children With ASD in Different Sex Groups

The results in **Table 5** showed that the effect of serum VA level was positively correlated with the general quotient ($\beta = 0.082$, 95% CI: 4.595 to 37.411, $P < 0.05$), gross motor quotient ($\beta = 0.070$, 95% CI: 2.058 to 39.480, $P < 0.05$), adaptive behavior ($\beta = 0.066$, 95% CI: 1.445 to 38.275, $P < 0.05$), language quotient ($\beta = 0.089$, 95% CI: 9.034 to 55.210, $P < 0.05$) and personal-social quotient ($\beta = 0.085$, 95% CI: 6.018 to 42.748, $P < 0.05$) in ASD children. We further analyzed the association between serum VA levels and development quotients in children of different sexes, and found that the serum VA level was positively associated with the general quotient ($\beta = 0.073$, 95% CI: 0.625 to 36.679, $P < 0.05$), gross motor quotient ($\beta = 0.079$, 95% CI: 2.599 to 43.533, $P < 0.05$), language quotient ($\beta = 0.080$, 95% CI: 3.391 to 54.324, $P < 0.05$) and personal-social quotient ($\beta = 0.078$, 95% CI: 2.009 to 42.391, $P < 0.05$) in boys with ASD. However, in girls with ASD, we only found that the VA level was positively correlated with the adaptive behavior quotient ($\beta = 0.151$, 95% CI: 3.835 to 97.569, $P < 0.05$).

DISCUSSION

In this study, we conducted a nationwide multicenter cross-sectional survey focused on the serum VA levels in ASD children

TABLE 3 | Comparison of the levels of serum VA between children with ASD and TD in different gender.

Group	VA mean(SD)	Unadjusted	*P*	Adjusted	*P*
		***OR* (95% CI)**		***OR* (95% CI)**	
Boys					
TD (*N* = 822)	0.353 (0.076)	reference	0.011	reference	0.007
ASD (*N* = 1,087)	0.344 (0.075)	0.207 (0.062 to 0.693)		0.190 (0.056 to 0.639)	
Girls					
TD (*N* = 430)	0.354 (0.071)	reference	0.294	reference	0.441
ASD (*N* = 244)	0.360 (0.069)	3.343 (0.351 to 31.794)		2.443 (0.252 to 23.665)	
Gender					
Boys with ASD (*N* = 1,087)	0.344 (0.075)	reference	0.003	reference	0.004
Girls with ASD (*N* = 244)	0.360 (0.069)	17.214 (2.591 to 114.375)		16.559 (2.471 to 110.959)	

TD, typically developing; ASD, autism spectrum disorder. OR, odds ratio; CI, confidence interval.
Uni-variate Logistics Regression was used for unadjusted model and Binary Logistics Regression was used for adjusted model (adjusted for age).

TABLE 4 | Effect of serum VA level on the scores of ABC, SRS, CARS and communication warming behavior of CBNS-R2016 in the ASD children at different gender groups.

Scale scores	Boys	*P*	Girls	*P*	Total	*P*
	β (95% CI)		**β (95% CI)**		**β (95% CI)**	
ABC scale scores	*n* = 895		*n* = 205		*n* = 1,100	
Sensory	0.040 (−1.865 to 7.589)	0.235	−0.102 (−18.562 to 3.068)	0.159	0.017 (−3.115 to 5.512)	0.586
Relating	−0.023 (−8.864 to 4.336)	0.501	−0.044 (−20.943 to 10.985)	0.539	−0.026 (−8.681 to 3.466)	0.400
Body and object use	0.048 (−1.767 to 10.949)	0.157	−0.108 (−28.857 to 3.785)	0.131	0.018 (−4.161 to 7.715)	0.557
Language	0.020 (−4.119 to 7.856)	0.540	0.008 (−14.694 to 16.438)	0.912	0.019 (−3.799 to 7.381)	0.530
Social self-help	0.011 (−3.769 to 5.205)	0.754	−0.135 (−21.288 to 0.446)	0.060	−0.015 (−5.184 to 3.094)	0.620
Total score	0.027 (−11.571 to 27.586)	0.422	−0.101 (−83.115 to 13.719)	0.159	0.004 (−16.792 to 3.094)	0.886
SRS scale scores	*n* = 912		*n* = 198		*n* = 1,110	
Social awareness	−0.078 (−6.353 to −0.566)	0.019	−0.013 (−6.782 to 5.615)	0.853	−0.065 (−5.478 to −0.243)	0.032
Social cognition	−0.057 (−7.227 to 0.485)	0.087	−0.133 (−15.714 to 0.379)	0.062	−0.066 (−7.356 to −0.411)	0.028
Social communication	−0.106 (−19.593 to −4.678)	0.001	−0.136 (−33.890 to 0.466)	0.056	−0.107 (−19.274 to −5.611)	<0.000
Social motivation	−0.058 (−8.188 to 0.496)	0.082	−0.030 (−12.326 to 8.004)	0.675	−0.049 (−7.298 to 0.670)	0.103
Autistic mannerisms	−0.045 (−8.622 to 1.557)	0.174	−0.074 (−17.647 to 5.418)	0.297	−0.050 (−8.582 to 0.688)	0.095
Total score	−0.090 (−45.386 to −7.307)	0.007	−0.109 (−75.921 to 9.442)	0.126	−0.090 (−43.795 to −9.113)	0.003
CARS scale scores	*n* = 924		*n* = 195		*n* = 1,119	
Total score	−0.106 (−15.360 to −3.738)	0.001	−0.211 (−36.867 to −7.157)	0.004	−0.126 (−17.000 to −6.182)	<0.000
Communication Warning	*n* = 785		*n* = 172		*n* = 957	
Behavior	−0.080 (−43.289 to −2.905)	0.025	−0.116 (−79.743 to 11.344)	0.140	−0.080 (−41.721 to −5.012)	0.013

β (95% CI), regression coefficient (95% confidence interval).
Multivariate Linear Regression Models were employed to analyze the association between serum VA level and scores of scales, with age as covariant.

aged 2–7 years and their relationship with the core symptoms and neurodevelopmental level of ASD children of different sexes. Most studies, to date, have used small single-center samples to investigate the VA levels in ASD children, ignoring the influence of sex. Moreover, there is no relevant report on whether there are still sex differences in VA levels of children with ASD. To the best of our knowledge, this study is the first national cross-sectional survey to focus on the role of VA in sex differences in children with ASD in China. Our results highlight the need for sex stratification to investigate the effects of VA on core symptoms and neurodevelopment in children with ASD. We found that the effects of VA on core symptoms and neurodevelopment were more significant in boys with ASD, which may provide a new idea for the study of the mechanism of VA and ASD.

VA has been shown to be critical for cognitive development and normal neurological function of ASD patients. Several studies have reported VAD-related genetic and molecular abnormalities associated with children with ASD, including reduced serum levels of RA (26, 27), beta-carotene (28), and RALDH1 (29). We previously found that among children with ASD suffering from multivitamin deficiency, the VAD rate was the highest, at up to 77.9% (30) in Chongqing,

TABLE 5 | Effect of serum VA level on the scores of CBNS-R2016 in the ASD children at different gender groups.

CNBS-R2016	Boys (n = 767)	P	Girls (n = 176)	P	Total (n = 943)	P
	β (95% CI)		β (95% CI)		β (95% CI)	
Gross motor	0.079 (2.599 to 43.533)	0.027	0.032 (−38.166 to 58.457)	0.679	0.070 (2.058 to 39.480)	0.030
Fine motor	0.024 (−12.868 to 26.136)	0.504	0.069 (−23.753 to 62.839)	0.374	0.032 (−8.870 to 26.413)	0.329
Adaptive behavior	0.048 (−6.032 to 34.234)	0.170	0.151 (3.835 to 97.569)	0.034	0.066 (1.445 to 38.275)	0.035
Language	0.080 (3.391 to 54.324)	0.026	0.141 (−4.341 to 109.497)	0.070	0.089 (9.034 to 55.210)	0.006
Personal-social	0.078 (2.009 to 42.391)	0.031	0.104 (−14.632 to 18.023)	0.179	0.085 (6.018 to 42.748)	0.009
General quotient	0.073 (0.625 to 36.679)	0.043	0.120 (−7.931 to 74.712)	0.113	0.082 (4.595 to 37.411)	0.012

β (95% CI), regression coefficient (95% confidence interval).

CNBS-2016R, Children Neuropsychological and Behavior Scale-Revision 2016.

Multivariate Linear Regression Models were employed to analyze the association between serum VA level and scores of CNBS-R2016 scale, with age as covariant.

China. These findings are consistent with the present study. Disordered or atypical eating patterns (including food refusal, diet restriction/pickiness, *etc.*) may have a causative role in serum VAD in ASD children (31). As reported (32) that serum VA levels in children with ASD were lower than those in TD children may be due to their specific dietary patterns, when ASD symptoms worsen, they may in turn aggravate abnormal eating behaviors in children with ASD, further exacerbating nutritional deficiencies (32, 33). However, such explanations do not fully explain the sex differences in VA levels in ASD children. We further performed stratified analysis by sex and found that, compared with the TD children group, the boys in the ASD children group had serum VAD, and VA levels in children with ASD were significantly lower in boys than in girls. Importantly, we previously observed a positive association between maternal micronutrient supplementation and VA levels in ASD children, and low serum VA levels in ASD children were associated with significantly lower rates of multivitamin supplementation during pregnancy, suggesting that the VAD may have started at the embryonic stage (19). For the prevention of ASD, it is necessary to balance nutrition during pregnancy and strengthen the prevention of VAD.

We also found that the serum VA level was negatively associated with the multiple subscale scores of SRS, including the SRS total score, the CARS total scores, and the scores of communication warning behavior of CBNS-R2016, which is consistent with the previous results of a small sample clinical study conducted by our research group (24). However, sex-stratified analysis was not performed in previous studies. In this study, as shown in **Tables 3**–**5**, we found that boys with ASD who had more severe clinical symptoms are more likely to have serum VAD. In addition, the serum VA level was positively correlated with the general quotient, gross motor quotient, language quotient, and personal-social quotient of boys with ASD, but no difference was found in girls. These findings indicate that there are sex differences in the effects of VA on clinical core symptoms and neurodevelopment in children with ASD. As is well known, ASD has a higher incidence (approximately 4.2:1) in males than in females (2), but the mechanism underlying the sex bias remains unknown. This led us to speculate that the effect of VA on the core symptoms and neurodevelopment of ASD children may be related to genetic and environmental factors.

VA can regulate central nervous system development through its active metabolite RA, which may regulate RORA through its RARs (34). As Valerie et al. reported, RORA may exhibit sex-related differences in gene expression in the human brain that are dependent on both brain region as well as stage of development (35). They also speculated that the involvement of RORA deficiency in the higher testosterone levels associated with increased risk for ASD. Another study reported that some children with ASD have mutations in RARα that disrupt downstream RA signaling, and fragile X mutations have also been found to impair RA signaling, as the fragile X mental retardation (FMR) protein interacts with RARα in RA-dependent homeostatic synaptic plasticity and homeostasis synaptic plasticity (36). Furthermore, RORA as a novel ASD candidate gene, which has been shown to be differentially regulated by male and female hormones, may contribute to sex bias in ASD (37). We previously found that RORA mRNA levels are significantly increased in children with ASD after the VA treatment (38). VAD caused by multiple factors during embryo or postnatal stage can lead to disturbance of RA signaling pathway. Therefore, the association between VA and ASD can be complex. As it is a cross-sectional survey, the causal relationship between VA and ASD cannot be determined. Further research is needed to confirm whether genetic or molecular abnormalities related to VA can lead to disruption of synaptic plasticity and affect the development of ASD and the sex bias. Thus, in addition to eating habits, we should also pay more attention to the sex bias of VAD, lifestyle and clinical manifestations in children with ASD and as well as to boys with ASD.

However, this study has several limitations that should be pointed out. Although this study is a national multicenter clinical survey, the causal relationship between VA and ASD cannot be determined as it is a cross-sectional survey. In addition, due to the large sample size of this study and the data collection from multiple cities, it is difficult to conduct a homogeneous dietary survey. Also, this study failed to collect data on dietary VA intake of ASD children. Moreover, although we found in this study that serum VA levels in ASD children were more significantly decreased in boys, and

VA had a more significant effect on core symptoms and neurodevelopment in ASD boys, we only found such a clinical phenomenon and further mechanism research is necessary. More basic studies are needed to clarify the possible role of VA in sex bias in ASD children. Meanwhile, prospective cohort studies are necessary to explore the relationship between VA and the development of ASD, as well as prospective randomized controlled trials to further characterize the outcome of VA treatment.

In conclusion, we found that ASD children have lower serum VA levels than TD children, and the difference is more obvious in ASD boys. Serum VA status is associated with clinical symptoms and neurodevelopmental levels of ASD children, especially in boys with ASD. Therefore, we should pay more attention to the VA status of children with ASD and timely supplement as needed, particularly in ASD boys. Additionally, research should be conducted on the mechanisms of VA in ASD and its impact on ASD sex bias.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Children's Hospital of Chongqing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TY: conducted data collection and analysis, drafted, and revised the manuscript. TYL and JC: conceived and designed the research, revised the article, and conducted general supervision. LC, YD, FYJ, YH, LJW, XYK, MJY, JZ, LL, QH, JJC, SFF, YCW, QW, and CHJ: performed data collection, and analysis and interpretation. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (Nos. 81771223, 81770526, and 31971089) from TYL and JC, the Guangzhou Key Project in Early diagnosis and treatment of autism spectrum disorders (202007030002), the Guangdong Key Project in Development of new tools for diagnosis and treatment of Autism (2018B030335001) from TYL.

ACKNOWLEDGMENTS

We thank all the families and children who participated in the survey. For data collection and collation, we would like to thank the following members of the participating units for their help: Jun-yan Feng, Qiu Li, Jiang Zhu, Wei Wei, Lin Tian, Jin-zhu Zhao, Ming-yang Zou, Zhao-chuan Yang, Liang Xu, Bo Xiao, Yun Zhang, Tao-tao Jiang, Yan-jun Zhao, and Xi-yue Xiong.

REFERENCES

1. Hyman SL, Levy SE, Myers SM, COUNCIL ON CHILDREN WITH DISABILITIES, SECTION ON DEVELOPMENTAL AND BEHAVIORAL PEDIATRICS. Identification, evaluation, and management of children with autism spectrum disorder. *Pediatrics*. (2020) 145:e20193447. doi: 10.1542/peds.2019-3447
2. Maenner MJ, Shaw KA, Bakian AV, Bilder DA, Durkin MS, Esler A, et al. Prevalence and characteristics of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, United States, 2018, morbidity and mortality weekly report. *Surveill Summ*. (2021) 70:1–16. doi: 10.15585/mmwr.ss7011a1
3. Zhou H, Xu X, Yan W, Zou X, Wu L, Luo X, et al. Prevalence of autism spectrum disorder in China: a nationwide multi-center population-based study among children aged 6 to 12 years. *Neurosci Bull*. (2020) 36:961–71. doi: 10.1007/s12264-020-00530-6
4. Beggiato A, Peyre H, Maruani A, Scheid I, Rastam M, Amsellem F, et al. Gender differences in autism spectrum disorders: divergence among specific core symptoms. *J Int Soc Autism Res*. (2017) 10:680–9. doi: 10.1002/aur.1715
5. Mandy W, Chilvers R, Chowdhury U, Salter G, Seigal A, Skuse D. Sex differences in autism spectrum disorder: evidence from a large sample of children and adolescents. *J Autism Dev Disord*. (2012) 42:1304–13. doi: 10.1007/s10803-011-1356-0
6. May T, Cornish K, Rinehart NJ. Gender profiles of behavioral attention in children with autism spectrum disorder. *J Atten Disord*. (2016) 20:627–35. doi: 10.1177/1087054712455502
7. Watkins EE, Zimmermann ZJ, Poling A. The gender of participants in published research involving people with autism spectrum disorders. *Res Autism Spec Disord*. (2014) 8:143–6. doi: 10.1016/j.rasd.2013.10.010
8. Xiong X, Liu D, He W, Sheng X, Zhou W, Xie D, et al. Identification of gender-related metabolic disturbances in autism spectrum disorders using urinary metabolomics. *Int J Biochem Cell Biol*. (2019) 115:105594. doi: 10.1016/j.biocel.2019.105594
9. Aita C, Mizoguchi Y, Yamamoto M, Seguch IY, Yatsuga C, Nishimura T, et al. Oxytocin levels and sex differences in autism spectrum disorder with severe intellectual disabilities. *Psychiatry Res*. (2019) 273:67–74. doi: 10.1016/j.psychres.2018.12.139
10. Shearer KD, Stoney PN, Morgan PJ, McCaffery PJ. A vitamin for the brain. *Trends Neurosci*. (2012) 35:733–41. doi: 10.1016/j.tins.2012.08.005
11. Blomhoff R, Green MH, Berg T, Norum KR. Transport and storage of vitamin A. *Science*. (1990) 250:399–404. doi: 10.1126/science.2218545
12. Shibata M, Pattabiraman K, Lorente-Galdos B, Andrijevic D, Kim SK, Kaur N, et al. Regulation of prefrontal patterning and connectivity by retinoic acid. *Nature*. (2021) 598:483–8. doi: 10.1038/s41586-021-03953-x
13. Riebold M, Mankuta D, Lerer E, Israel S, Zhong S, Nemanov L, et al. All-trans retinoic acid upregulates reduced CD38 transcription in lymphoblastoid cell lines from Autism spectrum disorder. *Mol Med*. (2011) 17:799-806. doi: 10.2119/molmed.2011.00080
14. Gordon I, Vander Wyk BC, Bennett RH, Cordeaux C, Lucas MV, Eilbott JA, et al. Oxytocin enhances brain function in children with autism. Proc Natl Acad Sci USA. (2013) 110:20953–8. doi: 10.1073/pnas.1312857110
15. Maes M, Anderson G, Betancort Medina SR, Seo M, Ojala JO. Integrating autism spectrum disorder pathophysiology: mitochondria, vitamin A, CD38, oxytocin, serotonin and melatonergic alterations in the placenta and gut. *Curr Pharm Des*. (2019) 25:4405–20. doi: 10.2174/1381612825666191102165459
16. Lai X, Zhang Q, Zhu J, Yang T, Guo M, Li Q, et al. A weekly vitamin A supplementary program alleviates social impairment in Chinese children with

autism spectrum disorders and vitamin A deficiency. *Eur J Clin Nutr*. (2021) 75:1118–25. doi: 10.1038/s41430-020-00827-9
17. Lai X, Wu X, Hou N, Liu S, Li Q, Yang T, et al. Vitamin A deficiency induces autistic-like behaviors in rats by regulating the RARβ-CD38-oxytocin axis in the hypothalamus. *Mol Nutr Food Res.* (2018) 62:5–15. doi: 10.1002/mnfr.201700754
18. WHO. *Global Prevalence of Vitamin A Deficiency in Populations at Risk 1995-2005. WHO Global Database on Vitamin A Deficiency*. Geneva, Switzerland: World Health Organization (2009). Available online at: http://apps.who.int/iris/bitstream/handle/10665/44110/9789241598019_eng.pdf?Sequence=1 (accessed December 2, 2017).
19. Tan M, Yang T, Zhu J, Li Q, Lai X, Li Y, et al. Maternal folic acid and micronutrient supplementation is associated with vitamin levels and symptoms in children with autism spectrum disorders. *Reprod Toxicol*. (2020) 91:109–15. doi: 10.1016/j.reprotox.2019.11.009
20. Rellini E, Tortolani D, Trillo S, Carbone S, Montecchi F. Childhood Autism Rating Scale (CARS) and Autism Behavior Checklist (ABC) correspondence and conflicts with DSM-IV criteria in diagnosis of autism. *J Autism Dev Disord*. (2004) 34:703–8. doi: 10.1007/s10803-004-5290-2
21. Cen CQ, Liang YY, Chen QR, Chen KY, Deng HZ, Chen BY, et al. Investigating the validation of the Chinese mandarin version of the social responsiveness scale in a mainland China child population. *BMC Psychiatry*. (2017) 17:51. doi: 10.1186/s12888-016-1185-y
22. Schopler E, Reichler RJ, DeVellis RF, Daly K. Toward objective classification of childhood autism: Childhood Autism Rating Scale (CARS). *J Autism Dev Disord*. (1980) 10:91–103. doi: 10.1007/BF02408436
23. Li HH, Feng JY, Wang B, Zhang Y, Wang CX, Jia FY. Comparison of the children neuropsychological and behavior scale and the Griffiths mental development scales when assessing the development of children with autism. *Psychol Res Behav Manage*. (2019) 12:973–81. doi: 10.2147/PRBM.S225904
24. Guo M, Li L, Zhang Q, Chen L, Dai Y, Liu L, et al. Vitamin and mineral status of children with autism spectrum disorder in Hainan Province of China: associations with symptoms. *Nutr Neurosci*. (2020) 23:803–810. doi: 10.1080/1028415X.2018.1558762
25. WHO. *Indicators for Assessing Vitamin A Deficiency and Their Application in Monitoring and Evaluating Intervention Programmes*. Geneva: WHO (1996).
26. Zhou W, Li S. Decreased levels of serum retinoic acid in Chinese children with autism spectrum disorder. *Psychiatry Res*. (2018) 269:469–73. doi: 10.1016/j.psychres.2018.08.091
27. Hao Z, Wu Q, Li Z, Li Y, Li Q, Lai X, et al. Maternal exposure to triclosan constitutes a yet unrecognized risk factor for autism spectrum disorders. *Cell Res*. (2019) 29:866–9. doi: 10.1038/s41422-019-0220-1
28. Avraham Y, Berry EM, Donskoy M, Ahmad WA, Vorobiev L, Albeck A, et al. Beta-carotene as a novel therapy for the treatment of "Autistic like behavior" in animal models of Autism. *Behav Brain Res.* (2019) 364:469–79. doi: 10.1016/j.bbr.2017.09.041
29. -Pavăl D, Rad F, Rusu R, Niculae A, Colosi HA, Dobrescu I, et al. Low retinal dehydrogenase 1 (RALDH1) level in prepubertal boys with autism spectrum disorder: a possible link to dopamine dysfunction?. *Clin Psychopharmacology Neurosci*. (2017) 15:229–36. doi: 10.9758/cpn.2017.15.3.229
30. Liu X, Liu J, Xiong X, Yang T, Hou N, Liang X, et al. Correlation between nutrition and symptoms: nutritional survey of children with autism spectrum disorder in Chongqing, China. *Nutrients*. (2016) 8:294–309. doi: 10.3390/nu8050294
31. Mayes SD, Zickgraf H. Atypical eating behaviors in children and adolescents with autism, ADHD, other disorders, and typical development. *Res Autism Spec Disord*. (2019) 64:76–83. doi: 10.1016/j.rasd.2019.04.002
32. Mandecka A, Regulska-Ilow B. How nutritional status, diet and dietary supplements can affect autism. A review. *Roczniki Państwowego Zakładu Higieny*. (2013) 64:1–12.
33. Peretti S, Mariano M, Mazzocchetti C, Mazza M, Pino MC, Verrotti Di Pianella A, et al. Diet: the keystone of autism spectrum disorder?. *Nutr Neurosci*. (2019) 22:825–39. doi: 10.1080/1028415X.2018.1464819
34. Stehlin-Gaon C, Willmann D, Zeyer D, Sanglier S, van Dorsselaer A, Renaud JP, et al. All-trans retinoic acid is a ligand for the orphan nuclear receptor ROR beta. *Nat Struct Biol*. (2003) 10:820–5. doi: 10.1038/nsb979
35. Hu VW, Sarachana T, Sherrard RM, Kocher KM. Investigation of sex differences in the expression of RORA and its transcriptional targets in the brain as a potential contributor to the sex bias in autism. *Mol Autism*. (2015) 6:7. doi: 10.1186/2040-2392-6-7
36. Zhang Z, Marro SG, Zhang Y, Arendt KL, Patzke C, Zhou B, et al. The fragile X mutation impairs homeostatic plasticity in human neurons by blocking synaptic retinoic acid signaling. *Sci Transl Med*. (2018) 10:452–85. doi: 10.1126/scitranslmed.aar4338
37. Sarachana T, Hu VW. Differential recruitment of coregulators to the RORA promoter adds another layer of complexity to gene (dys) regulation by sex hormones in autism. *Mol Autism*. (2013) 4:39. doi: 10.1186/2040-2392-4-39
38. J-Liu, Liu X, Xiong XQ, Yang T, Cui T, Hou NL, et al. Effect of vitamin A supplementation on gut microbiota in children with autism spectrum disorders - a pilot study. *BMC Microbiol*. (2017) 17:204. doi: 10.1186/s12866-017-1096-1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Yang, Chen, Dai, Jia, Hao, Li, Zhang, Wu, Ke, Yi, Hong, Chen, Fang, Wang, Wang, Jin, Chen and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

PERSPECTIVE
published: 28 April 2022
doi: 10.3389/fnut.2022.876112

Unhealthy Dieting During the COVID-19 Pandemic: An Opinion Regarding the Harmful Effects on Brain Health

*Iván Rentería[1], Patricia Concepción García-Suárez[1,2], José Moncada-Jiménez[3], Juan Pablo Machado-Parra[1], Barbara Moura Antunes[1], Fabio Santos Lira[4] and Alberto Jiménez-Maldonado[1]**

[1] Facultad de Deportes, Universidad Autónoma de Baja California, Ensenada, Mexico, [2] Department of Health, Sports and Exercise Sciences, University of Kansas, Lawrence, KS, United States, [3] Human Movement Sciences Research Center (CIMOHU), University of Costa Rica, San José, Costa Rica, [4] Exercise and Immunometabolism Research Group, Department of Physical Education, Paulista State University, UNESP, Presidente Prudente, São Paulo, Brazil

Since 2020, the world has been suffering from a pandemic that has affected thousands of people regardless of socio-economic conditions, forcing the population to adopt different strategies to prevent and control the advance of the disease, one of which is social distancing. Even though social distancing is a safe strategy to reduce the spread of COVID-19, it is also the cause of a rising sedentary behavior. This behavior develops an excess of fat tissue that leads to metabolic and inflammatory disruption related to chronic diseases and mental health disorders, such as anxiety, depression, and sleep issues. Furthermore, the adoption of dietary patterns involving the consumption of ultra-processed foods, higher in fats and sugars, and the reduction of fresh and healthy foods may play a role in the progress of the disease. In this perspective, we will discuss how an unhealthy diet can affect brain function and, consequently, be a risk factor for mental health diseases.

Keywords: COVID-19 pandemic, mental health, brain function, nutrition, brain

OPEN ACCESS

Edited by:
Andrew Scholey, Swinburne University of Technology, Australia

Reviewed by:
Michael W. Greene, Auburn University, United States

****Correspondence:***
Alberto Jiménez-Maldonado
jimenez.alberto86@uabc.edu.mx

Specialty section:
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

Received: *15 February 2022*
Accepted: *29 March 2022*
Published: *28 April 2022*

Citation:
Rentería I, García-Suárez PC, Moncada-Jiménez J, Machado-Parra JP, Antunes BM, Lira FS and Jiménez-Maldonado A (2022) Unhealthy Dieting During the COVID-19 Pandemic: An Opinion Regarding the Harmful Effects on Brain Health. Front. Nutr. 9:876112. doi: 10.3389/fnut.2022.876112

INTRODUCTION

The COVID-19 outbreak started in March of 2020; this human threat substantially modified the lifestyle of people around the world. Quarantine and social distancing were the two well-known initial preventive care measures imposed by governments worldwide to minimize the spread of infection of COVID-19. Because of the fast virus spread, schools were closed, national and international travel was restricted or forbidden, and other social activities, such as amateur and professional sports tournaments and musicals, were canceled. In addition, hundreds of countries kept their population in lockdown at home in isolation indefinitely to reduce the risk of transmission of the COVID-19. Although social distancing is a safe strategy to reduce the spread of the COVID-19, the lockdown increased sedentary behavior [might be defined as an energy expenditure of $\leq$1.5 metabolic equivalents of task (METs)] (1), mental and physical health problems (anxiety, depression, and others) (2, 3), and sleep and circadian rhythm disruption in the population (4, 5). The latter impacts body composition by promoting the greater intake of high energy-dense food types (5, 6). On the other hand, circadian misalignment can be achieved by alteration of the sleep and feed patterns (specially the increase in high-fat food intake), and potentially leading to cardiovascular disease (7). Furthermore, circadian clock genes trigger the onset of metabolic disorders, including metabolic syndrome (MetS) (7, 8).

The gain of excessive adipose tissue leads to local and systemic pro-inflammatory conditions, impairing glucose metabolism, and the onset of metabolic disorders (e.g., type-2 diabetes -T2D-), altering the functionality of organs and systems evenly. Moreover, the pro-inflammatory state *per se* harms the structure of brain topological integration and function. Similarly, poor diet quality, defined as the diet with a reduced variety and nutritional deficiency, does not align with international guidelines (9), represents another factor that generates dysfunctional brain activity (10). Unfortunately, during the COVID-19 lockdown, the population adopted unhealthy diet patterns from previous bad habits or eating behaviors or by emerging social conditions (e.g., reduction of income as a direct consequence of a sharp raising in the unemployment rate) (11, 12). Thereby, scientists and international organizations recommended maintaining a healthy diet focusing on strengthening the immune system and coping with the COVID-19 infection (13–15). However, as mentioned above, an unhealthy diet is a factor that negatively affects brain function. Therefore, the present perspective article briefly discusses how a current poor diet in the population during the COVID-19 lockdown might affect brain health.

ULTRA-PROCESSED FOOD-BASED DIET: A RISK FACTOR FOR BRAIN DYSFUNCTION DURING COVID-19

According to information provided by international organizations and scientists, ultra-processed foods (UPFs) have undergone excessive industrial manufacture. As a result, UPFs are deficient in dietary fiber, protein, and micronutrients, these products contain little to no whole foods, (16–18). Furthermore, UPFs are energy-dense products that contain artificial components that modify textures, flavors, and colors, producing palatable and more attractive foods (17). The UPFs are typically ready for consumption like soft drinks, sugar drinks, fatty or salty snack products, ice cream, French fries, burgers, desserts, and more products offered as a whole variety of fast foods (16, 17, 19).

The excessive consumption of UPFs is considered the primary source of non-communicable diseases (i.e., obesity, MetS, T2D, etc.) (19). In addition, during the COVID-19 lockdown, individuals have reported higher UPFs consumption in contrast to pre-pandemic times (20–24).

Currently, some studies pointed out the excess of dietary fats can promote changes in gut-microbiota and favor augmented lipopolysaccharides (LPS) extravasation to blood (25). Augmented LPS in blood lead to Toll-Like Receptor 4 (TLR-4) activation via binding the cellular membrane, stimulating pro-inflammatory signaling cascades, increasing cytokine synthesis (TNFα, interleukin -IL- 1B, IL-6, and interferon γ –IFNγ-). This constant cycle (higher dietary fat intake and blood LPS) favors the development of chronic metabolic disruptions, like insulin resistance (26). Recently, Teixeira et al. (27) demonstrated an increased microbial translocation and hyper inflammation in patients with severe COVID-19, provoking higher monocyte activation, which may be associated with worsening outcomes, including death.

Linked with the preponderance to UPFs ingestion, the SARS-Cov2 virus directly and indirectly affects at-risk populations (e.g., hypertensive patients, aged people). Social distancing has also caused the world population's physical activity reduction (2) both lifestyle habits induce body weight gain. In agreement, recent work reported that obesity prevalence has raised during the ongoing social distancing (28, 29). In obesity, immune, adipose tissue, skeletal muscle, and liver engage in a particular crosstalk leading to IR (30–38).

There is evidence that IR leads to hyperglycemia and a parallel increase in pancreatic β-cell insulin secretion (i.e., hyperinsulinemia) (39, 40). These conditions often lead to a cascade of metabolic risk factors collectively referred to as MetS, characterized by central obesity, IR, dyslipidemia, and hypertension (41), and it is known to increase T2D risk by over 2-fold (42–44). Contrary to MetS, T2D is mainly impaired insulin secretion resulting from IR (42).

Besides the pathological effects of MetS and T2D on peripheral organs, recent evidence also suggests a negative impact on brain function and surrounding areas (45–48), such as the blood-brain barrier (BBB) (49–52). The BBB regulates the molecular exchange between the peripheral blood and the brain (53, 54). Conceptual models suggest that chronic peripheral inflammation due to T2D and MetS increases the BBB permeability to leucocytes and external molecules into the brain (41, 42). Thus, the cerebral response begins with an inflammatory response (43, 44), followed by a pro-inflammatory response that alters endothelial cells (ECs), increasing the BBB permeability (55, 56). Other studies show that T2D increases the inflammatory profile of ECs and BBB permeability, a response closely associated with cognitive impairment (57, 58).

In addition, MetS depicts elevated serum triacylglycerol (TGs) and low high-density lipoprotein (HDL-c) concentrations (33). Nevertheless, cross-sectional studies have reported equivocal findings regarding the association between high serum TGs and cognitive function in humans (59, 60). Some authors report an adverse effect of TGs on cognitive function (46, 60), while others suggest a positive outcome on brain function (45). In this regard, it is worth indicating that the former study was in a Chinese sample, whereas the other studies were in the western populations. This evidence suggests racial/ethnic disparities in the effects of TGs on cognitive function. Finally, IR in the brain induced by TGs was also demonstrated (47).

Another concern in MetS is the continuous hyperglycemic state that facilitates the non-enzymatic interaction between glucose and proteins (48, 49). Glycated protein generates advanced glycation end-products (AGEs). These molecules have been associated with reduction in BBB integrity (39, 49). Moreover, AGEs activate the synthesis of pro-inflammatory cytokines in the BBB's ECs, causing a pro-inflammatory feedback loop (42). Chronic hyperglycemia triggers several metabolic signaling mechanisms that induce inflammation, apoptosis, and the synthesis of reactive oxygen species (ROS) (50). Additionally, studies performed in obese rodents show that ROS increases BBB permeability, reducing the expression of proteins

associated with tight junctions (37, 51). On the other hand, hyperglycemia *per se* harms the brain in patients with T2D (31, 52, 53). At least two mechanisms negatively contribute to this: 1) hyperglycemia is associated with brain atrophy (53), 2), hyperglycemia increases the risk for stroke, leading to brain acidosis (52, 54). Furthermore, hyperglycemia increases the activity of excitatory neurotransmitters (e.g., glutamate), resulting in a higher calcium concentration in neural cytosol to induce cell death (54). Finally, hyperglycemia is a factor that reduces the topological integration in brain (45), which possibly contributes to cognitive impairment in T2D (45) (**Figure 1**).

POOR DIET QUALITY: A RISK FACTOR FOR BRAIN INTEGRITY DURING COVID-19

The human body is a structure that requires energy for anabolic and catabolic processes. In this sense, ingesting food was initially considered a survival practice. However, anthropologists proved hypotheses concerning the diet role in Hominids evolution (61–65). For instance, cooking foods improved digestion capacity (62, 66). Additionally, cuisine foods increased the availability of the nutrients present in plants and meat (66). The previous conditions and others facilitated the brain evolution (i.e., encephalization) (62, 67). Therefore, the diet components have played a relevant role throughout the Homo evolution in conjunction with food processing. In this sense, fatty acids, mainly the long-chain polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA), and arachidonic acid (AA), have provided essential effects on brain evolution (63, 68). Moreover, AA is a lipid that strengthens synaptic transmission (69, 70). However, the AA is also a precursor of molecules linked with inflammatory responses such as prostaglandins and pro-inflammatory cytokines, such as TNFα and IL-1B (71, 72). Another example of dietary adaptations is the lactose tolerance of some populations. The latter is due to milk consumption after the weaning period, keeping the lactase enzyme active (73). As can be noted, the diet helps humans deal with the context of living, allowing us to say that we are what we eat. Therefore, the population who show a poor-quality diet will have few tools to cover all the surrounding challenges.

The COVID-19 lockdown disrupted the dietary patterns in the world population (74, 75), affecting low-and-middle-income countries (11, 12, 76, 77). Therefore, besides the enhancing effect on the UPFs consumption, the COVID-19 outbreak reduced food security, and consequently, the dietary quality (11, 12, 75, 78, 79). Food security is a complex phenomenon that implicates time, physical and economic access to sufficient healthy food to satisfy the nutritional needs and food preferences for a healthy lifestyle (80). The opposite condition is known as food insecurity (FI) (80, 81), which is related to malnutrition (i.e., undernutrition and micronutrient deficiency) (81). During the COVID-19 lockdown, unemployment growth and increased food prices were the main factors reducing food affordability (11). In addition, the lockdown restrictions reduced the food supply chain (78), which in turn reduced the ingestion of fresh products such as fruit and vegetables (75). Together with the prior information, other authors have reported that the population with high FI scores showed higher anxiety levels (78), independent of the socio-economic factors (82). Besides the FI, an inverse relationship is reported between quality diet and anxiety levels in individuals undergoing lockdown (29).

The nutrient deficiency intake might impact brain function. For instance, the PUFAs role on neural membrane integrity, gray matter, and hippocampal volume (83), makes them an important nutrient whose low ingestion contributes to a reduced brain plasticity (83–85). Together with lipid actions, other molecules are also essential to strengthen brain function. Concretely, polyphenols found in fruits and vegetables also have positive effects (86). Resveratrol for example, a phytoalexin present in grapes, berries, tomatoes, nuts, and cocoa (87), demonstrated positive effects on brain function and structure (88, 89). Chronic consumption of resveratrol led to a better cognitive performance (i.e., improving memory) and mood in postmenopausal women (89), the hypothetical mechanism explaining these responses was a better cerebral perfusion modulation in the participants (89). A similar effect was reported in healthy men (90); moreover, this polyphenol enhances the functional connectivity from the hippocampus to frontal, parietal, and occipital areas, improvement in the memory retention correlated with a topological shift in brain, and glucose metabolism in healthy older adults (91).

Conversely, protein malnutrition (PMN) is a risk factor for neuroinflammation and oxidative stress (10, 92, 93). Moreover, the PMN in pregnant women affects brain development and cognition considerably in the offspring (10). The previous findings emphasize the impact of the diet on brain integrity during the COVID-19 outbreak (**Figure 1**).

Poor diet, nutrient availability, and quality will also impact the gut microbiome and, eventually, brain health. Intestinal content and the brain represent a dynamic bidirectional communication described as the "gut (microbiota)-brain axis" (94). The human gut microbiome includes different types of bacteria responsible for several functions such as energy metabolism, immunity, vitamin synthesis, hormone, and neurotransmitter production, and it also influences human behavior (94–96). Environmental factors (e.g., diet changes caused by the COVID-19 pandemic, medication, exercise) can potentially change the gut microbiome rapidly. In addition, special conditions might lead to a microbial imbalance (i.e., dysbiosis), a factor contributing or associated to the development of some diseases like inflammatory bowel disease (97), atopic diseases (e.g., eczema, asthma, food allergies) (98), type-1 diabetes (99), schizophrenia, and other cognitive disorders (94, 100, 101). Changes in the gut microbiome have shown concomitant changes in brain structure, function, and behaviors (e.g., stress, anxiety, depression) (94, 101). Indeed, the effect of gut microbiome diversity on brain function is partially accounted for by vitamin-mediated neuronal function, neurotransmitter composition, and short-chain fatty acid (sCFas) metabolites (94, 102).

Previous evidence suggests that vagal afferent sensory neurons are microbiota-mediated, regulating information transmission through the kynurenine pathway (103). Probiotic supplementation impacts the central nervous system, and

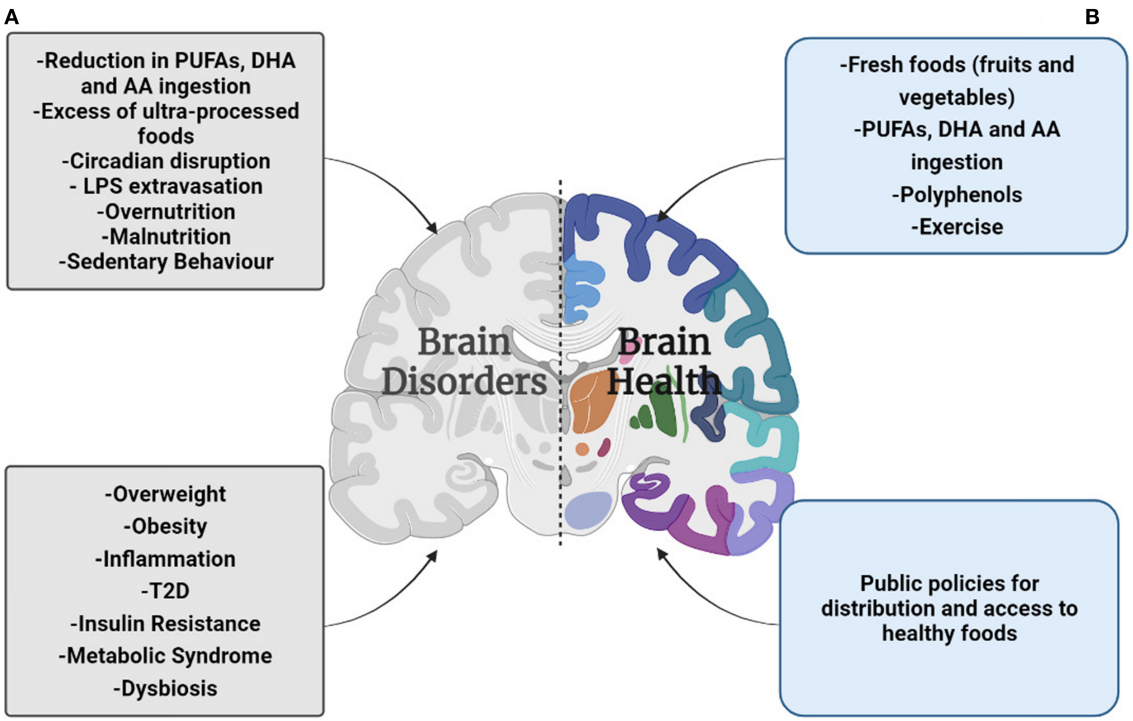


FIGURE 1 | Summary overview of the malnutrition effects on brain health during the COVID-19 pandemic. **(A)** The preponderance by the UPF's ingestion, circadian disruption, and a sedentary lifestyle will facility the gain of body weight, leading to suffering overweight/obesity, and in a worst-case scenario suffer metabolic syndrome and T2D. Moreover, overweight/obesity increases the risk of low-grade chronic inflammation; the pro-inflammatory cytokines hinder the blood-brain barrier function deteriorating brain health. Additionally, the higher rate of unemployment and the rising price of food have reduced the affordability of fresh products, and protein based-products, resulting in a poor quality diet. This condition is a factor that reduces brain integrity that leads to suffering brain disorders. **(B)** The public services focused to facilitate the practice of physical exercise during the lockdown, and better distribution and access to healthy food will strengthen the brain health and reduce the risk to suffer brain disorders.

research has shown its effects on anxiety disorders (i.e., anxiolytic effect) (102, 104). Although specific probiotic species affecting brain health are currently under study, recent evidence suggests that anxiety and depressive disorders correlate to higher pro-inflammatory species and lower abundance of sCFas-producing species (105).

CONCLUSIONS AND FINAL REMARKS

Social distancing was a strategy implemented worldwide by several governments to reduce the risk of COVID-19 infection. However, this outbreak has impacted the household economy considerably, reducing food affordability and, consequently, the food quality. Although different documents highlight the diet's relevance to strengthening the immune system, there is a lack of emphasis on the diet's role in maintaining brain integrity and functionality during the COVID-19 outbreak. In the current work, we discussed how overweight and obesity impact brain function. Even though this effect is widely reported, the social distancing during COVID-19 increased the risk of suffering obesity. We also discussed how undernutrition is a condition with deleterious effects on brain integrity. Although the mobility restriction is less severe today, the economic impact of the COVID-19 pandemic is still present in society; furthermore, it is projected to reach the pre-pandemic levels until 2023 (106). This scenario impairs individuals' availability to secure adequate nutrients and causes changes in the gut microbiome, resulting in vulnerable brain health and increasing the risk of suffering anxiety, cognitive deficiency, mental disorders, and impaired mood. Finally, different authors indicated that the brain is directly and indirectly affected by COVID-19 (107–109). Therefore, we believe that a fragile brain resulting from malnutrition (i.e., over-nutrition and undernutrition) could worsen the consequences after the COVID-19 infection. Consequently, we consider that governments worldwide must develop strategies to improve the diet quality in the population, mainly during the COVID-19 outbreak. If fulfilled, the possibility of increasing brain health in children, adults, and the elderly is nigh.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

IR, PG-S, and JM-P reviewed the literature, wrote the first draft, and finalized the manuscript. JM-J, BA, and FL finalized the manuscript, BA conceived and designed **Figure 1**. AJ-M conceived the article focus, reviewed the literature, and wrote the first draft. All authors approved the final version of the manuscript.

REFERENCES

1. Thivel D, Tremblay A, Genin PM, Panahi S, Rivière D, Duclos M. Physical activity, inactivity, and sedentary behaviors: definitions and implications in occupational health. *Front Public Health*. (2018) 6:288. doi: 10.3389/fpubh.2018.00288
2. Tison GH, Avram R, Kuhar P, Abreau S, Marcus GM, Pletcher MJ, et al. Worldwide effect of COVID-19 on physical activity: a descriptive study. *Ann Intern Med*. (2020) 173:767–70. doi: 10.7326/M20-2665
3. Balanzá-Martínez V, Kapczinski F, de Azevedo Cardoso T, Atienza-Carbonell B, Rosa AR, Mota JC, et al. The assessment of lifestyle changes during the COVID-19 pandemic using a multidimensional scale. *Rev Psiquiatr Salud Ment*. (2021) 14:1. doi: 10.1016/j.rpsm.2020.07.003
4. Morin CM, Carrier J, Bastien C, Godbout R. Sleep and circadian rhythm in response to the COVID-19 pandemic. *Can J Public Heal*. (2020) 111:654–7. doi: 10.17269/s41997-020-00382-7
5. Romdhani M, Rae DE, Nédélec M, Ammar A, Chtourou H, Al Horani R, et al. COVID-19 lockdowns: a worldwide survey of circadian rhythms and sleep quality in 3911 athletes from 49 countries, with data-driven recommendations. *Sport Med.* (2021) 8:1–16. doi: 10.1007/s40279-021-01601-y
6. Broussard JL, Cauter E Van. Disturbances of sleep and circadian rhythms: novel risk factors for obesity. *Curr Opin Endocrinol Diabetes Obes*. (2016) 23:353–9. doi: 10.1097/MED.0000000000000276
7. Machado RM, Koike MK. Circadian rhythm, sleep pattern, and metabolic consequences: an overview on cardiovascular risk factors. *Horm Mol Biol Clin Investig*. (2014) *18*:47–52. doi: 10.1515/hmbci-2013-0057
8. Škrlec I, Talapko J, DŽijan S, Cesar V, Lazić N, Lepeduš H. The association between circadian clock gene polymorphisms and metabolic syndrome: a systematic review and meta-analysis. *Biology (Basel) (2022) 1*1:1–14. doi: 10.3390/biology11010020
9. Wirt A, Collins CE. Diet quality - what is it and does it matter? *Public Health Nutr*. (2009) *12*(12). doi: 10.1017/S136898000900531X
10. Sinha S, Patro N, Patro IK. Maternal protein malnutrition: current and future perspectives of spirulina supplementation in neuroprotection. *Front Neurosci*. (2018) 12: 966. doi: 10.3389/fnins.2018.00966
11. Madzorera I, Ismail A, Hemler EC, Korte ML, Olufemi AA, Wang D, et al. Impact of COVID-19 on nutrition, food security, and dietary diversity and quality in Burkina Faso, Ethiopia, and Nigeria. *Am J Trop Med Hyg*. (2021) 105:2. doi: 10.4269/ajtmh.20-1617
12. Picchioni F, Goulao LF, Roberfroid D. The impact of COVID-19 on diet quality, food security and nutrition in low and middle income countries: a systematic review of the evidence. *Clin Nutr*. (2021) 1532–983. doi: 10.1016/j.clnu.2021.08.015
13. Eiser AR. Could dietary factors reduce COVID-19 mortality rates? *moderating the inflammatory state*. *J Altern Complement Med*. (2021):27. doi: 10.1089/acm.2020.0441
14. Calder PC. Nutrition, immunity and COVID-19. *BMJ Nutr Prev Heal*. (2020) 3:1. doi: 10.1136/bmjnph-2020-000085
15. Arshad MS, Khan U, Sadiq A, Khalid W, Hussain M, Yasmeen A, et al. Coronavirus disease (COVID-19) and immunity booster green foods: a mini review. *Food Sci Nutr*. (2020) 8. 3971–976. doi: 10.1002/fsn3.1719
16. Monteiro CA, Cannon G, Moubarac JC, Levy RB, Louzada MLC, Jaime PC. The un decade of Nutrition, the NOVA food classification and the trouble with ultra-processing. *Public Health Nutr*. (2018) 21: 5–17. doi: 10.1017/S1368980017000234
17. Monteiro CA, Cannon G, Lawrence M, Costa Louzada ML, Machado PP. *The NOVA Food Classification System and Its Four Food Groups. Ultra-Processed Foods, Diet Quality, and Health Using the NOVA Classification System*. Rome: FAO (2019) p. 6–9.
18. Fardet A, Rock E. Ultra-processed foods and food system sustainability: what are the links? *Sustainability (Switzerland)*. (2020) 12:6280. doi: 10.3390/su12156280
19. Pan American Health Organization. *Ultra-Processed Food and Drink Products in Latin America: Sales, Sources, Nutrient Profiles and Policy Implications*. Washington, DC: PAHO (2019) p. 72.
20. Lopez KV, Garduño AMJ, Regules AEO, Romero LMI, Martinez OAG, Pereira TSS. Lifestyle and nutrition changes during the SARS-CoV-2 (COVID-19) lockdown in México: an observational study. *Rev Esp Nutr Humana y Diet*. (2021) 25:1–21. doi: 10.14306/renhyd.25.S2.1099
21. Ruiz-Roso MB, Padilha P de C, Mantilla-Escalante DC, Ulloa N, Brun P, Acevedo-Correa D, et al. Covid-19 confinement and changes of adolescent's dietary trends in Italy, Spain, Chile, Colombia and Brazil. *Nutrients*. (2020) 12:6. doi: 10.3390/nu12061807
22. Clemente-Suárez VJ, Ramos-Campo DJ, Mielgo-Ayuso J, Dalamitros AA, Nikolaidis PA, Hormeño-Holgado A, et al. Nutrition in the actual covid-19 pandemic. a narrative review. *Nutrients.* (2021) 13:1924. doi: 10.3390/nu13061924
23. Zupo R, Castellana F, Sardone R, Sila A, Giagulli VA, Triggiani V, et al. Preliminary trajectories in dietary behaviors during the COVID-19 pandemic: a public health call to action to face obesity. *Int J Environ Res*. (2020) 17:7073. doi: 10.3390/ijerph17197073
24. Smaira FI, Mazzolani BC, Esteves GP, André HCS, Amarante MC, Castanho DF, et al. Poor eating habits and selected determinants of food choice were associated with ultraprocessed food consumption in Brazilian women during the COVID-19 pandemic. *Front Nutr*. (2021) 8:672372. doi: 10.3389/fnut.2021.672372
25. Lancaster GI, Langley KG, Berglund NA, Kammoun HL, Reibe S, Estevez E, et al. Evidence that TLR4 is not a receptor for saturated fatty acids but mediates lipid-induced inflammation by reprogramming macrophage metabolism. *Cell Metab*. (2018) 27:5. doi: 10.1016/j.cmet.2018.03.014
26. Lira FS, Rosa JC, Pimentel GD, Santos R V, Carnier J, Sanches PL, et al. Long-term interdisciplinary therapy reduces endotoxin level and insulin resistance in obese adolescents. *Nutr J*. (2012) 11:1. doi: 10.1186/1475-2891-11-74
27. Teixeira PC, Dorneles GP, Santana Filho PC, da Silva IM, Schipper LL, Postiga IAL, et al. Increased LPS levels coexist with systemic inflammation and result in monocyte activation in severe COVID-19 patients. *Int Immunopharmacol*. (2021) 100:108125. doi: 10.1016/j.intimp.2021.108125
28. Lange SJ, Kompaniyets L, Freedman DS, Kraus EM, Porter R, Blanck HM, et al. *Morbidity and Mortality Weekly Report Longitudinal Trends in Body Mass Index Before and During the Covid-19 Pandemic Among Persons Aged 2–19 Years-United States, 2018–2020*. Washington, DC: Morbidity and Mortality Weekly Reports (2018).
29. Kaufman-Shriqui V, Navarro DA, Raz O, Boaz M. Multinational dietary changes and anxiety during the coronavirus pandemic-findings from Israel. *Isr J Health Policy Res*. (2021) 10:1. doi: 10.1186/s13584-021-00461-1
30. Lorenzo C, Okoloise M, Williams K, Stern MP, Haffner SM. The metabolic syndrome as predictor of type 2 diabetes: the san antonio heart study. *Diabetes Care*. (2003) *26*:11. doi: 10.2337/diacare.26.11.3153
31. Kim DJ, Yu JH, Shin MS, Shin YW, Kim MS. Hyperglycemia reduces efficiency of brain networks in subjects with type 2 diabetes. *PLoS ONE*. (2016) 11:1–14. doi: 10.1371/journal.pone.0157268
32. Nägga K, Gustavsson A-M, Stomrud E, Lindqvist D, van Westen D, Blennow K, et al. Increased midlife triglycerides predict brain β-amyloid and tau pathology 20 years later. *Neurology*. (2018) 90:e73–81. doi: 10.1212/WNL.0000000000004749

33. Banks WA, Coon AB, Robinson SM, Moinuddin A, Shultz JM, Nakaoke R, et al. Triglycerides induce leptin resistance at the blood-brain barrier. *Diabetes*. (2004) *53*:1253–60. doi: 10.2337/diabetes.53.5.1253
34. Moheet A, Mangia S, Seaquist ER. Impact of diabetes on cognitive function and brain structure. *Ann N Y Acad Sci*. (2015) 1353:60–71. doi: 10.1111/nyas.12807
35. Rutkowsky JM, Lee LL, Puchowicz M, Golub MS, Befroy DE, Wilson DW, et al. Reduced cognitive function, increased bloodbrain-barrier transport and inflammatory responses, and altered brain metabolites in LDLr-/-And C57BL/6 mice fed a western diet. *PLoS ONE*. (2018) 13:191909. doi: 10.1371/journal.pone.0191909
36. Hargrave SL, Davidson TL, Zheng W, Kinzig KP. Western diets induce blood-brain barrier leakage and alter spatial strategies in rats. *Behav Neurosci*. (2016) 130:123–35. doi: 10.1037/bne0000110
37. Salameh TS, Mortell WG, Logsdon AF, Butterfield DA, Banks WA. Disruption of the hippocampal and hypothalamic blood-brain barrier in a diet-induced obese model of type II diabetes: prevention and treatment by the mitochondrial carbonic anhydrase inhibitor, topiramate. *Fluids Barriers CNS*. (2019) 16:1–17. doi: 10.1186/s12987-018-0121-6
38. Yoo DY, Yim HS, Jung HY, Nam SM, Kim JW, Choi JH, et al. Chronic type 2 diabetes reduces the integrity of the blood-brain barrier by reducing tight junction proteins in the hippocampus. *J Vet Med Sci*. (2016) 78:957–62. doi: 10.1292/jvms.15-0589
39. Bogush M, Heldt NA, Persidsky Y. Blood brain barrier injury in diabetes: unrecognized effects on brain and cognition. *J Neuroimmune Pharmacol*. (2017) 12:593–601. doi: 10.1007/s11481-017-9752-7
40. Shalev H, Serlin Y, Friedman A. Breaching the blood-brain barrier as a gate to psychiatric disorder. *Cardiovasc Psychiatry Neurol*. (2009) 2009:1–7. doi: 10.1155/2009/278531
41. Mauro C, De Rosa V, Marelli-Berg F, Solito E. Metabolic syndrome and the immunological affair with the blood-brain barrier. *Front Immunol*. (2015) 6:677. doi: 10.3389/fimmu.2014.00677
42. Van Dyken P, Lacoste B. Impact of metabolic syndrome on neuroinflammation and the blood–brain barrier. *Front Neurosci*. (2018) 12:930. doi: 10.3389/fnins.2018.00930
43. Takechi R, Lam V, Brook E, Giles C, Fimognari N, Mooranian A, et al. Blood-brain barrier dysfunction precedes cognitive decline and neurodegeneration in diabetic insulin resistant mouse model: an implication for causal link. *Front Aging Neurosci*. (2017) 9:399. doi: 10.3389/fnagi.2017.00399
44. Hwang IK, Choi JH, Nam SM, Park OK, Yoo DY, Kim W, et al. Activation of microglia and induction of proinflammatory cytokines in the hippocampus of type 2 diabetic rats. *Neurol Res*. (2014) 36:824–32. doi: 10.1179/1743132814Y.0000000330
45. Yin ZX, Shi XM, Kraus VB, Fitzgerald SM, Qian HZ, Xu JW, et al. High normal plasma triglycerides are associated with preserved cognitive function in chinese oldest-old. *Age Ageing*. (2012) 41:600–6. doi: 10.1093/ageing/afs033
46. Parthasarathy V, Frazier DT, Bettcher BM, Jastrzab L, Chao L, Reed B, et al. Triglycerides are negatively correlated with cognitive function in non-demented aging adults. *Neuropsychology*. (2017) 31:682–8. doi: 10.1037/neu0000335
47. Banks WA, Farr SA, Salameh TS, Niehoff ML, Rhea EM, Morley JE, et al. Triglycerides cross the blood – brain barrier and induce central leptin and insulin receptor resistance. *Nat Publ Gr*. (2017) 42:391–7. doi: 10.1038/ijo.2017.231
48. Singh VP, Bali A, Singh N, Jaggi AS. Advanced glycation end products and diabetic complications. *Korean J Physiol Pharmacol*. (2014) 18:1–14. doi: 10.4196/kjpp.2014.18.1.1
49. Ferreira LSS, Fernandes CS, Vieira MNN, De Felice FG. Insulin resistance in Alzheimer ' s disease. *Front Neurosci*. (2018) 12:830. doi: 10.3389/fnins.2018.00830
50. de Oliveira Sá G, dos Santos Neves V, de Oliveira Fraga SR, Souza-Mello V, Barbosa-da-Silva S. High-intensity interval training has beneficial effects on cardiac remodeling through local renin-angiotensin system modulation in mice fed high-fat or high-fructose diets. *Life Sci*. (2017) 189:8–17. doi: 10.1016/j.lfs.2017.09.012
51. Salameh TS, Shah GN, Price TO, Hayden MR, Banks WA. Blood-brain barrier disruption and neurovascular unit dysfunction in diabetic mice: protection with the mitochondrial carbonic anhydrase inhibitor topiramate. *J Pharmacol Exp Ther*. (2016) 359:452–9. doi: 10.1124/jpet.116.237057
52. Sommerfield AJ, Deary IJ, Frier BM. Acute hyperglycemia alters mood state and impairs cognitive performance in people with type 2 diabetes. *Diabetes Care*. (2004) 27:2335–40. doi: 10.2337/diacare.27.10.2335
53. Tiehuis AM, van der Graaf Y, Visseren FL, Vincken KL, Biessels GJ, Appelman APA, et al. Diabetes increases atrophy and vascular lesions on brain MRI in patients with symptomatic arterial disease. *Stroke*. (2008) 39:1600–3. doi: 10.1161/STROKEAHA.107.506089
54. Kagansky N, Levy S, Knobler H. The role of hyperglycemia in acute stroke. *Arch Neurol*. (2001) 58:1209–12. doi: 10.1001/archneur.58.8.1209
55. Boulé NG, Haddad E, Kenny GP, Wells GA, Sigal RJ. Effects of exercise on glycemic control and body mass in type 2 diabetes mellitus: a meta-analysis of controlled clinical trials. *JAMA*. (2001) 286:1218–27. doi: 10.1001/jama.286.10.1218
56. Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, et al. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy. *Diabetes Care*. (2009) 32:1. doi: 10.2337/dc08-9025
57. Colberg SR, Sigal RJ, Yardley JE, Riddell MC, Dunstan DW, Dempsey PC, et al. Physical activity/exercise and diabetes: a position statement of the American Diabetes Association. *Diabetes Care*. (2016) 39:2065–79. doi: 10.2337/dc16-1728
58. Johnson NA, Sachinwalla T, Walton DW, Smith K, Armstrong A, Thompson MW, et al. Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. *Hepatology*. (2009) 50:4. doi: 10.1002/hep.23129
59. Fisher G, Brown AW, Bohan Brown MM, Alcorn A, Noles C, Winwood L, et al. High intensity interval- vs moderate intensity-training for improving cardiometabolic health in overweight or obese males: a randomized controlled trial. *PLoS ONE*. (2015) 10:138853. doi: 10.1371/journal.pone.0138853
60. Weston M, Taylor KL, Batterham AM, Hopkins WG. Effects of low-volume high-intensity interval training (HIT) on fitness in adults: a meta-analysis of controlled and non-controlled trials. *Sports Med*. (2014) 44:1005–17. doi: 10.1007/s40279-014-0180-z
61. Laland KN, Odling-Smee J, Myles S. How culture shaped the human genome: Bringing genetics and the human sciences together. *Nat Rev Genet*. (2010) 11:137–48. doi: 10.1038/nrg2734
62. Wrangham RW, Jones JH, Laden G, Pilbeam D, Conklin-Brittain N. The raw and the stolen. *Curr Anthropol*. (1999) 40:5. doi: 10.1086/300083
63. Leonard WR, Snodgrass JJ, Robertson ML. Effects of brain evolution on human nutrition and metabolism. *Annu Rev Nutr*. (2007) 27:311–27. doi: 10.1146/annurev.nutr.27.061406.093659
64. James WPT, Johnson RJ, Speakman JR, Wallace DC, Frühbeck G, Iversen PO, et al. Nutrition and its role in human evolution. *J Intern Med*. (2019) 285:533–49. doi: 10.1111/joim.12878
65. Luca F, Perry GH, Di Rienzo A. Evolutionary adaptations to dietary changess. *Annu Rev Nutr*. (2010) 30:291–314. doi: 10.1146/annurev-nutr-080508-141048
66. Wrangham R. The evolution of human nutrition. *Curr Bio*. (2013) 23:354–5. doi: 10.1016/j.cub.2013.03.061
67. Burini RC, Leonard WR. The evolutionary roles of nutrition selection and dietary quality in the human brain size and encephalization. *Nutrire*. (2018) 43:19. doi: 10.1186/s41110-018-0078-x
68. Cordain L, Watkins BA, Mann NJ. Fatty acid composition and energy density of foods available to African hominids. evolutionary implications for human brain development. *World Rev Nutr Diet*. (2001) 90:144–61. doi: 10.1159/000059813
69. Nishizaki T, Nomura T, Matsuoka T, Enikolopov G, Sumikawa K. Arachidonic acid induces a long-lasting facilitation of hippocampal synaptic transmission by modulating PKC activity and nicotinic ACh receptors. *Mol Brain Res*. (1999) 69:263–72. doi: 10.1016/S0169-328X(99)00117-5

70. Rickman C, Davletov B. Arachidonic acid allows SNARE complex formation in the presence of munc18. *Chem Biol*. (2005) 12:545–53. doi: 10.1016/j.chembiol.2005.03.004
71. Anthonsen MW, Solhaug A, Johansen B. Functional coupling between secretory and cytosolic phospholipase a 2 modulates tumor necrosis factor-α- and interleukin-1β -induced nf-κb activation. *J Biol Chem [Internet]*. (2001) *276*:30527–36. doi: 10.1074/jbc.M008481200
72. Song C, Manku MS, Horrobin DF. Long-chain polyunsaturated fatty acids modulate interleukin-1β-induced changes in behavior, monoaminergic neurotransmitters, and brain inflammation in rats. *J Nutr*. (2008) 138:954–63. doi: 10.1093/jn/138.5.954
73. Armelagos GJ. Brain evolution, the determinates of food choice, and the omnivore's dilemma. *Crit Rev Food Sci Nutr*. (2014) 54:10. doi: 10.1080/10408398.2011.635817
74. Kaku K, Kadowaki T, Seino Y, Okamoto T, Shirakawa M, Sato A, et al. Efficacy and safety of ipragliflozin in Japanese patients with type 2 diabetes and inadequate glycaemic control on sitagliptin. *Diabetes Obes Metab*. (2021) 23:2099–108. doi: 10.1111/dom.14448
75. Bennett G, Young E, Butler I, Coe S. The impact of lockdown during the covid-19 outbreak on dietary habits in various population groups: a scoping review. *Front Nutr*. (2021) 8:626432. doi: 10.3389/fnut.2021.626432
76. Laborde D, Herforth A, Headey D, de Pee S. COVID-19 pandemic leads to greater depth of unaffordability of healthy and nutrient-adequate diets in low- and middle-income countries. *Nat Food*. (2021) 2:7. doi: 10.1038/s43016-021-00323-8
77. Merino J, Joshi AD, Nguyen LH, Leeming ER, Mazidi M, Drew DA, et al. Diet quality and risk and severity of COVID-19: a prospective cohort study. *Gut*. (2021) 70:11. doi: 10.1136/gutjnl-2021-325353
78. Gaitán-Rossi P, Vilar-Compte M, Teruel G, Pérez-Escamilla R. Food insecurity measurement and prevalence estimates during the COVID-19 pandemic in a repeated cross-sectional survey in Mexico. *Public Health Nutr*. (2021) 24:3. doi: 10.1017/S1368980020004000
79. Headey D, Heidkamp R, Osendarp S, Ruel M, Scott N, Black R, et al. Impacts of COVID-19 on childhood malnutrition and nutrition-related mortality. *Lancet*. (2020) 396:513–82. doi: 10.1016/S0140-6736(20)31647-0
80. Jones AD, Ngure FM, Pelto G, Young SL. What are we assessing when we measure food security? a compendium and review of current metrics. *Adv Nutr*. (2013) 4:481–505. doi: 10.3945/an.113.004119
81. FAO, IFAD, UNICEF W. *The State of Food Security and Nutrition in the World (2020) Transforming Food Systems for Affordable Healthy Diets. IEEE Journal of Selected Topics in Applied Earth Observations and Remote Sensing*. (2020).
82. Jones AD. Food insecurity and mental health status: a global analysis of 149 countries. *Am J Prev Med*. (2017) 53:2. doi: 10.1016/j.amepre.2017.04.008
83. Witte AV, Kerti L, Hermannstädter HM, Fiebach JB, Schreiber SJ, Schuchardt JP, et al. Long-chain omega-3 fatty acids improve brain function and structure in older adults. *Cereb Cortex*. (2014) 24:11. doi: 10.1093/cercor/bht163
84. Nurk E, Drevon CA, Refsum H, Solvoll K, Vollset SE, Nygård O, et al. Cognitive performance among the elderly and dietary fish intake: the hordaland health study. *Am J Clin Nutr*. (2007) 86:5. doi: 10.1093/ajcn/86.5.1470
85. Bos DJ, van Montfort SJT, Oranje B, Durston S, Smeets PAM. Effects of omega-3 polyunsaturated fatty acids on human brain morphology and function: what is the evidence? *Eur Neuropsychopharmacol*. (2016) 26:3. doi: 10.1016/j.euroneuro.2015.12.031
86. Duthie GG, Gardner PT, Kyle JAM. Plant polyphenols: are they the new magic bullet? *Proc Nutr Soc*. (2003) 62:3. doi: 10.1079/PNS2003275
87. Sebastià N, Montoro A, León Z, Soriano JM. Searching trans-resveratrol in fruits and vegetables: a preliminary screening. *J Food Sci Technol*. (2017) 54:3. doi: 10.1007/s13197-016-2474-7
88. Köbe T, Witte AV, Schnelle A, Tesky VA, Pantel J, Schuchardt JP, et al. Impact of resveratrol on glucose control, hippocampal structure and connectivity, and memory performance in patients with mild cognitive impairment. *Front Neurosci*. (2017) 11:105. doi: 10.3389/fnins.2017.00105
89. Evans HM, Howe PRC, Wong RHX. Effects of resveratrol on cognitive performance, mood and cerebrovascular function in post-menopausal women; a 14-week randomised placebo-controlled intervention trial. *Nutrients*. (2017) 9:1. doi: 10.3390/nu9010027
90. Kennedy DO, Wightman EL, Reay JL, Lietz G, Okello EJ, Wilde A, et al. Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation. *Am J Clin Nutr*. (2010) 91:6. doi: 10.3945/ajcn.2009.28641
91. Veronica Witte A, Kerti L, Margulies DS, Flöel A. Effects of resveratrol on memory performance, hippocampal functional connectivity, and glucose metabolism in healthy older adults. *J Neurosci*. (2014) *34*:23. doi: 10.1523/JNEUROSCI.0385-14.2014
92. Khare M, Mohanty C, Das BK, Jyoti A, Mukhopadhyay B, Mishra SP. Free radicals and antioxidant status in protein energy malnutrition. *Int J Pediatr*. (2014) 6:254396. doi: 10.1155/2014/254396
93. Verma A, Saini T, Meena K. Evaluation of oxidative stress in severe acute malnourished children at malnutrition treatment centre of Sardar Patel Medical College, Bikaner, Rajasthan, India. *Int J Res Med Sci*. (2016) 4:6. doi: 10.18203/2320-6012.ijrms20161796
94. Morais LH, Schreiber HL, Mazmanian SK. The gut microbiota–brain axis in behaviour and brain disorders. Nat Rev Microbiol. (2021) 19:241–55. doi: 10.1038/s41579-020-00460-0
95. Amon P, Sanderson I. What is the microbiome? *Arch Dis Child Educ Pract Ed*. (2017) 102:5. doi: 10.1136/archdischild-2016-311643
96. Galland L. The gut microbiome and the brain. *J Med Food*. (2014) 17:1261–72. doi: 10.1089/jmf.2014.7000
97. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut*. (2006) 55:2. doi: 10.1136/gut.2005.073817
98. Riiser A. The human microbiome, asthma, and allergy. *Allergy Asthma Clin Immunol*. (2015) 11:35. doi: 10.1186/s13223-015-0102-0
99. Charbonneau MR, Blanton L V, Digiulio DB, Relman DA, Lebrilla CB, Mills DA, et al. A microbial perspective of human developmental biology. *Nature*. (2016) 535:48–55. doi: 10.1038/nature18845
100. Ding HT, Taur Y, Walkup JT. Gut microbiota and autism: key concepts and findings. *J Autism Dev Disord*. (2017) 47:480–9. doi: 10.1007/s10803-016-2960-9
101. Li S, Song J, Ke P, Kong L, Lei B, Zhou J, et al. The gut microbiome is associated with brain structure and function in schizophrenia. *Sci Rep*. (2021) 11:1. doi: 10.1038/s41598-021-89166-8
102. Hasan Mohajeri M, La Fata G, Steinert RE, Weber P. Relationship between the gut microbiome and brain function. *Nutr Rev*. (2018) 76:7. doi: 10.1093/nutrit/nuy009
103. Kennedy PJ, Cryan JF, Dinan TG, Clarke G. Kynurenine pathway metabolism and the microbiota-gut-brain axis. *Neuropharmacology*. (2017) 112:399–12. doi: 10.1016/j.neuropharm.2016.07.002
104. Kane L, Kinzel J. The effects of probiotics on mood and emotion. *JAAPA*. (2018) 3:1–3. doi: 10.1097/01.JAA.0000532122.07789.f0
105. Simpson CA, Diaz-Arteche C, Eliby D, Schwartz OS, Simmons JG, Cowan CSM. The gut microbiota in anxiety and depression – asystematic review. *Clin Psychol Rev*. (2021) 83:101943. doi: 10.1016/j.cpr.2020.101943
106. OECD. *Real GDP Forecast (Indicator)*. Paris: Organisation for Economic Co-operation and Development (2022).
107. Kaushik P, Kaushik M, Parveen S, Tabassum H, Parvez S. Cross-talk between key players in patients with covid-19 and ischemic stroke: a review on neurobiological insight of the pandemic. *Mol Neurobiol*. (2020) 57:4921–8. doi: 10.1007/s12035-020-02072-4
108. Ganti L, Serrano E, Toklu HZ. Can neuron specific enolase be a diagnostic biomarker for neuronal injury in COVID-19? *Cureus*. (2020) 12:e11033. doi: 10.7759/cureus.11033
109. Baig AM, Khaleeq A, Ali U, Syeda H. Evidence of the COVID-19 virus targeting the CNS: tissue distribution, host-virus interaction, and

proposed neurotropic mechanisms. *ACS Chem Neurosci*. (2020) 11:995–8. doi: 10.1021/acschemneuro.0c00122

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Rentería, García-Suárez, Moncada-Jiménez, Machado-Parra, Antunes, Lira and Jiménez-Maldonado. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

REVIEW
published: 28 April 2022
doi: 10.3389/fnut.2022.800901

Lactate Is Answerable for Brain Function and Treating Brain Diseases: Energy Substrates and Signal Molecule

Ming Cai[1,2†], *Hongbiao Wang*[3†], *Haihan Song*[4], *Ruoyu Yang*[5], *Liyan Wang*[5], *Xiangli Xue*[6], *Wanju Sun*[4*] and *Jingyun Hu*[4*]

[1] Department of Rehabilitation Medicine, Shanghai University of Medicine and Health Sciences Affiliated Zhoupu Hospital, Shanghai, China, [2] Bio-X Institutes, Shanghai Jiao Tong University, Shanghai, China, [3] Department of Physical Education, Shanghai University of Medicine and Health Sciences, Shanghai, China, [4] Central Lab, Shanghai Pudong New Area People's Hospital, Shanghai, China, [5] College of Rehabilitation Sciences, Shanghai University of Medicine and Health Sciences, Shanghai, China, [6] Key Laboratory of Exercise and Health Sciences of Ministry of Education, Shanghai University of Sport, Shanghai, China

OPEN ACCESS

Edited by:
Andrew Scholey,
Swinburne University of Technology, Australia

Reviewed by:
Romana Stark,
Monash University, Australia
George A. Brooks,
University of California, Berkeley, United States

***Correspondence:**
Wanju Sun
sunwanju2021@163.com
Jingyun Hu
jingyunhu121@126.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

Received: 24 October 2021
Accepted: 18 March 2022
Published: 28 April 2022

Citation:
Cai M, Wang H, Song H, Yang R, Wang L, Xue X, Sun W and Hu J (2022) Lactate Is Answerable for Brain Function and Treating Brain Diseases: Energy Substrates and Signal Molecule. Front. Nutr. 9:800901. doi: 10.3389/fnut.2022.800901

Research to date has provided novel insights into lactate's positive role in multiple brain functions and several brain diseases. Although notable controversies and discrepancies remain, the neurobiological role and the metabolic mechanisms of brain lactate have now been described. A theoretical framework on the relevance between lactate and brain function and brain diseases is presented. This review begins with the source and route of lactate formation in the brain and food; goes on to uncover the regulatory effect of lactate on brain function; and progresses to gathering the application and concentration variation of lactate in several brain diseases (diabetic encephalopathy, Alzheimer's disease, stroke, traumatic brain injury, and epilepsy) treatment. Finally, the dual role of lactate in the brain is discussed. This review highlights the biological effect of lactate, especially L-lactate, in brain function and disease studies and amplifies our understanding of past research.

Keywords: lactate, brain function, brain diseases, energy substrates, signal molecule

INTRODUCTION

Brain lactate, as a well-known metabolite, primarily roots in astrocytic glycolysis from blood glucose, glycogen, and blood lactate. Recently, the role of the "good guy" has gradually superseded the traditional concept of metabolic waste in medical literature in neuroscience (1). The most interesting dimension of this is the physiological character of lactate's role in mediating brain function (2). These canonical function involves learning and memory (3), cerebral blood flow (4), neurogenesis (5, 6) and cerebral microangiogenesis (7), energy metabolism (8), neuronal activity (9–11), and neuroprotection (12–15). Therefore, lactate is competent to be a potential therapy for ameliorating the pathological process of some brain diseases associated with impaired brain function. In mammals, lactate exists as two enantiomers. The structure of asymmetrical C2 carbon leads to the two stereoisomers of lactate that are designated as L-lactate and D-lactate (16). L-lactate is the major enantiomer found in the brain and blood whereas D-lactate is normally present in very

low concentrations under healthy physiological conditions (17). D-lactate is also considered as the rivalrous inhibitor of L-lactate since it competitively inhibits L-lactate transport (18). In different brain disease patterns, L- and D-lactate is reported to exert a similar or distinct effect on brain function. The involving mechanisms are far more complex than originally thought. For the most part, L-lactate can be utilized as a preferred energy substrate of neurons for meeting the energy demand (19, 20) or act as the novel hormone-like effect called lactormone (21, 22). But current research about D-lactate's role in brain function and brain-related disease is sparse and debatable. D-lactate-mediated mechanisms are also unclear.

In this narrative review, we aim to provide a comprehensive and profound summary of the role of lactate in brain function and related diseases. Consequently, we expound the food source of lactate intake, discuss the lactate enantiomers and their metabolism manner in the brain, compare the influence of L- and D-lactate on brain functions, expound on the effect of L- and D-lactate replenishment on several common brain diseases, and summarize the mechanisms of L-lactate.

LACTATE ENANTIOMERS

Lactate in mammals exists as two enantiomers: the most common form of L-isomers (known as L-lactate) is produced during mammalian glucose metabolism, and a quite small quantity of D-isomers (known as D-lactate) is generated from carbohydrates by bacterial metabolism (23, 24). The L-isomer of lactate is believed to have biological metabolic activity, while the D-isomer is too low in the body to activate the relevant enzymes for catabolism (25).

The Formation of Lactate in the Brain

L-Lactate

In the brain, the main source of L-lactate is astrocytic glycolysis from blood glucose, glycogen, and blood lactate (26, 27). In astrocytes, glucose can be converted directly to L-lactate by glycolysis or be stored in the form of glycogen (28). Glycogen is almost exclusively localized in astrocytes (20). As the neuronal activity intensifies, astrocytic glycogen is mobilized to supply neurons in case of neuronal glucose dissatisfying the energetic demand (29). In consequence, besides maintaining the astrocytes itself energy demand, L-lactate also supports neuronal activity by providing ATP (30, 31). Furthermore, the elevated blood L-lactate can also cross the blood-brain barrier into the brain *via* the monocarboxylate transporter 1 (MCT1) in some conditions (32, 33), such as vigorous exercise (34, 35) and fermentative (36) or fiber-containing foods (37).

D-Lactate

D-lactate, as the stereoisomer of L-lactate, is barely found in the brain and does not participate in energy production (23). A tiny amount of methylglyoxal (MG), the metabolic intermediary product, can be produced during glycolysis (38). The glyoxalase system, mostly located in astrocytes, allows bulk MG to convert into endogenic D-lactate or glutathione (GSH) (39).

The Metabolism of Lactate in the Brain

L-Lactate

The hypothesis of astrocyte-neuron lactate shuttle (ANLS) describing L-lactate shuttling between astrocytes and neurons is linked to glutamatergic signaling by Pellerin and Magistretti (40). A model opens the new insight for the L-lactate role in the brain and perfectly elaborates the mechanism of L-lactate how to serve as energy substrates. The ANLS switches on glutamate released by neuronal terminals and then taken up by astrocytes *via* the excitatory amino acid transporters 1 and 2 (EAAT1 and 2) to convert into glutamine or glutathione, which is activated by a gradient. This process stimulates the Na^+-K^+ pump to favor astrocytic mitochondrial acidification for launching glycolysis (21). Phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (Pfkfb3) is a key positive modulator of glycolysis. Its expression and activity are high in astrocytes but not in neurons. These cell-specific expression and activity profiles result in the capacity of glycolysis being more active in astrocytes than neurons (26). The stored glycogen first decomposes into glucose-6-Phosphate (glucose-6P) catalyzed by glycogen phosphorylase. Then the glucose-6P gives rise to two molecules of pyruvate. Finally, pyruvate can be either converted to acetyl coenzyme A (acetyl-CoA) to be used directly by the astrocytic TCA cycle or transformed into L-lactate by lactate dehydrogenase 5 (LDH5) (26, 39).

Monocarboxylate transporters (MCTs) belong to the SLC16 gene family and facilitate the transmembrane H+-linked monocarboxylate, like lactate, pyruvate, and ketone bodies, to shuttle between astrocytes and neurons for the brain metabolic demands (26, 41). MCT1 is mainly located at the membrane of astrocytes, oligodendrocytes, and endothelial cells of blood vessels (42–44). The *Km* value of MCT1 for L-lactate approximates 3.5 mM. MCT4 is widely expressed at the membrane of astrocytes to cooperate with MCT1 in L-lactate efflux transport, in which the *Km* value for L-lactate is 34 mM. Compared with MCT1 and MCT4, MCT2 is specific in the membrane of neurons (45, 46) and has a higher affinity for L-lactate with the *Km* value of 0.7 mM (20, 47). The L-lactate from astrocytes or blood will be released into intercellular substances through MCT1 and/or MCT4, transported by MCT2 into neurons (30, 48), and then oxidized at the Krebs cycle (21).

The L-lactate oxidation in neuronal cytoplasm into pyruvate by LDH1 is the first step to metabolic removal (1, 38). The newly generated pyruvate later crosses the mitochondrial inner membrane in favor of presumptive pyruvate transporters MPC (MPC1 and MPC2) and/or MCT1 (16, 49), to transform into acetyl-CoA for entering the tricarboxylic acid cycle (TCA) and therefore producing 14–17 ATPs per lactate molecule for maintaining neuronal activity (26). But the remarkable thing is that the MPC is only identified in yeast, drosophila, and humans (49). The molecular masses of MPC1 (15 kD) and MPC2 (14 kD) are akin to the findings in the experiment performed in rat liver and heart (50). That is to say, deficient evidence shows that the MPC exists in the neurons so far. The localization of MCT1 in mitochondrial inner membrane is not only verified in rat heart and muscle (51–53) but also in the rat cortical, hippocampal, and thalamic neurons (54). Besides deductive pyruvate transporters,

L-lactate may be directly oxidized by mitochondria (in heart, muscle, liver, spermatozoa, and brain) (22, 55) in the wake of the concept "cytosol-mitochondria lactate shuttle" proposed by Brooks' team (53, 56–58). In this model, L-lactate enters into the mitochondrial intermembrane space through porins in the outer mitochondrial membrane. Then it will be oxidized by putative mitochondrial LDH (mLDH) (located at the inner mitochondrial membrane) into pyruvate (59). Several studies have confirmed that L-lactate can be taken up and metabolized by the mLDH in both astrocytes (human astrocytoma cells) (60) and neurons (rat cerebellar granule cells, cortex neurons, and hippocampal neurons) (54, 61). The evidence implies that cytosol-mitochondria lactate shuttle may occur in the brain (**Figure 1**).

D-Lactate

D-lactate exerts metabolically inert in mammals (62) because of the absence of specific cytosolic lactate dehydrogenase (D-LDH) to metabolize D-lactate into pyruvate (63, 64). This enzyme barely exists in mammals (24). Some evidence shows that MCTs can transport D-lactate to inhibit L-lactate out of astrocytes or into neurons despite the *Km* value of MCTs for D-lactate being far higher than L-lactate (65, 66). The rate of oxidation of D-lactate in the brain is considerably slower than that of L-lactate due to the rather low expression levels of D-LDH in the brain (36, 67). Since interfering with more efficient energy substrates (pyruvate and L-lactate) for mitochondrial use, the physiological function of D-lactate is now known as the competitive inhibitor of L-lactate (18). Moreover, D-lactate is confirmed to interfere with the pyruvate metabolism in the brain, which eventually impairs in state 3 and state 4 of mitochondrial respiration (63), its accumulation may have an adverse impact on energy metabolism and thus lead to toxicity (**Figure 1**).

LACTATE INTAKES FROM THE FOOD

Lactate, as the predominant end-product of lactose fermentation in the food (68), is produced by the lactic acid bacteria, namely, gram-positive and catalase-negative microorganisms. Such microorganisms are involved in the fermentation of a range of foods and beverages, such as dairy products, meat, fish, vegetable, sourdough, wine, and cider (68), which creates the specific flavors and aromas for the food and benefits human health (69). People can uptake lactate from cheese, yogurt, wine, fermented vegetables (69), and fermented oyster extract (70). Thereby, lactate will appear in the gut *via* the consumption of fermented foods. In addition, prebiotic, fiber-containing foods also are the way of the lactate intake, such as broccoli, brussels sprouts, cabbage, cauliflower, collard greens, kale, radish, and rutabaga (37). Since gut microbiota is likely to produce a racemic mixture, it is not surprising that L- and D-lactate are generated simultaneously following the above food intake (37). The disposal of lactate in the lower gut can be converted to acetate, butyrate, propionate, and succinate. Alternatively, the unverified idea of "gut-soma lactate shuttle" is another way for the disposal of lactate in the gut, in which the lactate production in the gut releases into the systemic circulation (37). The discovery of the sodium-dependent monocarboxylate transporter (SMCT) (including SMCT1 and SMCT2) is favorable evidence for supporting this hypothesis, which is located in the mouse digestive tract and involved in the transport of food-derived monocarboxylates, such as lactate (71). The other implicit clue is the phenomenon of the rise in the blood L-lactate after a carbohydrate diet (37, 72). Tappy's team finds that dietary fructose or the co-mixture (fructose and glucose) facilitate the L-lactate release into the systemic circulation (73, 74). Furthermore, glucose rooted in the oxidation of carbohydrate only provides 10–20% energy, while other carbohydrate energy sources like glycogen and L-lactate accounts for 70–80% in the condition of exercise (75). That is to say when blood glucose is supported by hepatic glycogenolysis and gluconeogenesis, L-lactate plays important role in carbohydrate energy substrate distribution (72). As blood L-lactate can be transported into the brain *via* the MCT1 (32, 33), net L-lactate uptake directly provides 12% of brain fuel (37, 75, 76). Besides, gluconeogenesis provides 45% of brain fuel. Thereby, L-lactate comprises 57% of the total brain energy source (75). Regarding D-lactate, it can be excreted in urine by renal (64) or feces by gut (37) and cannot be detected in the blood under normal physiological conditions (64). Its excessive accumulation can result in acidosis and irritation of the lower bowel. Furthermore, the release of D-lactate into circulation also cause neurotoxic effect (37). The clinical presentation of D-lactic acidosis is characterized by episodes of encephalopathy and metabolic acidosis (24).

THE ROLE OF LACTATE IN REGULATING BRAIN FUNCTIONS

Lactate is reported to participate in the regulation of various brain functions in the terms of learning and memory (3), cerebral blood flow (4), neurogenesis (5, 6) and cerebral microangiogenesis (7), energy metabolism (8), neuronal activity (9–11), and neuroprotection (12–15). L-lactate and D-lactate are reported to exert similar or distinct effects on those functions.

The Distinction Between L-Lactate and D-Lactate for Influencing Learning and Memory

The Effect of L-Lactate on Learning and Memory

The glycogen is necessary for long-term potentiation maintenance in the mouse Shaffer collateral-CA1 synapse (77). Shima et al. find that 4-week exercise can increase glycogen reserve, along with the elevated transport rate of L-lactate into the neurons, to ameliorate memory dysfunction in diabetic rats (78). Extracellular L-lactate rapidly increases in the rat hippocampus during spontaneous alternation. Intrahippocampal 50 nM L-lactate can not only enhance the memory in this task but will rescue impaired memory in the condition of glycogenolysis inhibition (27). This evidence shows that the use of L-lactate metabolically coupled astrocytes and neurons depends on the character of high-energy demands for memory consolidation and storage (30). The provision of L-lactate by astrocytes is proved to be a more generally important element of learning and memory

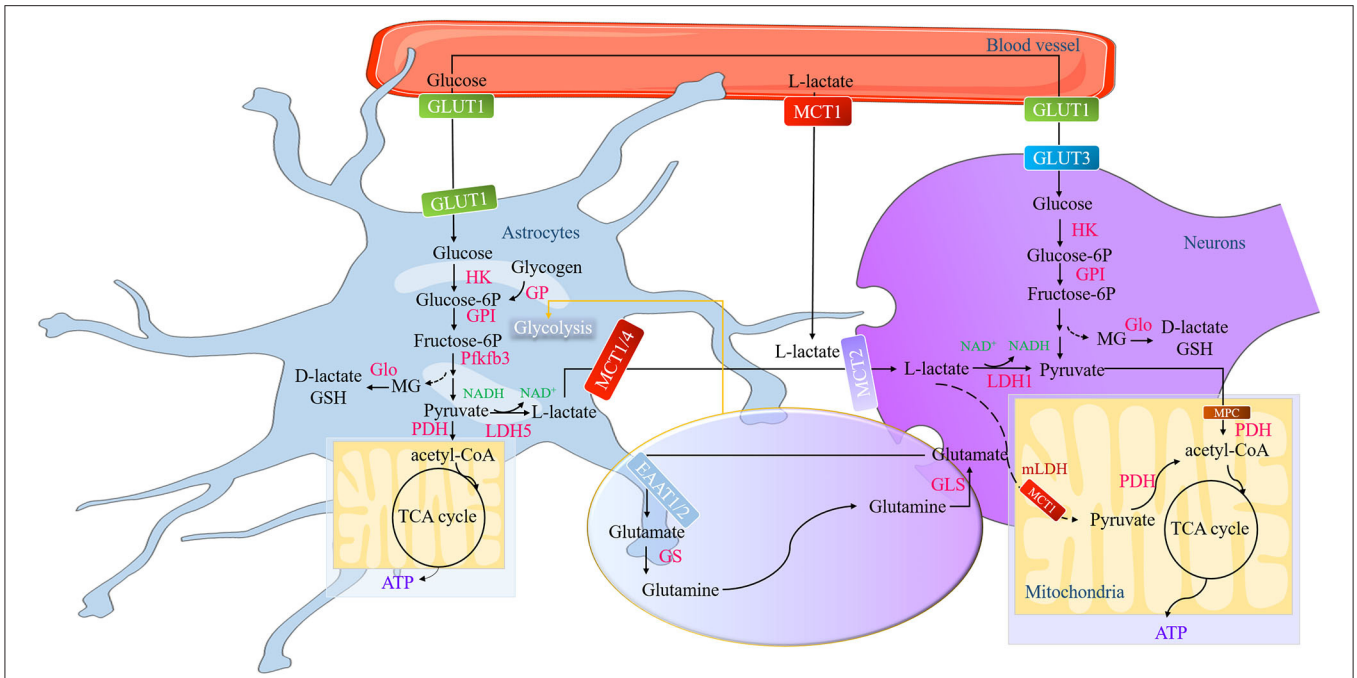


FIGURE 1 | The formation and metabolism of lactate in the brain. The action of the glutamate-glutamine cycle switches on the glycolysis in the astrocytes, a process of glutamate released by neuronal terminals and then is taken up by astrocytes *via* the EAATs to convert into glutamine or GSH. Pfkfb3 (a key positive modulator of glycolysis) is a high expression in astrocytes and low in neurons, while the activity of PDH is low in astrocytes and low in neurons. These characteristics determine that astrocytes have strong glycolysis ability and neurons have strong aerobic oxidation ability. Astrocytic glycogen first decomposes into glucose-6P catalyzed by GP and finally into pyruvate through a series of catalytic reactions. During this process, a tiny amount of MG generates and then converts into endogenic D-lactate or GSH. The newly formed pyruvate can be either converted to acetyl-CoA for the astrocytic TCA cycle or transformed into L-lactate by LDH5. The L-lactate in the astrocytes and in the brain blood vessel is transported into the neurons through MCTs (MCT1, MCT2, and MCT4) and then oxidized at the Krebs cycle. EAATs, excitatory amino acid transporters; GSH, glutathione; Pfkfb3, phosphofructokinase-2/fructose-2,6-bisphosphatase 3; PDH, pyruvate dehydrogenase; Glucose-6P, glucose-6-Phosphate; GP, glycogen phosphorylase; MG, methylglyoxal; acetyl-CoA, acetyl coenzyme A; lactate dehydrogenase 5, LDH5; Monocarboxylic acid transporters, MCTs.

processing than memory consolidation (29). Harris et al. find that the L-lactate produced by glycolysis, is required for memory acquisition but not for established memory in mice at the age of 9 months (79), suggesting that the main function of L-lactate is regulating the process of learning (80). Recent studies reveal the fact that L-lactate transporters are necessary for L-lactate mediating the memory process, especially MCT2. Inhibition of MCT1 or MCT2 can impair the rat reconsolidation of cocaine memory (81) or the mouse long-term memory formation (82). L-lactate replenishment will reverse the memory impairment in MCT1 or MCT4 knockdown of rat hippocampus (77). By contrast, L-lactate replenishment or even glucose supplement will not rescue the impaired memory once blocking MCT2 activity, which transports L-lactate into neurons (27, 77). In these studies, L-lactate in neurons is seen as the pyruvate donor, which produces ATP for neuronal energy demand (see section "Serves as an energy substrate for neurons"). Therefore, the transfer of L-lactate from astrocytes into neurons to support neuronal functions is necessary for memory consolidation (77, 83). The mechanisms may involve sustaining synaptic transmission and function (84–88) and regulating synaptic plasticity-related genes and proteins expression (89, 90). In facilitating the synaptic transmission and function, Tang et al. find that injections of 2 mM L-lactate into the locus coeruleus can activate an unidentified G_s receptor to evoke the NEergic neuronal excitability for norepinephrine release (85). Schurr et al. first find that L-lactate can replace glucose as a sole energy substrate for sustaining the normal synaptic function in rat hippocampal slices for hours (87). Lucas et al. declare that the physiological concentration of L-lactate can be utilized by presynaptic terminals as the energy for meeting the demand of maintaining functional presynaptic release sites (84). Herrera-Lo'pez et al. find that 1–2 mM L-lactate can induce glutamatergic synapse potentiation to promote the memory formation process in rat hippocampal CA3 pyramidal cells (86). As for modulating the synaptic plasticity genes and proteins expression, Margineanu et al. find that 20 mM L-lactate treatment can promote various plastic plasticity and plastic activity-related genes in mouse cortical neurons through RNA sequence (89). Yang et al. find that L-lactate from 2.5 to 20 mM will potentiate the NMDAR activity to improve the immediate early genes (IEGs) expression in mouse cortical neurons, which benefits the form of learning and memory (90). Hayek et al. find that intraperitoneal injection physiological concentration of L-lactate (117 or 180 mg/Kg) for 1 month can promote the proteins and genes expression of brain-derived neurotrophic factor (BDNF) and IEGs to improve the mouse learning and memory ability (3) (**Figure 2**).

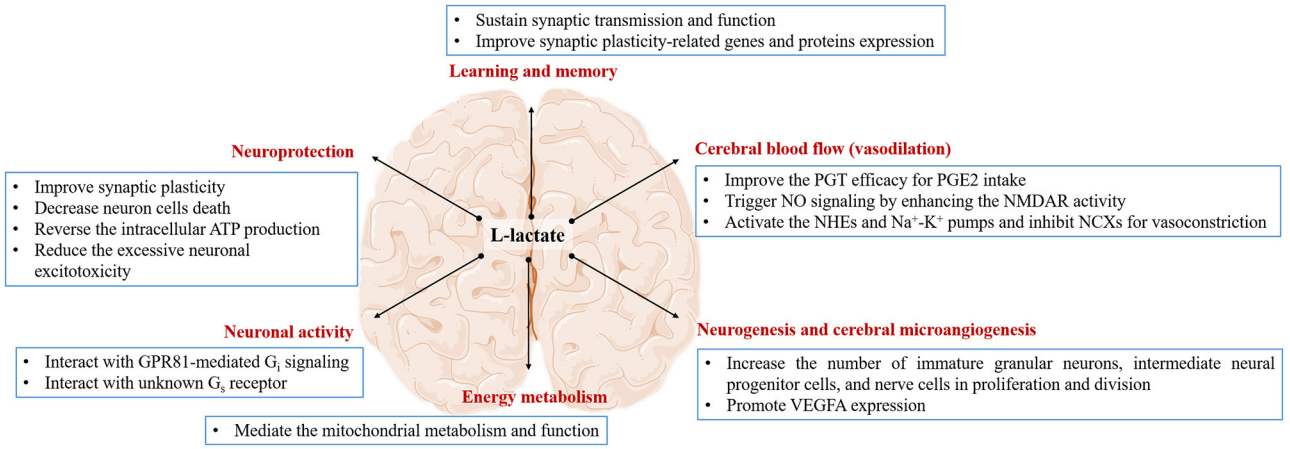


FIGURE 2 | The effect of L-lactate and its possible mechanism on the brain. L-lactate is capable of regulating brain function in the terms of learning and memory, cerebral blood flow, neurogenesis and cerebral microangiogenesis, energy metabolism, neuronal activity, and neuroprotection *via* different pathways.

The Effect of D-Lactate on Learning and Memory

D-lactate is proved to impair the neonatal chick memory process in the way of intracranial injection (23, 91). The reasons ascribe inhibition of L-lactate uptake into neurons and interference with the astrocytic metabolism. Baker and Edwards find that bilateral administration of D-lactate in the lower dosage range of 1.75–2.25 mM inhibits memory retention after discrimination avoidance task from 40 min and onward, and the inhibitory effect sustains 140 min. And the scope of the validity period is from 10 min before to 20 min after the task (91). This time window is similar to the dosage of 10 nM (23). The observation indicates that the inhibitory effect of D-lactate on memory is valid for a certain period (23). Further to support this idea is the study of rodents. Michael et al. find that intracranial injection of a higher concentration of D-lactate (18 mM) at 30 min before the Y maze task seems to not affect memory retention (92). Scavuzzo et al. even find that subcutaneous injections of D-lactate (1 g/kg) impair memory at 15 min before the inhibitory avoidance (IA) task, whereas it significantly enhances the rat memory at 2 min after the training (80) (**Table 1**). Thereby, it is still debatable that the different concentration and time-point of D-lactate usage lead to the distinct results for memory up to date. The conflict results are puzzling what is the physiological role of D-lactate in learning and memory. In short, it will be interesting to figure out the mechanism of D-lactate on the memory at the level of molecules and physiology. More interestingly, the results of intracranial unilateral injection by Gibbs and Hertz also show that D-lactate injection into left intermediate medial mesopallium inhibits memory formation from 10 min before task, whereas injection into the right hemisphere works from 10 min after task (23), implying that the time window of D-lactate on inhibiting memory formation varies from the part of the brain hemisphere.

L-Lactate Increases Cerebral Blood Flow (Vasodilation)

For the last few years, the function of L-lactate regulating cerebral blood flow (CBF) has been proposed in several studies (4, 95–98). First, a hypoxia-induced increase of L-lactate can improve the prostaglandin transporter (PGT) efficacy for vasodilator prostaglandin E2 (PGE2) intake in the brain (4). Second, exercise is reported to trigger the N-methyl D-aspartate (NMDA) receptor/nitric oxide (NO) signaling for realizing the hippocampal functional hyperemia during neural activation (99). Previous evidence indicates the effect of L-lactate on enhancing the NMDAR activity in cortex neurons (90). Moreover, when the porcine second-order retinal arterioles are pressurized to no flow, 10 mM L-lactate can induce the release of NO synthase to activate the downstream guanylyl cyclase/cGMP signaling, which opens the K_{ATP} channels for vasodilation in retinal arterioles (100). Contemplating all this, it is possible for L-lactate to dilate the brain microvessels for increasing CBF *via* NO biological effect. During hypoglycemia, the CBF increase can be considered neuroprotective since it is an attempt to increase capillary glucose concentration for improved glucose supply to the brain (101). Third, L-lactate has the autoregulatory vascular function in switching the pericyte response from contraction to dilation in the retinal vasculature. In the normal physiological state, L-lactate activates the Na^+/H^+ exchangers, inhibits Na^+/Ca^{2+} exchangers, and excites Na^+-K^+ pumps to trigger pericyte vasoconstriction regardless of the L-lactate concentration (**Figure 2**). While under the hypoxic condition, 20 mM L-lactate induces mural cell relaxation throughout the retinal vasculature, from the arterioles to the capillaries (102). Exploring the L-lactate-induced vascular reaction when dealing with the physiological and pathological situation, may be crucial for aiding in our understanding of neurovascular coupling, enhancing brain functions, and even treating multiple brain diseases.

TABLE 1 | The effect of D-lactate on the brain function.

Brain function	Model	Method of administration	Concentration	Intervention time	Effect
Learning and memory	Neonatal chick (91)	Tail intravenous injection	1.75–2.25 mM	The scope of validity period from 10 min before to 20 min after the discrimination avoidance task	Impair memory
	Neonatal chick (23)	Intracranial injection	10 nM	The scope of validity period from 10 min before to 20 min after the discrimination avoidance task Injection at 20 min before or 25 min after the task	Impair memory No adverse effect on memory
	9∼10-week-old rats (92)	Intracranial injection	18 mM	Injection at 30 min before the Y maze task	No adverse effect on memory retention
	6∼11-week-old rats (80)	Subcutaneous injection	1 g/kg	Injection within 2 min following IA task Injection at 15 min prior to IA task	Enhance memory Impair memory
Neuronal activity	Glutamatergic neurons and GABAergic neurons (9)		5 mM		Decrease the spontaneous calcium spiking frequency
Neuroprotection	tMCAO model Hippocampal slices in OGD model (12)	Intravenous injection	1 μmol/g 4 mM	Injection at 45 min after tMCAO 48 h after OGD	Decrease the infarct volume Reduce neuronal cell death
	NMDAR-induced neuronal excitotoxicity (93)		10 mM	22 min before adding glutamate.	No alteration in excitotoxicity
	Glutamate-induced neurotoxicity model (94)	Infusion	6 mM	Combination with containing glutamate perfusion medium	Aggravate the cortex lesion area

L-Lactate Benefits the Process of Neurogenesis and Cerebral Microangiogenesis

Neurogenesis is important for memory and learning. Lambertus et al. find that the 7-week exercise or 18 mM L-lactate can induce neurogenesis in the mouse ventricular-subventricular zone but not in the hippocampus (5). Notably, the author mentioned that neurogenesis in this zone contributes to the olfactory memory, which may provide contextual clues to the spatial-visual memory controlled by the hippocampus (5). Controversially, Vachnish et al. find that 6-week 13–17 mM L-lactate can promote mice hippocampal neurogenesis (6). The distinct results are probably related to the selection of neurogenic markers. The former refers to DCX and Ki-67 to represent neurogenesis, which shows immature granular neurons and intermediate neural progenitor cells, respectively. The latter chooses BrdU as the neurogenic marker, which shows nerve cells in proliferation and division. The different markers are on behalf of different stages of neurogenesis (103). The above evidence suggests that the neurogenic effect of L-lactate is tissue-specific. For example, the effect of L-lactate on the ventricular-subventricular zone depends on the GPR81 action but the hippocampus depends on the MCT2 metabolic action. Since neurogenesis is highly correlated with cerebral microangiogenesis, there is no doubt that angiogenesis also may be influenced by L-lactate in the brain. The direct evidence is supported by Moland's team. The study declares that 7-week 18 mM L-lactate is capable of increasing the density of microvessels in the dentate gyrus of the hippocampus along with a higher VEGFA expression. Furthermore, this effect depends on the GPR81 action (7) (**Figure 2**). In the further study, figuring out the approaches of L-lactate how to deal with neurogenesis and cerebral microangiogenesis (metabolic pathway, lactormone action, or both), may be worthiness for understanding the physiological role of L-lactate in brain function regulation.

L-Lactate Influences Brain Energy Metabolism

Mitochondria are best known for their role in the generation of ATP that supplies eukaryotes with energy to serve their cellular needs (104). Recent studies report that L-lactate can also mediate various mitochondria-related genes. A novel discovery is first reported by Brooks's team in L6 cells (105). They find that 20 mM L-lactate can upregulate 79 genes involved in mitochondrial metabolism (MFN1, MFN2, PGC-1α, NRF2,

LDHb, ATP5g1, NADH-dh, SDH, and TIM) and oxidative stress (GPX1, Glrx2, Glrx5, Prdx2, and Txndc12) (105). The evidence is also verified in the brain. For example, intraperitoneal injection of 18 mM L-lactate for 14 consecutive days will promote the PRC mRNA expression and the mtDNA levels (8). 20 mM L-lactate pretreats SY5Y cells can reverse high-concentration hydrogen peroxide (H_2O_2)-induced oxidative stress injury, including NRF2 expression improvement and mitochondrial membrane potential potentiation (106). The biological effect of L-lactate mediating mitochondrial metabolism and function is also in the primary mouse neurons. A total of 15–20 mM L-lactate can improve mitochondrial fusion (OPA1, MFN1, and MFN2), inhibit mitochondrial fission (DRP1 and FIS1), and promote biogenesis (PGC-1α, NRF2, TFAM, and mtDNA) (3, 107) (**Figure 2**). Besides, "cytosol-mitochondria lactate shuttle" also elaborates the role of L-lactate as the substrate source of mitochondrial ATP production (53, 56–58). Thus, L-lactate ought to have a close relationship with mitochondria in the brain. Considering the fact that multiple brain diseases in a large part are also associated with the energy crisis, the application of L-lactate in an animal-related experiment to shed light on the effect and mitochondria mechanism on brain energy metabolism as early as possible should have a great significance of the novel treatment in clinic brain diseases.

L-Lactate and D-Lactate Influence Neuronal Activity

The previous study demonstrates that lactate interacting with its receptor GPR81 operates negative feedback on neuronal activity. Application of 5 mM L- or D-lactate reversibly diminishes the calcium transient frequency by more than 50% in both principal and GABAergic neurons (**Table 1**). Moreover, the activation of GPR81 can also mimic a similar potency as the lactate (9). In fact, lactate can bind to GPR81 to couple with the $G_{i\alpha}$ subunit to inhibit the intracellular adenylate cyclase (AC)-cyclic adenosine monophosphate (cAMP) cascade signal that contributes to the reduction of exocytosis (10, 11). In addition, the GPR81 activation can bind to the $G_{i\beta}$ subunit to regulate the activity of phospholipase C (PLC) and thus inhibit neuronal excitability (10) by inducing a hyperpolarization for potassium (K^+) indrawal or activating the GABA receptor for exocytosis reduction (9). Interestingly, 5 mM L-lactate and 0.56 mM 3,5-dihydroxybenzoic acid (3,5-DHBA) (one of the GPR81 agonists) reduce the firing frequency of CA1 pyramidal cells, whereas a higher level of L-lactate (30 mM) and 3,5-DHBA (3.1 mM) increase the firing frequency (11). In the locus coeruleus, 2 mM L-lactate has rather an excitatory effect on the NEergic neurons by activating an unidentified Gs receptor, which will be abolished by the D-lactate (85) (**Figure 2**). Given these, we speculate that the effect of L-lactate on neuronal activity may depend on the L-lactate concentration, the types of neurons, and the types of receptors. Knowledge of the physiological effects of different concentrations and lactate isomers on the activity of different neurons, application of lactate is a potential therapeutic way to improve the complex neurological symptoms of epilepsy, which disease is characterized by neuronal hyperexcitability and sudden, synchronized electrical discharges (47, 108). In fact, some studies have focused on the application of L-lactate in treating epilepsy (see section Epilepsy).

The Neuroprotective Role of L-Lactate and D-Lactate

Lactate has been reported the role of neuroprotection in multiple brain disease models, however, the effect of the isomer of L- and D-lactate is split depending on the disease patterns. As known, transient middle cerebral artery occlusion (tMCAO) *in vivo* and oxygen and glucose deprivation (OGD) *in vitro* are the desired models for stroke. In the tMCAO model, L-lactate treatment can ameliorate neurological deficits after ischemia by inducing sustained neuroprotection for up to two weeks (14, 15). And as for the D-lactate treatment, it is also reported to exert neuroprotection by decreasing the infarct volume and ameliorating neurological deficits by working as the energy substrate or activating the receptor GPR81 (12). In the OGD model, the effect of ameliorating neuronal injury is possessed by both L-lactate and D-lactate (12–14). Hence, L- and D-lactate seem to exert a neuroprotective role in stroke.

In the human and rodent traumatic brain injury (TBI) model, evidence shows that endogenous produced brain L-lactate or exogenous L-lactate replenishment can facilitate neurologic recovery by improving synaptic plasticity (109) and regulating the brain metabolic state (76, 110–114) (see section "TBI"). However, the level of blood D-lactate is adopted as the biomarker of TBI degree. This is because that D-lactate is one of the microbe-dependent metabolites which will pass through the destroyed intestinal barrier into the blood when TBI causes gut dysbiosis (115, 116).

In addition, L-lactate but not D-lactate can reduce the excessive NMDAR-induced neuronal excitotoxicity, which is common in some acute brain pathologies. 10 mM L-lactate can improve the intracellular ATP production to stimulate metabotropic purinergic receptor P2Y for activating the PI3K pathway and opening the K_{ATP} channels, as a result of decreasing the intracellular Ca^{2+} concentration (93) (**Figure 2**). Furthermore, in the high concentration of glutamate-induced neurotoxicity model, infusion of 6 mM L-lactate can obviously reduce the rat cortex lesion from 6.05 ± 0.64 mm^3 to 4.16 ± 0.43 mm^3. On the contrary, the same concentration of D-lactate aggravates the lesion area from 2.7 ± 0.4 mm^3 to 4.4 ± 0.5 mm^3 (18) (**Table 1**). With a view to the metabolism of lactate and the universality of neuroprotective effects in the brain, L-lactate is a preferred research direction and practical value of the application and treatment in neuroprotection.

LACTATE IS THE POTENTIAL THERAPY FOR MULTIPLE BRAIN DISEASES

In the preceding decades, lactate was long considered as a waste product or even a sign of cerebral harm. As the proposal of the novel opinion such as "lactate shuttles" (37) and "lactormone" (22), these theories transform our stereotype and gradually unlock the mystery of lactate in physiological and pathological

states. Recently, lactate (especially L-lactate) is postulated to protect the brain in several pathologic conditions, such as diabetic encephalopathy, Alzheimer's disease (AD), stroke, TBI, and epilepsy.

Diabetic Encephalopathy

Diabetes mellitus often hurts brain health, which accompanies the symptoms of brain functional decline (117–119), especially cognitive impairment and vascular dementia (120). This symptom is called "diabetic encephalopathy" (121). Evidence shows that the brain lactate falls in response to hypoglycemia in diabetes (122, 123). Intravenous injection of L-lactate is capable of reducing the symptomatic and adrenaline responses to hypoglycemia and simultaneously alleviating hypoglycemia-induced cognitive dysfunction in diabetic patients (124–127). In addition, intracerebroventricular administration of 200 nM L-lactate can also mitigate the deterioration of memory consolidation *via* recovering the amplitude of sharp-wave ripples (SWRs) in diabetic mice (128). These results directly demonstrate that the L-lactate supplement benefits diabetic brain function. Another indirect evidence is that an increase of L-lactate transporter MCTs in the cortex and/or the hippocampus, especially MCT2 (transport L-lactate into neurons), is observed to be capable of ameliorating the cognitive impairment in diabetic encephalopathy (32, 78, 129). It is conceivable that L-lactate uptake enhancement supported by MCTs may improve diabetic encephalopathy. However, some studies show the phenomenon that the L-lactate increases in the brain regions of corpus callosum and hippocampus in diabetic encephalopathy (130–132). One possibility is the low utilization rate of L-lactate during diabetic encephalopathy (133, 134). The convincing evidence listed is that the L-lactate is secreted to increase in astrocytes but not in neurons in the condition of a high-glucose environment (134, 135), implying that the transport of L-lactate into neurons is hampered and thus the elevated brain L-lactate is observed in diabetic encephalopathy.

AD

Alzheimer's disease (AD) is one of the most common forms of brain disease (136), characterized by a series of neuropathological changes, such as cognitive dysfunction. Cognitive impairment in AD is associated with recessionary energy metabolism (137). Whereas, L-lactate seems like an important energy substrate for memory formation in connection with energy supply (19, 27). In fact, decreased L-lactate content is observed in the cerebral cortex and hippocampus in AD rodent models (138–140). Nevertheless, elevated L-lactate levels occur in aging models (94, 141, 142) and AD patients (143). The increased lactate dehydrogenase A (LDH-A)/lactate dehydrogenase B (LDH-B) gene activity ratios (94) and downregulated MCTs (MCT1, MCT2, and MCT4) are due for the opposite results (139, 140). This is because that increased brain L-lactate production owing to the LDH-A/LDH-B ratio but blockage of L-lactate transport from glia to neurons, resulting in L-lactate deficit in neurons. Inferior L-lactate utilization rate leads to energy deficiency in neurons and thereby exacerbates the progression of neuronal injury, including cognitive impairment during the pathological process of AD or aging.

Regarding D-lactate, a study reports that D-LDH, located at the inner side of the rat liver mitochondrial inner membrane (MIM), oxidizes D-lactate to pyruvate in the mitochondrial matrix (144). This process will improve oxidative phosphorylation (OXPHOS) efficiency, synthesis, and efflux of biosynthetic precursors from mitochondria, balance the cytosolic and mitochondrial GSH pools, and enhance the NADPH production in the cytosol. All of these can contribute to mitochondrial energy production (145). Nevertheless, the D-LDH isoforms in both human and mouse brains show a rather weak signal (67). Based on this evidence, we speculate that moderate D-lactate combined with D-LDH supplement may target a novel mitochondrial energetic mechanism for AD treatment.

Stroke

Stroke, one of the important factors causing neurological morbidity and mortality, is most characterized by ischemia and infarcts involving the territory of the middle cerebral artery (146). Berthet et al. adopt different concentrations of L-lactate treatment after OGD in the rat hippocampal slices. The results show that, immediately after OGD, the low dose treatment of L-lactate (4 mM) significantly inhibits the hippocampal neuron death, while L-lactate at the 20 mM dose aggravates the neuronal injury (14). Whereas, during OGD, only a high dose of L-lactate at 20 mM is the capacity of reversing the ATP decline to prevent cell death in primary cultured neurons and N2A cells, but not the low dose at 1 mM (13). Hence, the neuroprotective effect of L-lactate on stroke may be related to the time points for intervention or the types of neuron cells. Further study shows that L-lactate can reduce the size of the lesion area to promote neurological rehabilitation in the way of intracerebroventricular injection of 100 mM L-lactate (14) or tail vein injection of 200 mM L-lactate (15) immediately after tMCAO. They speculate that the ischemia-induced lactate increase may initially have a neuroprotective effect on the brain, but also may cause lactic acidosis when the lactate concentration reaches higher levels (14). Therefore, different concentrations of L-lactate may have different regulatory effects on brain metabolism and function under different physiological metabolic states. The complex metabolic characteristics of L-lactate bring immense challenges for revealing its role in brain function (14). Soon afterward, their team finds that similar neuroprotection occurs in D-lactate. The results suggest that intravenous injection of 1 μmol/g D-lactate will decrease about half of the infarct volume in tMCAO of the mice. Moreover, in the OGD model, 4 mM D-lactate treatment can also reduce about 50% neuronal cell death in the rat hippocampal slices (12). Future studies need further excellently clarify the mechanism of how lactate performs neuroprotection although it has fully considered the dual role of lactate as the energy substrate (pyruvate replacement) or signal molecular (receptor agonist replacement). So far, two results are responsible for explaining the neuroprotective effect of lactate on stroke. First, the accumulated lactate is transported by MCTs into the neurons and utilized as the alternative energy substrate immediately postischemia (147) in favor of sustaining neuronal integrity (148) and delaying the neuronal damage (149).

Second, lactate may interact with the known receptor-GPR81 or unknown putative G_s-type receptors to resist ischemic injury. It is worth noting that the evidence and characterization of GPR81 are still disputed in stroke. For example, Castillo et al. find the 3,5-DHBA treatment, one of the GPR81 agonists, can reduce neuronal cell death in OGD (12). In contrast with the study, Shen et al. find that inhibition of the GPR81 activity can promote the ERK1/2 signal to copy with apoptosis in neurons (13). Moreover, a newly published study also reports that the GPR81 agonists (3Cl-5OH-BA and 3,5-DHBA) do exert no neuroprotective effect on stroke mice whether intravenous administration or intracerebroventricular administration (150). Applying the genetic tools for GPR81 knockout may be a preferred protocol to definite the role and the mechanism of this receptor in the stroke.

TBI

Traumatic brain injury (TBI) is a type of brain injury acquired from an external force that inflicts devastating effects on the brain vasculature and neurons (116). It is always reported to face an energy crisis in the brain in the fact of glucose uptake suppression following cerebral injury. Although glucose is the preferred fuel of the brain, it may result in odious insults, such as hyperglycemia and infection or mortality events increment (151–153). As in previous studies, many TBI patients have observed the increased net L-lactate uptake after-hours postinjury (76, 154) without hyperglycemia (110), implying the protective function of L-lactate in TBI. Increasing blood arterial L-lactate to supraphysiologic range (4–5 mmol/l) in the early phase of brain injury can accelerate the recovery of neural function through cerebral perfusion and brain glucose availability improvement (155). Brooks and Martin find that endogenous L-lactate, induced by TBI, can support brain metabolism indirectly or directly (76). On the one hand, endogenous lactate is reported to be related to accelerating hepatic and renal gluconeogenesis to make glucose available for essential organs like the brain. On the other hand, elevated blood L-lactate may provide substrate directly to the injured brain in link with the L-lactate shuttle (114). Through isotope tracer, ∼70% carbohydrate (direct lactate uptake and indirect glucose uptake from lactate) is provided for the TBI brain (113), suggesting the importance of endogenous L-lactate generation as the energy supply in TBI. In addition, exogenous intraperitoneal injection of 500 mg/kg L-lactate before 30 min of TBI is reported to alleviate the brain injury-induced neurological deficits by promoting the neuronal plasticity proteins (PSD95, GAP43, and BDNF) (109). Intravenous infusion of 5 mM L-lactate is also verified to utilize with sparing of cerebral glucose along with a reduction of brain glutamate and intracranial pressure after TBI, which implies the benefit of cerebral metabolic and hemodynamic effects (110). A total of 100 mM L-lactate infusion is shown reduced lesion volume at day 2 after cerebral cortical impact through slightly regulating cerebral blood flow (111). Hence, L-lactate, as the alternative source of glucose, has dream prospects to improve the outcome of posttraumatic brain damage.

Recently, the lactate receptor GPR81 is also reported to potentially participate in the pathological process of TBI. The evidence is that the increased GPR81 gene expression lasts at least 28 days after TBI injury in lesion areas of the cerebral cortex and hippocampus (156). Coincidentally, L-lactate further enhances the expression of GPR81 in the ipsilateral cortex and hippocampus 24 h after TBI (109). These results show that L-lactate may not only be the brain fuel, but also the possible upstream molecule that interacts with the receptor to activate or inhibit the downstream molecular signals for neuroprotection in the TBI.

Epilepsy

Epilepsy is a common neurological emergency with considerable associated healthcare costs, morbidity, and mortality, which is often causing neuronal and glial damage (157). During epilepsy, L-lactate is observed to rise in the brain regions of cerebral gray and white matter (158), cerebrospinal fluid (159), cerebral cortex (160), and hippocampus (161). Previously, one research has proved that the elevated L-lactate level in tissues can persist for approximately 1 h (161). In some cases, the L-lactate level can even reach 6 mM (162). Jorwal and Sikdar find that 6 mM L-lactate application reduces the spike frequency and hyperpolarizes the subicular neurons in rat hippocampal slices, suggesting an anticonvulsant effect of L-lactate. Further electrophysiological recordings reveal that L-lactate induces the $G_{i\beta\gamma}$ subunit activity to expedite the inwardly rectifying potassium (GIRK) channel. They also point out an interesting phenomenon that normal brain concentration of L-lactate (2 mM) and even up to 6 mM have few effects on rat hippocampus neural activity in the absence of epileptiform activity. This fact reminds us that the important role of elevated brain L-lactate in epilepsy (163). It is worth thinking that since there is no negative effect of excessive brain L-lactate on the mean spike frequency and membrane potential of subicular pyramidal neurons in the normal physical condition, what physiological effects can this L-lactate have after being metabolized or working as the neurotransmitter? Considering the fact that and concentration of 6 mM L-lactate is sufficient to activate its receptor GPR81, which is also known to couple to $G_{i\beta\gamma}$ subunit to inhibit the neuronal activity (10). Another function of GPR81 is to couple to the $G_{i\alpha}$ subunit to inhibit the AC-cAMP cascade signal, as a result, the decrease of neuronal excitement (11). However, unlike L-lactate, 3,5-DHBA does not result in hyperpolarizing subicular neurons (10). Therefore, it is likely that L-lactate may interact with other unknown G_i-type proteins. L-lactate-receptor signals may be the novel target for antiepileptic treatment.

THE NEUROBIOLOGICAL ROLE OF L-LACTATE IN THE BRAIN

Serves as an Energy Substrate for Neurons

Astrocytic derived-L-lactate has been proposed to serve as an energy pool for neurons (40). In the presence or absence of oxygen, glycogen in the astrocytes can be broken down into L-lactate (30). An interesting phenomenon that manifests the role of L-lactate in neuronal energy metabolism is that the levels of glycogen in various brain regions (cortices,

hippocampus, brainstem, epencephalon, and hypothalamus) are constant during the first hour of moderate-intensity exercise, while the levels of glycogen will decrease approximately 50% in the several hours that follow. Accompanied with the decreased glycogen levels are the elevated L-lactate levels in these regions (164). A similar phenomenon exists in the exhaustive swimming model, especially in the hippocampus (165). L-lactate oxidation metabolism is preferentially located in neurons by using the 3-[^{13}C] lactate label (166). Furthermore, when increasing the plasma L-lactate levels, the cerebrum tends to reduce glucose utilization and prefers to utilize L-lactate by using the 1-[^{11}C]L-lactate and [^{18}F]fluorodeoxyglucose (FDG) for local cerebral metabolism measurement (148). The formed L-lactate in the brain is a crucial aerobic energy substrate that enables neurons to endure activation (167). Another evidence for L-lactate is the energy substrate of neurons is that the capacity of sustaining the presynaptic transmission function in the lack of glucose. The interesting note is that the transport of L-lactate into neurons is significant for ATP production to satisfy the energy demand under physiological or hypoxic conditions (28, 84, 168) (**Figure 3**).

Signal Pathways Regulation as a Lactormone

In addition to being an energy substrate, L-lactate is also suggested its role as a signal regulator molecule or neurotransmitter since the proposal of "lactormone" (22) and the confirmation of its endogenous receptor GPR81 in mice brain (169).

Influences the Intracellular NADH/NAD$^+$ Ratio

The catalytic action of LDH1 in neurons allows L-lactate to convert into pyruvate. This process of reducing NAD$^+$ to NADH is supposed to influence NMDAR excitability (16, 93, 170). It is L-lactate, but not pyruvate, that generates NADH to enhance the NMDAR activity, which increases the intracellular Ca^{2+} and then promotes the expression of plasticity-associated genes (90). Along with the changes of redox state (NADH/NAD$^+$ ratio), L-lactate is permissible to activate the NAD$^+$-dependent histone deacetylase Sirtuin1 (SIRT1) (3, 171). SIRT1 deacetylates and amplifies the activity of the peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α), which is known to mediate the BDNF expression (3) and mitochondrial biogenesis (172, 173). For example, Hayek et al. find that L-lactate can induce the expression of SITRT1 to activate the PGC1-1α, which will contribute to BDNF signals and some immediate early gene (IEGs) as a result in the mouse neurons (3). Our experiment also finds that a high concentration of L-lactate promotes the PGC-1-NRF2 signal axis and subsequently boosts mitochondrial biogenesis in mouse primary hippocampal cells (107) (**Figure 3**). However, Lezi et al. find that 14 consecutive-day-intraperitoneal injections of 18 mM L-lactate do not affect the PCG-1α-mediated mitochondrial biogenesis in the mouse brain (8). This may relate to the short administration time or the examination of the whole brain.

Activates Reactive Oxygen Species-Related Signals

It is well-known that excessive reactive oxygen species (ROS) production is considered a cause of several pathological conditions, which are harmful to the cells. Yet, low ROS production may be relevant to cellular activity under physiological conditions (174). Moreover, the moderate elevation of ROS can even protect against oxidative damage through the induction of antioxidant and detoxification enzymes (175).

Hashimoto et al. first report that L-lactate is able to stimulate some of the ROS-sensitive transcription factors' activity in L6 cells. Their research also reports that the PGC1α, known as a master coordinator of mitochondrial biogenesis, is activated in a high concentration of L-lactate treatment which is believed to be regulated by ROS and H_2O_2. Then the activated PGC1α interacts with transcription factors for mitochondrial gene expression, including cAMP-response element-binding protein (CREB) and nuclear respiratory factor (NRF)-2 in L6 cells (105). L-lactate metabolism also potentiates the phosphorylation of adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) to activate PCG-1α signals for mitochondrial biogenesis and inhibits the phosphorylation of mTOR for mitophagy *via* mild production of ROS in skin fibroblasts (175). Recently, a similar phenomenon occurs in neurons. In SY5Y cells, 20 mM L-lactate is proved to induce moderate ROS for antioxidant stress and cellular homeostasis improvement, for instance, activating mTOR signaling for cell growth and survival, phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (AKT) signaling for neuroprotection, mild endoplasmic reticulum stress (ERS) for protein homeostasis, and NRF2 signaling for ROS detoxification (**Figure 3**). Interestingly, it is an extremely high concentration of L-lactate (100 mM) but not lower or physiological concentration of L-lactate (50 and 10 mM) exerts antiaging phenotypes when *Caenorhabditis elegans* suffer from oxidative stress. Furthermore, 10 mM L-lactate suffice to resist stress and increase their lifespan (106).

Noteworthy, it is not known how L-lactate is ready-witted enough to mediate and maintain moderate ROS production for safeguarding cells when a high concentration of H_2O_2 provokes excessive ROS. One of the reasons may be associated with the potential ability of L-lactate sensing and decreasing the mitochondrial H_2O_2 production accurately to ensure moderate ROS production (175). Another hypothesis of L-lactate mitochondrial metabolism may explain the ROS signals activation. Bari et al. find that L-lactation can generate the H_2O_2 production *via* the inner membrane of putative L-lactate oxidase (LOX) in the pure rat liver mitochondria (176). Hence, L-lactate metabolism in mitochondria is likely to produce partial ROS and then activate its related signals if there exists analogous oxidase located at the mitochondrial inner membrane in the neurons. The evidence supports this hypothesis is that a mitochondrial lactate oxidation complex (mLOC) (including MCT1 or MCT2, LDH, and COX) is present in the neurons discovered by Hashimoto et al. (54). However, how L-lactate metabolism in neurons produces and regulates ROS in the subsequent process remains to be further investigated.

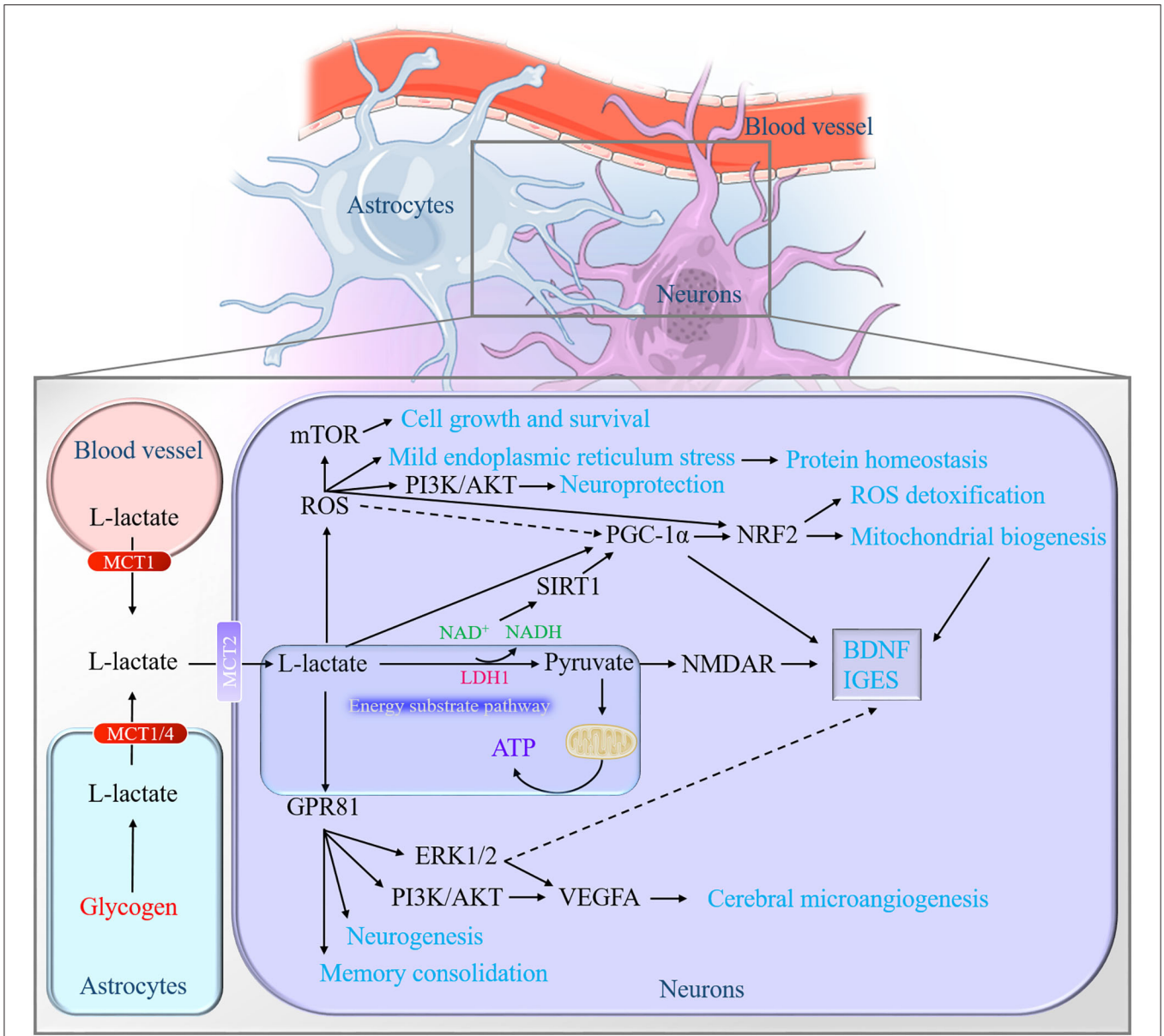


FIGURE 3 | Graphical summary of the neurobiological role of L-lactate in the brain. On the one hand, L-lactate serves as an energy pool for neurons to facilitate mitochondrial energy production through converting into pyruvate. On the other hand, L-lactate may act as the role like the hormone, defined as lactormone. It triggers multiple downstream signaling cascades *via* influencing the intracellular NADH/NAD^+ ratio, mediating the ROS generation, and activating the receptor GPR81. NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; GPR81, G_i-protein-coupled receptor 81.

Interacts With the Receptor GPR81

GPR81 is a type of G protein (G_i) coupled receptor and involves the metabolic process of some tissues and cells (177–179). So far, recent research has revealed that GPR81 regulates multiple signal pathways in tissues and cells, such as the extracellular regulated protein kinases (ERK1/2) (7, 180–182), nod-like receptor family pyrin domain-containing 3 (NLRP3)/ nuclear factor kappa-B (NF-κB) inflammation (183, 184), peroxisome proliferator-activated receptor γ (PPARγ) (185, 186) and Wnt signaling pathway (187, 188).

In the brain, it expresses in the regions of the pituitary (177), hippocampus, cerebellum, and brain stem (169). Cell localization indicates that it widely distributes at neuronal synaptic membranes (169), implying that GPR81 may have an important role in regulating brain function. It is known as the endogenic lactate receptor in 2008 (189, 190). The efficiency of lactate on GPR81 has a wide range from 0.2 to 30 mM (191). Furthermore, partial activation of GPR81 by lactate requires only 0.2–1 mM (192). Therefore, lactate absolutely has the ability to activate the GPR81 in the physiological state

(human blood lactate concentration range is 0.5–2 mM) (193). Needless to say, in the conditions of intensive exercise and hypoxia, the blood lactate level can even reach 20–30 mM (194, 195). In neuronal activity, the activation of GPR81 inhibits the excitability of neurons (9, 10). In cellular energy, the astrocytic glycolysis rates accelerate ATP production to meet the energy requirements of neurons when the GPR81 agonists stimulate the cells (196). In terms of cerebral microangiogenesis and neurogenesis, L-lactate and exercise enhance the PI3K/AKT and ERK1/2 signaling pathway to promote VEGFA protein expression and vascular density in the dentate gyrus of mice *via* GPR81 (7). In addition, the neurogenesis effect of both L-lactate and exercise on the ventriculosubventricular region is dependent on the GPR81 in the mice (5). As for the learning and memory, intraperitoneal injection of GPR81 agonist 3,5-DHBA before the inhibitory avoidance task will impair the memory of rats, while the injection after the training contributes to memory consolidation. The distinct effects may be attributed to the reasons that the activation of GPR81 in the forebrain after training, can promote slow-wave activity (SWA) and enhance the consolidation of previous experience, but it also impairs the ongoing learning process at the same time (80). The results suggest that GPR81 activation at different stages of training may have different functions of influencing learning and memory. Synaptic plasticity, induced by appropriate synapses, is both necessary and sufficient for the information storage of memory (197). Previous studies revealed that L-lactate could promote plastic plasticity-related proteins and genes expression *via* enhancing the NMDAR-mediated phosphorylation of ERK1/2 (89, 90). The interesting thing is the fact that GPR81 is upstream of the ERK1/2 (7) (**Figure 3**). Hence, GPR81 is likely to act as a sensor for L-lactate metabolism in neurons and participate in L-lactate-regulated synaptic plasticity (198).

CONCLUSION

To summarize the current evidence, this review shows that lactate participates in coordinating various brain functions in both the healthy and diseased states. The involving mechanisms are far more complex than originally thought and further knowledge of the lactate operating, as outlined in this article, is a drop in the bucket to our understanding of brain physiological and pathological mysteries. L-lactate not only serves as the energy substrate but also acts as lactormone in the regulation of downstream cascaded signaling pathways. Considering the consensus that lactate is a common metabolite and readily available (food intake or exercise), knowledge of lactate's role in the brain may provide precise tactics for brain diseases involving lactate metabolism. It also may set new insight and ideas for studying the relationship between lactate metabolism and brain function improvement.

AUTHOR CONTRIBUTIONS

MC and HW drafted the manuscript. HS, RY, and LW assisted with drafting the tables and figures. XX suggested valuable advice for the article. WS and JH conceptualized the article and revised the final version. All authors read and approved the final manuscript.

FUNDING

This work was sponsored by Shanghai Sailing Program (22YF1441600), the grant of the funding of Youth Fund Project of Research Planning Foundation on Humanities and Social Sciences of the Ministry of Education (20YJCZH001), China Postdoctoral Science Foundation (2021M702128), and the Scientific Research Foundation of SUMHS (SSF-21-03-008).

REFERENCES

1. Mason S. Lactate shuttles in neuroenergetics-homeostasis, allostasis and beyond. *Front Neurosci*. (2017) 11:43. doi: 10.3389/fnins.2017.00043
2. Todd JJ. Lactate: valuable for physical performance and maintenance of brain function during exercise. *Biosci Horiz*. (2014). doi: 10.1093/biohorizons/hzu001
3. El Hayek L, Khalifeh M, Zibara V, Abi Assaad R, Emmanuel N, Karnib N, et al. Lactate mediates the effects of exercise on learning and memory through SIRT1-dependent activation of hippocampal brain-derived neurotrophic factor (BDNF). *J Neurosci*. (2019) 39:2369–82. doi: 10.1523/JNEUROSCI.1661-18.2019
4. Gordon GR, Choi HB, Rungta RL, Ellis-Davies GC, MacVicar BA. Brain metabolism dictates the polarity of astrocyte control over arterioles. *Nature*. (2008) 456:745–9. doi: 10.1038/nature07525
5. Lambertus M, Overberg LT, Andersson KA, Hjelden MS, Hadzic A, Haugen OP, et al. L-lactate induces neurogenesis in the mouse ventricular-subventricular zone via the lactate receptor HCA1. *Acta Physiol*. (2021) 231:e13587. doi: 10.1111/apha.13587
6. Lev-Vachnish Y, Cadury S, Rotter-Maskowitz A, Feldman N, Roichman A, Illouz T, et al. L-Lactate promotes adult hippocampal neurogenesis. *Front Neurosci*. (2019) 13:403. doi: 10.3389/fnins.2019.00403
7. Morland C, Andersson KA, Haugen OP, Hadzic A, Kleppa L, Gille A, et al. Exercise induces cerebral VEGF and angiogenesis via the lactate receptor HCAR1. *Nat Commun*. (2017) 8:15557. doi: 10.1038/ncomms15557
8. E L, Lu J, Selfridge JE, Burns JM, Swerdlow RH. Lactate administration reproduces specific brain and liver exercise-related changes. *J Neurochem*. (2013) 127:91–100. doi: 10.1111/jnc.12394
9. Bozzo L, Puyal J, Chatton JY. Lactate modulates the activity of primary cortical neurons through a receptor-mediated pathway. *PLoS ONE*. (2013) 8:e71721. doi: 10.1371/journal.pone.0071721
10. de Castro Abrantes H, Briquet M, Schmuziger C, Restivo L, Puyal J, Rosenberg N, et al. The lactate receptor HCAR1 modulates neuronal network activity through the activation of galpha and gbetagamma subunits. *J Neurosci*. (2019) 39:4422–33. doi: 10.1523/JNEUROSCI.2092-18.2019
11. Herrera-Lopez G, Galvan EJ. Modulation of hippocampal excitability via the hydroxycarboxylic acid receptor 1. *Hippocampus*. (2018) 28:557–67. doi: 10.1002/hipo.22958
12. Castillo X, Rosafio K, Wyss MT, Drandarov K, Buck A, Pellerin L, et al. A probable dual mode of action for both L- and D-lactate neuroprotection in cerebral ischemia. *J Cereb Blood Flow Metab*. (2015) 5:1561–9. doi: 10.1038/jcbfm.2015.115
13. Shen Z, Jiang L, Yuan Y, Deng T, Zheng YR, Zhao YY, et al. Inhibition of G protein-coupled receptor 81 (GPR81) protects against ischemic brain injury. *CNS Neurosci Ther*. (2015) 21:271–9. doi: 10.1111/cns.12362

14. Berthet C, Lei H, Thevenet J, Gruetter R, Magistretti PJ, Hirt L. Neuroprotective role of lactate after cerebral ischemia. *J Cereb Blood Flow Metab.* (2009) 29:1780–9. doi: 10.1038/jcbfm.2009.97
15. Berthet C, Castillo X, Magistretti PJ, Hirt L. New evidence of neuroprotection by lactate after transient focal cerebral ischaemia: extended benefit after intracerebroventricular injection and efficacy of intravenous administration. *Cerebrovasc Dis.* (2012) 34:329–35. doi: 10.1159/000343657
16. Ferguson BS, Rogatzki MJ, Goodwin ML, Kane DA, Rightmire Z, Gladden LB. Lactate metabolism: historical context, prior misinterpretations, and current understanding. *Eur J Appl Physiol.* (2018) 118:691–728. doi: 10.1007/s00421-017-3795-6
17. Kondoh Y, Kawase M, Kawakami Y, Ohmori S. Concentrations of D-lactate and its related metabolic intermediates in liver, blood, and muscle of diabetic and starved rats. *Res Exp Med.* (1992) 192:407–14. doi: 10.1007/BF02576298
18. Ros J, Pecinska N, Beat Alessandri, Landolt H, Fillenz M. Lactate reduces glutamate-induced neurotoxicity in rat cortex. *J Neurosci Res.* (2001) 66:790–4. doi: 10.1002/jnr.10043
19. Bouzier-Sore AK, Voisin P, Canioni P, Magistretti PJ, Pellerin L. Lactate is a preferential oxidative energy substrate over glucose for neurons in culture. *J Cereb Blood Flow Metab.* (2003) 23:1298–306. doi: 10.1097/01.WCB.0000091761.61714.25
20. Pellerin L, Magistretti PJ. Sweet sixteen for ANLS. *J Cereb Blood Flow Metab.* (2012) 32:1152–66. doi: 10.1038/jcbfm.2011.149
21. Sobral-Monteiro-Junior R, Maillot P, Gatica-Rojas V, Avila WRM, de Paula AMB, Guimaraes ALS, et al. Is the "lactormone" a key-factor for exercise-related neuroplasticity? A hypothesis based on an alternative lactate neurobiological pathway. *Med Hypotheses.* (2019) 123:63–6. doi: 10.1016/j.mehy.2018.12.013
22. Brooks GA. Cell-cell and intracellular lactate shuttles. *J Physiol.* (2009) 587:5591–600. doi: 10.1113/jphysiol.2009.178350
23. Gibbs ME, Hertz L. Inhibition of astrocytic energy metabolism by d-lactate exposure impairs memory. *Neurochem Int.* (2008) 52:1012–8. doi: 10.1016/j.neuint.2007.10.014
24. Uribarri J, Oh MS, Carroll HJ. D-lactic acidosis. A review of clinical presentation, biochemical features, and pathophysiologic mechanism. *Medicine.* (1998) 77:73–82. doi: 10.1097/00005792-199803000-00001
25. Robergs RA, Ghiasvand F, Parker D. Biochemistry of exercise-induced metabolic acidosis. *Am J Physiol Regul Integr Comp Physiol.* (2004) 287:R502–16. doi: 10.1152/ajpregu.00114.2004
26. Magistretti PJ, Allaman I. A cellular perspective on brain energy metabolism and functional imaging. *Neuron.* (2015) 86:883–901. doi: 10.1016/j.neuron.2015.03.035
27. Newman LA, Korol DL, Gold PE. Lactate produced by glycogenolysis in astrocytes regulates memory processing. *PLoS ONE.* (2011) 6:e28427. doi: 10.1371/journal.pone.0028427
28. Sickmann HM, Walls AB, Schousboe A, Bouman SD, Waagepetersen HS. Functional significance of brain glycogen in sustaining glutamatergic neurotransmission. *J Neurochem.* (2009) 109(Suppl. 1):80–6. doi: 10.1111/j.1471-4159.2009.05915.x
29. Gold PE, Newman LA, Scavuzzo CJ, Korol DL. Modulation of multiple memory systems: from neurotransmitters to metabolic substrates. *Hippocampus.* (2013) 23:1053–65. doi: 10.1002/hipo.22182
30. Steinman MQ, Gao V, Alberini CM. The role of lactate-mediated metabolic coupling between astrocytes and neurons in long-term memory formation. *Front Integr Neurosci.* (2016) 10:10. doi: 10.3389/fnint.2016.00010
31. Matsui T, Omuro H, Liu YF, Soya M, Shima T, McEwen BS, et al. Astrocytic glycogen-derived lactate fuels the brain during exhaustive exercise to maintain endurance capacity. *Proc Natl Acad Sci.* (2017) 114:6358–63. doi: 10.1073/pnas.1702739114
32. Aveseh M, Nikooie R, Sheibani V, Esmaeili-Mahani S. Endurance training increases brain lactate uptake during hypoglycemia by up regulation of brain lactate transporters. *Mol Cell Endocrinol.* (2014) 394:29–36. doi: 10.1016/j.mce.2014.06.019
33. Masaki Takimoto TH. Acute exercise increases brain region-specific expression of MCT1, MCT2, MCT4, GLUT1, and COX IV proteins. *J Appl Physiol.* (2014) 116:1238–50. doi: 10.1152/japplphysiol.01288.2013
34. Hashimoto T, Tsukamoto H, Takenaka S, Olesen ND, Petersen LG, Sorensen H, et al. Maintained exercise-enhanced brain executive function related to cerebral lactate metabolism in men. *Faseb J.* (2018) 32:1417–27. doi: 10.1096/fj.201700381RR
35. van Hall G, Stromstad M, Rasmussen P, Jans O, Zaar M, Gam C, et al. Blood lactate is an important energy source for the human brain. *J Cereb Blood Flow Metab.* (2009) 29:1121–9. doi: 10.1038/jcbfm.2009.35
36. Tubbs PK. The metabolism of D-alpha-hydroxy acids in animal tissues. *Ann N Y Acad Sci.* (1965) 119:920–6. doi: 10.1111/j.1749-6632.1965.tb47452.x
37. Brooks GA. The science and translation of lactate shuttle theory. *Cell Metab.* (2018) 27:757–85. doi: 10.1016/j.cmet.2018.03.008
38. Adeva-Andany M, López-Ojén M, Funcasta-Calderón R, Ameneiros-Rodríguez E, Donapetry-García C, Vila-Altesor M, et al. Comprehensive review on lactate metabolism in human health. *Mitochondrion.* (2014) 17:76–100. doi: 10.1016/j.mito.2014.05.007
39. Finsterwald C, Magistretti PJ, Lengacher S. Astrocytes: new targets for the treatment of neurodegenerative diseases. *Curr Pharm Des.* (2015) 21:3570–81. doi: 10.2174/1381612821666150710144502
40. Pellerin L, Magistretti PJ. Glutamate uptake into astrocytes stimulates aerobic glycolysis: A mechanism coupling neuronal activity to glucose utilization. *Proc Nat Acad Sci.* (1994) 9:10625–9. doi: 10.1073/pnas.91.22.10625
41. Baltazar F, Afonso J, Costa M, Granja S. Lactate beyond a waste metabolite: metabolic affairs and signaling in malignancy. *Front Oncol.* (2020) 10:231. doi: 10.3389/fonc.2020.00231
42. Pierre K, Pellerin L, Debernardi R, Riederer BM, Magistretti PJ. Cell-specific localization of monocarboxylate transporters, MCT1 and MCT2, in the adult mouse brain revealed by double immunohistochemical. *Neuroscience Vol.* (2000) 100:617–27. doi: 10.1016/S0306-4522(00)00294-3
43. Gerhart DZ, Enerson BE, Zhdankina OY, Leino RL, Drewes LR. Expression of monocarboxylate transporter MCT1 by brain endothelium and glia in adult and suckling rats. *Am J Physiol.* (1997) 273:E207–213. doi: 10.1152/ajpendo.1997.273.1.E207
44. Hanu R, McKenna M, O'Neill A, Resneck WG, Bloch RJ. Monocarboxylic acid transporters, MCT1 and MCT2, in cortical astrocytes *in vitro* and *in vivo*. *Am J Physiol Cell Physiol.* (2000) 278:C921–30. doi: 10.1152/ajpcell.2000.278.5.C921
45. Rafiki A, Boulland JL, Halestrap AP, Ottersen OP, Bergersen L. Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. *Neuroscience.* (2003) 122:677–88. doi: 10.1016/j.neuroscience.2003.08.040
46. Bergersen LH. Is lactate food for neurons? Comparison of monocarboxylate transporter subtypes in brain and muscle. *Neuroscience.* (2007) 145:11–9. doi: 10.1016/j.neuroscience.2006.11.062
47. Dienel GA. The metabolic trinity, glucose–glycogen–lactate, links astrocytes and neurons in brain energetics, signaling, memory, and gene expression. *Neurosci Lett.* (2017) 637:18–25. doi: 10.1016/j.neulet.2015.02.052
48. Pierre K, Pellerin L. Monocarboxylate transporters in the central nervous system: distribution, regulation and function. *J Neurochem.* (2005) 94:1–14. doi: 10.1111/j.1471-4159.2005.03168.x
49. Bricker DK, Taylor EB, Schell JC, Orsak T, Boutron A, Chen YC, et al. A mitochondrial pyruvate carrier required for pyruvate uptake in yeast, Drosophila, and humans. *Science.* (2012) 337:96–100. doi: 10.1126/science.1218099
50. Thomas AP, Halestrap AP. Identification of the protein responsible for pyruvate transport into rat liver and heart mitochondria by specific labelling with [3H]N-phenylmaleimide. *Biochem J.* (1981) 196:471–9. doi: 10.1042/bj1960471
51. Brooks GA, Brown MA, Butz CE, Sicurello JP, Dubouchaud H. Cardiac and skeletal muscle mitochondria have a monocarboxylate transporter MCT1. *J Appl Physiol (1985).* (1999) 87:1713–8. doi: 10.1152/jappl.1999.87.5.1713
52. Butz CE, McClelland GB, Brooks GA. *MCT1 confirmed in rat striated muscle mitochondria. J Appl Physiol (1985).* (2004) 97:1059–66. doi: 10.1152/japplphysiol.00009.2004
53. Hashimoto T, Hussien R, Brooks GA. Colocalization of MCT1, CD147, and LDH in mitochondrial inner membrane of L6 muscle cells: evidence of a mitochondrial lactate oxidation complex. *Am J Physiol Endocrinol Metab.* (2006) 290:E1237–44. doi: 10.1152/ajpendo.00594.2005
54. Hashimoto T, Hussien R, Cho HS, Kaufer D, Brooks GA. Evidence for the mitochondrial lactate oxidation complex in rat neurons: demonstration

of an essential component of brain lactate shuttles. *PLoS ONE.* (2008) 3:e2915. doi: 10.1371/journal.pone.0002915
55. Passarella S, de Bari L, Valenti D, Pizzuto R, Paventi G, Atlante A. Mitochondria and L-lactate metabolism. *FEBS Lett.* (2008) 582:3569–76. doi: 10.1016/j.febslet.2008.09.042
56. Stainsby WN, Brooks GA. Control of lactic acid metabolism in contracting muscles and during exercise. *Exerc Sport Sci Rev.* (1990) 18:29–63. doi: 10.1249/00003677-199001000-00005
57. Hashimoto T, Brooks GA. Mitochondrial lactate oxidation complex and an adaptive role for lactate production. *Med Sci Sports Exerc.* (2008) 40:486–94. doi: 10.1249/MSS.0b013e31815fcb04
58. Brooks GA. Intra- and extra-cellular lactate shuttles. *Med Sci Sports Exerc.* (2000) 32:790–9. doi: 10.1097/00005768-200004000-00011
59. Rogatzki MJ, Ferguson BS, Goodwin ML, Gladden LB. Lactate is always the end product of glycolysis. *Front Neurosci.* (2015) 9:22. doi: 10.3389/fnins.2015.00022
60. Lemire J, Mailloux RJ, Appanna VD. Mitochondrial lactate dehydrogenase is involved in oxidative-energy metabolism in human astrocytoma cells (CCF-STTG1). *PLoS ONE.* (2008) 3:e1550. doi: 10.1371/journal.pone.0001550
61. Atlante A, de Bari L, Bobba A, Marra E, Passarella S. Transport and metabolism of L-lactate occur in mitochondria from cerebellar granule cells and are modified in cells undergoing low potassium dependent apoptosis. *Biochim Biophys Acta.* (2007) 1767:1285–99. doi: 10.1016/j.bbabio.2007.08.003
62. Tekkök SB, Brown AM, Westenbroek R, Pellerin L, Ransom BR. Transfer of glycogen-derived lactate from astrocytes to axons via specific monocarboxylate transporters supports mouse optic nerve activity. *J Neurosci Res.* (2005) 81:644–52. doi: 10.1002/jnr.20573
63. Ling B, Peng F, Alcorn J, Lohmann K, Bandy B, Zello GA. D-Lactate altered mitochondrial energy production in rat brain and heart but not liver. *Nutr Metab.* (2012) 9:6. doi: 10.1186/1743-7075-9-6
64. Connor H, Woods HF, Ledingham JGG. Comparison of the kinetics and utilisation of D(-)-and L(+)-sodium lactate in normal man. *Ann Nutr Metab.* (1983) 27:481–7. doi: 10.1159/000176723
65. Jackson VN, Halestrap AP. The kinetics, substrate, and inhibitor specificity of the monocarboxylate (Lactate) transporter of rat liver cells determined using the fluorescent intracellular pH indicator, 2′,7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein. *J Biol Chem.* (1996) 271:861–8. doi: 10.1074/jbc.271.2.861
66. Halestrap AP. The monocarboxylate transporter family–Structure and functional characterization. *IUBMB Life.* (2012) 64:1–9. doi: 10.1002/iub.573
67. Flick MJ, Konieczny SF. Identification of putative mammalian D-lactate dehydrogenase enzymes. *Biochem Biophys Res Commun.* (2002) 295:910–6. doi: 10.1016/S0006-291X(02)00768-4
68. Liu S. Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. *Int J Food Microbiol.* (2003) 83:115–31. doi: 10.1016/S0168-1605(02)00366-5
69. Sousa MA, Rama GR, Volken de Souza CF, Granada CE. Acid lactic lactobacilli as a biotechnological toll to improve food quality and human health. *Biotechnol Prog.* (2020) 36:e2937. doi: 10.1002/btpr.2937
70. Reid SNS, Park JH, Kim Y, Kwak YS, Jeon BH. *In vitro* and *in vivo* effects of fermented oyster-derived lactate on exercise endurance indicators in mice. *Int J Environ Res Public Health*. (2020). 17:8811. doi: 10.3390/ijerph17238811
71. Teramae H, Yoshikawa T, Inoue R, Ushida K, Takebe K, Nio-Kobayashi J, et al. The cellular expression of SMCT2 and its comparison with other transporters for monocarboxylates in the mouse digestive tract. *Biomed Res.* (2010) 31:239–49. doi: 10.2220/biomedres.31.239
72. Brooks GA, Arevalo JA, Osmond AD, Leija RG, Curl CC, Tovar AP. Lactate in contemporary biology: a phoenix risen. *J Physiol.* (2022) 600:1229–51. doi: 10.1113/JP280955
73. Lecoultre V, Benoit R, Carrel G, Schutz Y, Millet GP, Tappy L, et al. Fructose and glucose co-ingestion during prolonged exercise increases lactate and glucose fluxes and oxidation compared with an equimolar intake of glucose. *Am J Clin Nutr.* (2010) 92:1071–9. doi: 10.3945/ajcn.2010.29566
74. Theytaz F, de Giorgi S, Hodson L, Stefanoni N, Rey V, Schneiter P, et al. Metabolic fate of fructose ingested with and without glucose in a mixed meal. *Nutrients.* (2014) 6:2632–49. doi: 10.3390/nu6072632
75. Brooks GA. The precious few grams of glucose during exercise. *Int J Mol Sci.* (2020) 21:5733. doi: 10.3390/ijms21165733
76. Brooks GA, Martin NA. Cerebral metabolism following traumatic brain injury: new discoveries with implications for treatment. *Front Neurosci.* (2014) 8:408. doi: 10.3389/fnins.2014.00408
77. Suzuki A, Stern SA, Bozdagi O, Huntley GW, Walker RH, Magistretti PJ, et al. Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell.* (2011) 144:810–23. doi: 10.1016/j.cell.2011.02.018
78. Shima T, Matsui T, Jesmin S, Okamoto M, Soya M, Inoue K, et al. Moderate exercise ameliorates dysregulated hippocampal glycometabolism and memory function in a rat model of type 2 diabetes. *Diabetologia*. (2017) 60:597–606. doi: 10.1007/s00125-016-4164-4
79. Harris RA, Lone A, Lim H, Martinez F, Frame AK, Scholl TJ, et al. Aerobic glycolysis is required for spatial memory acquisition but not memory retrieval in mice. *eNeuro*. (2019). doi: 10.1523/ENEURO.0389-18.2019
80. Scavuzzo CJ, Rakotovao I, Dickson CT. Differential effects of L- and D-lactate on memory encoding and consolidation: Potential role of HCAR1 signaling. *Neurobiol Learn Mem.* (2020) 168:107151. doi: 10.1016/j.nlm.2019.107151
81. Zhang Y, Xue Y, Meng S, Luo Y, Liang J, Li J, et al. Inhibition of lactate transport erases drug memory and prevents drug relapse. *Biol Psychiatry*. (2016) 79:928–9. doi: 10.1016/j.biopsych.2015.07.007
82. Tadi M, Allaman I, Lengacher S, Grenningloh G, Magistretti PJ. Learning-induced gene expression in the hippocampus reveals a role of neuron - astrocyte metabolic coupling in long term memory. *PLoS ONE.* (2015) 10:e0141568. doi: 10.1371/journal.pone.0141568
83. Alberini CM, Cruz E, Descalzi G, Bessières B, Gao V. Astrocyte glycogen and lactate: New insights into learning and memory mechanisms. *Glia.* (2018) 66:1244–62. doi: 10.1002/glia.23250
84. Lucas SJ, Michel CB, Marra V, Smalley JL, Hennig MH, Graham BP, et al. Glucose and lactate as metabolic constraints on presynaptic transmission at an excitatory synapse. *J Physiol.* (2018) 596:1699–721. doi: 10.1113/JP275107
85. Tang F, Lane S, Korsak A, Paton JF, Gourine AV, Kasparov S, et al. Lactate-mediated glia-neuronal signalling in the mammalian brain. *Nat Commun.* (2014) 5:3284. doi: 10.1038/ncomms4284
86. Herrera-Lo'pez G, Griego E, Galva'n EJ. Lactate induces synapse-specific potentiation on CA3 pyramidal cells of rat hippocampus. *PLoS ONE*. (2020) 15:e0242309. doi: 10.1371/journal.pone.0242309
87. Schurr A, West CA, Rigor BM. Lactate-supported synaptic function in the rat hippocampal slice preparation. *Science*. (1988) 240: 1326–8. doi: 10.1126/science.3375817
88. Brown AM, Tekkök SB, Ransom BR. Glycogen regulation and functional role in mouse white matter. *J Physiol.* (2003) 549:501–12. doi: 10.1113/jphysiol.2003.042416
89. Margineanu MB, Mahmood H, Fiumelli H, Magistretti PJ. L-lactate regulates the expression of synaptic plasticity and neuroprotection genes in cortical neurons: a transcriptome analysis. *Front Mol Neurosci.* (2018) 11:375. doi: 10.3389/fnmol.2018.00375
90. Yang Y, Ruchti E, Petit JM, Jourdain P, Magistretti PJ. Lactate promotes plasticity gene expression by potentiating NMDA signaling in neurons. *Proc Natl Acad Sci U S A*. (2014) 111:12228–33. doi: 10.1073/pnas.1322912111
91. Baker KD, Edwards TM. D-Lactate inhibition of memory in a single trial discrimination avoidance task in the young chick. *Neurobiol Learn Mem.* (2007) 88:269–76. doi: 10.1016/j.nlm.2007.06.004
92. Ragozzino ME, Hellems K, Robert C, Lennartz, Gold PE. Pyruvate infusions into the septal area attenuate spontaneous alternation impairments induced by intraseptal morphine injections. *Behav Neurosci.* (1995) 109:T074–1080. doi: 10.1037/0735-7044.109.6.1074
93. Jourdain P, Allaman I, Rothenfusser K, Fiumelli H, Marquet P, Magistretti PJ. L-Lactate protects neurons against excitotoxicity: implication of an ATP-mediated signaling cascade. *Sci Rep*. (2016) 6:21250. doi: 10.1038/srep21250
94. Ross JM, Oberg J, Brene S, Coppotelli G, Terzioglu M, Pernold K, et al. High brain lactate is a hallmark of aging and caused by a shift in the lactate dehydrogenase A/B ratio. *Proc Natl Acad Sci U S A.* (2010) 107:20087–92. doi: 10.1073/pnas.1008189107
95. langer MB, Allaman I, Magistretti PJ. Brain energy metabolism_ focus on astrocyte neuron metabolic cooperation. *Cell Metab.* (2011) 14:724–38. doi: 10.1016/j.cmet.2011.08.016

96. Dienel GA, McKenna MC. A dogma-breaking concept: glutamate oxidation in astrocytes is the source of lactate during aerobic glycolysis in resting subjects. *J Neurochem.* (2014) 131:395–8. doi: 10.1111/jnc.12835
97. Dienel GA. Brain lactate metabolism: the discoveries and the controversies. *J Cereb Blood Flow Metab.* (2011) 32:1107–38. doi: 10.1038/jcbfm.2011.175
98. Vafaee MS, Vang K, Bergersen LH, Gjedde A. Oxygen consumption and blood flow coupling in human motor cortex during intense finger tapping: implication for a role of lactate. *J Cereb Blood Flow Metab.* (2012) 32:1859–68. doi: 10.1038/jcbfm.2012.89
99. Nishijima T, Okamoto M, Matsui T, Kita I, Soya H. Hippocampal functional hyperemia mediated by NMDA receptor/NO signaling in rats during mild exercise. *J Appl Physiol (1985).* (2012) 54:197–203. doi: 10.1152/japplphysiol.00763.2011
100. Hein TW, Xu W, Kuo L. Dilation of retinal arterioles in response to lactate: role of nitric oxide, guanylyl cyclase, and ATP-sensitive potassium channels. *Invest Ophthalmol Vis Sci.* (2006) 47:693–9. doi: 10.1167/iovs.05-1224
101. Falkowska A, Gutowska I, Goschorska M, Nowacki P, Chlubek D, Baranowska-Bosiacka I. Energy metabolism of the brain, including the cooperation between astrocytes and neurons, especially in the context of glycogen metabolism. *Int J Mol Sci.* (2015) 16:25959–81. doi: 10.3390/ijms161125939
102. Yamanishi S, Katsumura K, Kobayashi T, Puro DG. Extracellular lactate as a dynamic vasoactive signal in the rat retinal microvasculature. *Am J Physiol Heart Circ Physiol.* (2006) 290:H925–934. doi: 10.1152/ajpheart.01012.2005
103. Wojtowicz JM, Kee N. BrdU assay for neurogenesis in rodents. *Nat Protoc.* (2006) 1:1399–405. doi: 10.1038/nprot.2006.224
104. Roger AJ, Munoz-Gomez SA, Kamikawa R. The origin and diversification of mitochondria. *Curr Biol.* (2017) 27:R1177–92. doi: 10.1016/j.cub.2017.09.015
105. Hashimoto T, Hussien R, Oommen S, Gohil K, Brooks GA. Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis. *FASEB J.* (2007) 21:2602–12. doi: 10.1096/fj.07-8174com
106. Tauffenberger A, Fiumelli H, Almustafa S, Magistretti PJ. Lactate and pyruvate promote oxidative stress resistance through hormetic ROS signaling. *Cell Death Dis.* (2019) 10:653. doi: 10.1038/s41419-019-1877-6
107. Hu J, Cai M, Shang Q, Li Z, Feng Y, Liu B, et al. Elevated lactate by high-intensity interval training regulates the hippocampal bdnf expression and the mitochondrial quality control system. *Front Physiol.* (2021) 12:629914. doi: 10.3389/fphys.2021.629914
108. Lauritzen F, Eid T, Bergersen LH. Monocarboxylate transporters in temporal lobe epilepsy: roles of lactate and ketogenic diet. *Brain Struct Funct.* (2015) 220:1–12. doi: 10.1007/s00429-013-0672-x
109. Zhai X, Li J, Li L, Sun Y, Zhang X, Xue Y, et al. L-lactate preconditioning promotes plasticity-related proteins expression and reduces neurological deficits by potentiating GPR81 signaling in rat traumatic brain injury model. *Brain Res.* (2020) 1746:146945. doi: 10.1016/j.brainres.2020.146945
110. Bouzat P, Sala N, Suys T, Zerlauth JB, Marques-Vidal P, Feihl F, et al. Cerebral metabolic effects of exogenous lactate supplementation on the injured human brain. *Intensive Care Med.* (2014) 40:412–21. doi: 10.1007/s00134-013-3203-6
111. Alessandri B, Schwandt E, Kamada Y, Nagata M, Heimann A, Kempski O. The neuroprotective effect of lactate is not due to improved glutamate uptake after controlled cortical impact in rats. *J Neurotrauma.* (2012) 29:2181–91. doi: 10.1089/neu.2011.2067
112. Dienel GA. Lactate shuttling and lactate use as fuel after traumatic brain injury: metabolic considerations. *J Cereb Blood Flow Metab.* (2014) 34:1736–48. doi: 10.1038/jcbfm.2014.153
113. Glenn TC, Martin NA, Horning MA, McArthur DL, Hovda DA, Vespa P, et al. Lactate: brain fuel in human traumatic brain injury: a comparison with normal healthy control subjects. *J Neurotrauma.* (2015) 32:820–32. doi: 10.1089/neu.2014.3483
114. Glenn TC, Martin NA, McArthur DL, Hovda DA, Vespa P, Johnson ML, et al. Endogenous nutritive support after traumatic brain injury: peripheral lactate production for glucose supply via gluconeogenesis. *J Neurotrauma.* (2015) 32:811–19. doi: 10.1089/neu.2014.3482
115. Ma Y, Liu T, Fu J, Fu S, Hu C, Sun B, et al. Lactobacillus acidophilus exerts neuroprotective effects in mice with traumatic brain injury. *J Nutr.* (2019) 149:1543–52. doi: 10.1093/jn/nxz105
116. Li H, Sun J, Du J, Wang F, Fang R, Yu C, et al. Clostridium butyricum exerts a neuroprotective effect in a mouse model of traumatic brain injury via the gut-brain axis. *Neurogastroenterol Motil.* (2018) 30:e13260. doi: 10.1111/nmo.13260
117. Mijnhout GS, Scheltens P, Diamant M, Biessels GJ, Wessels AM, Simsek S, et al. Diabetic encephalopathy: a concept in need of a definition. *Diabetologia.* (2006) 49:1447–8. doi: 10.1007/s00125-006-0221-8
118. McCrimmon RJ, Ryan CM, Frier BM. Diabetes and cognitive dysfunction. *Lancet.* (2012) 379:2291–9. doi: 10.1016/S0140-6736(12)60360-2
119. Biessels GJ, van der Heide LP, Kamal A, Bleys RL, Gispen WH. Ageing and diabetes implications for brain function. *Eur J Pharmacol.* (2002) 441:1–14. doi: 10.1016/S0014-2999(02)01486-3
120. Yi SS. Effects of exercise on brain functions in diabetic animal models. *World J Diabetes.* (2015) 6:583–97. doi: 10.4239/wjd.v6.i4.583
121. Fazeli SA. Neuroprotection in diabetic encephalopathy. *Neurodegener Dis.* (2009) 6:213–8. doi: 10.1159/000278694
122. Wiegers EC, Rooijackers HM, Tack CJ, Heerschap A, de Galan BE, van der Graaf M. Brain lactate concentration falls in response to hypoglycemia in patients with type 1 diabetes and impaired awareness of hypoglycemia. *Diabetes.* (2016) 65:1601–5. doi: 10.2337/db16-0068
123. Wiegers EC, Rooijackers HM, Tack2 CJ, Groenewoud HJMM, Heerschap A, Galan BEd, et al. Effect of exercise-induced lactate elevation on brain lactate levels during hypoglycemia in patients with type 1 diabetes and impaired awareness of hypoglycemia. *Diabetes.* (2017) 66:3105–10. doi: 10.2337/db17-0794
124. King P, Kong MF, Parkin H, MacDonald IA, Barber C, Tattersall RB. Intravenous lactate prevents cerebral dysfunction during hypoglycaemia in insulin-dependent diabetes mellitus. *Clin Sci.* (1998) 94:157–63. doi: 10.1042/cs0940157
125. Maran A, Crepaldi C, Trupiani S, Lucca T, Jori E, Macdonald IA, et al. Brain function rescue effect of lactate following hypoglycaemia is not an adaptation process in both normal and type I diabetic subjects. *Diabetologia.* (2000) 43:733–41. doi: 10.1007/s001250051371
126. Wiegers EC, Rooijackers HM, Tack CJ, Philips BW, Heerschap A, van der Graaf M, et al. Effect of lactate administration on brain lactate levels during hypoglycemia in patients with type 1 diabetes. *J Cereb Blood Flow Metab.* (2019) 39:1974–82. doi: 10.1177/0271678X18775884
127. Chan O, Paranjape S, Horblitt A, Zhu W, Sherwin RS. Lactate-induced release of GABA in the ventromedial hypothalamus contributes to counterregulatory failure in recurrent hypoglycemia and diabetes. *Diabetes.* (2013) 62:4239–46. doi: 10.2337/db13-0770
128. Kobayashi R, Maruoka J, Norimoto H, Ikegaya Y, Kume K, Ohsawa M. Involvement of l-lactate in hippocampal dysfunction of type I diabetes. *J Pharmacol Sci.* (2019) 141:79–82. doi: 10.1016/j.jphs.2019.09.004
129. Mason GF, Petersen KF, Lebon V, Rothman DL, Shulman GI. increased brain monocarboxylic acid transport and utilization in type 1 diabetes. *Diabetes.* (2006) 55:929–34. doi: 10.2337/diabetes.55.04.06.db05-1325
130. Hackett MJ, Hollings A, Majimbi M, Brook E, Cochran B, Giles C, et al. Multimodal imaging analyses of brain hippocampal formation reveal reduced Cu and lipid content and increased lactate content in non-insulin-dependent diabetic mice. *ACS Chem Neurosci.* (2019) 10:2533–40. doi: 10.1021/acschemneuro.9b00039
131. Li J, Liu B, Cai M, Lin X, Lou S. Glucose metabolic alterations in hippocampus of diabetes mellitus rats and the regulation of aerobic exercise. *Behav Brain Res.* (2019) 364:447–56. doi: 10.1016/j.bbr.2017.11.001
132. Choi Y-S, Song JE, Lee JE, Kim E, Kim CH, Kim D-H, et al. Hyperpolarized [1-13C] lactate flux increased in the hippocampal region in diabetic mice. *Molecular Brain.* (2019) 12:88. doi: 10.1186/s13041-019-0505-9
133. De Feyter HM, Mason GF, Shulman GI, Rothman DL, Petersen KF. Increased brain lactate concentrations without increased lactate oxidation during hypoglycemia in type 1 diabetic individuals. *Diabetes.* (2013) 62:3075–80. doi: 10.2337/db13-0313

134. Zhao L, Dong M, Ren M, Li C, Zheng H, Gao H. Metabolomic analysis identifies lactate as an important pathogenic factor in diabetes-associated cognitive decline rats. *Mol Cell Proteomics*. (2018) 17:2335–46. doi: 10.1074/mcp.RA118.000690
135. Wang D, Zhao L, Zheng H, Dong M, Pan L, Zhang X, et al. Time-dependent lactate production and amino acid utilization in cultured astrocytes under high glucose exposure. *Mol Neurobiol*. (2018) 55:1112. doi: 10.1007/s12035-016-0360-y
136. Tapeinos C, Battaglini M, Ciofani G. Advances in the design of solid lipid nanoparticles and nanostructured lipid carriers for targeting brain diseases. *J Control Release*. (2017) 264:306–32. doi: 10.1016/j.jconrel.2017.08.033
137. Yin F, Sancheti H, Patil I, Cadenas E. Energy metabolism and inflammation in brain aging and Alzheimer's disease. *Free Radic Biol Med*. (2016) 100:108–22. doi: 10.1016/j.freeradbiomed.2016.04.200
138. Lu WT, Sun SQ, Li Y, Xu SY, Gan SW, Xu J, et al. Curcumin ameliorates memory deficits by enhancing lactate content and MCT2 expression in APP/PS1 transgenic mouse model of Alzheimer's disease. *Anat Rec*. (2019) 302:332–8. doi: 10.1002/ar.23969
139. Lu W, Huang J, Sun S, Huang S, Gan S, Xu J, et al. Changes in lactate content and monocarboxylate transporter 2 expression in Abeta(2)(5)(-)(3)(5)-treated rat model of Alzheimer's disease. *Neurol Sci*. (2015) 36:871–6. doi: 10.1007/s10072-015-2087-3
140. Zhang M, Cheng X, Dang R, Zhang W, Zhang J, Yao Z. Lactate deficit in an alzheimer disease mouse model: the relationship with neuronal damage. *J Neuropathol Exp Neurol*. (2018) 77:1163–76. doi: 10.1093/jnen/nly102
141. Datta S, Chakrabarti N. Age related rise in lactate and its correlation with lactate dehydrogenase (LDH) status in post-mitochondrial fractions isolated from different regions of brain in mice. *Neurochem Int*. (2018) 118:23–33. doi: 10.1016/j.neuint.2018.04.007
142. Long DM, Frame AK, Reardon PN, Cumming RC, Hendrix DA, Kretzschmar D, et al. Lactate dehydrogenase expression modulates longevity and neurodegeneration in Drosophila melanogaster. *Aging*. (2020) 12:10041–58. doi: 10.18632/aging.103373
143. Mullins R, Reiter D, Kapogiannis D. Magnetic resonance spectroscopy reveals abnormalities of glucose metabolism in the Alzheimer's brain. *Ann Clin Transl Neurol*. (2018) 5:262–72. doi: 10.1002/acn3.530
144. de Bari L, Atlante A, Guaragnella N, Principato G, Passarella S. D-Lactate transport and metabolism in rat liver mitochondria. *Biochem J*. (2002) 365:391–403. doi: 10.1042/bj20020139
145. de Bari L, Atlante A, Armeni T, Kalapos MP. Synthesis and metabolism of methylglyoxal, S-D-lactoylglutathione and D-lactate in cancer and Alzheimer's disease. Exploring the crossroad of eternal youth and premature aging. *Ageing Res Rev*. (2019) 53:100915. doi: 10.1016/j.arr.2019.100915
146. Maida CD, Norrito RL, Daidone M, Tuttolomondo A, Pinto A. Neuroinflammatory mechanisms in ischemic stroke: focus on cardioembolic stroke, background, and therapeutic approaches. *Int J Mol Sci*. (2020) 21:6454. doi: 10.3390/ijms21186454
147. Hyacinthe JN, Buscemi L, Le TP, Lepore M, Hirt L, Mishkovsky M. Evaluating the potential of hyperpolarised [1-(13)C] L-lactate as a neuroprotectant metabolic biosensor for stroke. *Sci Rep*. (2020) 10:5507. doi: 10.1038/s41598-020-62319-x
148. Wyss MT, Jolivet R, Buck A, Magistretti PJ, Weber B. *In vivo* evidence for lactate as a neuronal energy source. *J Neurosci*. (2011) 31:7477–85. doi: 10.1523/JNEUROSCI.0415-11.2011
149. Schurr A, Payne RS, Miller JJ, Tseng MT, Rigor BM. Rigor. Blockade of lactate transport exacerbates delayed neuronal damage in__a rat model of cerebral ischemia. *Brain Res*. (2001) 895:268–72. doi: 10.1016/S0006-8993(01)02082-0
150. Buscemi L, Blochet C, Magistretti PJ, Hirt L. Hydroxycarboxylic acid receptor 1 and neuroprotection in a mouse model of cerebral ischemia-reperfusion. *Front Physiol*. (2021) 12:689239. doi: 10.3389/fphys.2021.689239
151. Moro N, Ghavim S, Harris NG, Hovda DA, Sutton RL. Glucose administration after traumatic brain injury improves cerebral metabolism and reduces secondary neuronal injury. *Brain Res*. (2013) 1535:124–36. doi: 10.1016/j.brainres.2013.08.044
152. Bergsneider M, Hovda DA, Shalmon E, Kelly DF, Vespa PM, Martin NA, et al. Cerebral hyperglycolysis following severe traumatic brain injury in humans: a positron emission tomography study. *J Neurosurg*. (1997) 86:241–51. doi: 10.3171/jns.1997.86.2.0241
153. Glenn TC, Kelly DF, Boscardin WJ, McArthur DL, Vespa P, Oertel M, et al. Energy dysfunction as a predictor of outcome after moderate or severe head injury: indices of oxygen, glucose, and lactate metabolism. *J Cereb Blood Flow Metab*. (2003) 23:1239–50. doi: 10.1097/01.WCB.0000089833.23606.7F
154. Jalloh I, Helmy A, Shannon RJ, Gallagher CN, Menon DK, Carpenter KL, et al. Lactate uptake by the injured human brain: evidence from an arteriovenous gradient and cerebral microdialysis study. *J Neurotrauma*. (2013) 30:2031–7. doi: 10.1089/neu.2013.2947
155. Carteron L, Solari D, Patet C, Quintard H, Miroz JP, Bloch J, et al. Hypertonic lactate to improve cerebral perfusion and glucose availability after acute brain injury. *Crit Care Med*. (2018) 46:1649–55. doi: 10.1097/CCM.0000000000003274
156. Zhou J, Burns MP, Huynh L, Villapol S, Taub DD, Saavedra JM, et al. Temporal changes in cortical and hippocampal expression of genes important for brain glucose metabolism following controlled cortical impact injury in mice. *Front Endocrinol*. (2017) 8:231. doi: 10.3389/fendo.2017.00231
157. Betjemann JP, Lowenstein DH. Status epilepticus in adults. *Lancet Neurol*. (2015) 14:615–24. doi: 10.1016/S1474-4422(15)00042-3
158. Thoresen M, Hallstrom A, Whitelaw A, Puka-Sundvall M, Loberg EM, Satas S, et al. Lactate and pyruvate changes in the cerebral gray and white matter during posthypoxic seizures in newborn pigs. *Pediatr Res*. (1998) 44:746–54. doi: 10.1203/00006450-199811000-00018
159. Mariani CL, Nye CJ, Ruterbories L, Tokarz DA, Green L, Lau J, et al. Cerebrospinal fluid lactate concentrations in dogs with seizure disorders. *J Vet Intern Med*. (2020) 34:2562–70. doi: 10.1111/jvim.15953
160. Slais K, Vorisek I, Zoremba N, Homola A, Dmytrenko L, Sykova E. Brain metabolism and diffusion in the rat cerebral cortex during pilocarpine-induced status epilepticus. *Exp Neurol*. (2008) 209:145–54. doi: 10.1016/j.expneurol.2007.09.008
161. During MJ, Fried I, Leone P, Katz A, Spencer DD. Direct measurement of extracellular lactate in the human hippocampus during spontaneous seizures. *J Neurochem*. (1994) 62:2356–61. doi: 10.1046/j.1471-4159.1994.62062356.x
162. Cavus I, Kasoff WS, Cassaday MP, Jacob R, Gueorguieva R, Sherwin RS, et al. Extracellular metabolites in the cortex and hippocampus of epileptic patients. *Ann Neurol*. (2005) 57:226–35. doi: 10.1002/ana.20380
163. Jorwal P, Sikdar SK. Lactate reduces epileptiform activity through HCA1 and GIRK channel activation in rat subicular neurons in an in vitro model. *Epilepsia*. (2019) 60:2370–85. doi: 10.1111/epi.16389
164. Matsui T, Soya S, Okamoto M, Ichitani Y, Kawanaka K, Soya H. Brain glycogen decreases during prolonged exercise. *J Physiol*. (2011) 589:3383–93. doi: 10.1113/jphysiol.2011.203570
165. Matsui T, Soya S, Kawanaka K, Soya H. Brain glycogen decreases during intense exercise without hypoglycemia: the possible involvement of serotonin. *Neurochem Res*. (2015) 40:1333–40. doi: 10.1007/s11064-015-1594-1
166. Bouzier AK, Thiaudiere E, Biran M, Rouland R, Canioni P, Merle M. The metabolism of [3-(13)C]lactate in the rat brain is specific of a pyruvate carboxylase-deprived compartment. *J Neurochem*. (2000) 75:480–6. doi: 10.1046/j.1471-4159.2000.0750480.x
167. Schurr A, Miller JJ, Payne RS, Rigor BM. An increase in lactate output by brain tissue serves to meet the energy needs of glutamateactivated neurons. *J Neurosci*. (1999) 19:34–9. doi: 10.1523/JNEUROSCI.19-01-00034.1999
168. Genc S, Kurnaz IA, Ozilgen M. Astrocyte-neuron lactate shuttle may boost more ATP supply to the neuron under hypoxic conditions–in silico study supported by *in vitro* expression data. *BMC Syst Biol*. (2011) 5:162. doi: 10.1186/1752-0509-5-162
169. Lauritzen KH, Morland C, Puchades M, Holm-Hansen S, Hagelin EM, Lauritzen F, et al. Lactate receptor sites link neurotransmission, neurovascular coupling, and brain energy metabolism. *Cereb Cortex*. (2014) 24:2784–95. doi: 10.1093/cercor/bht136
170. Magistretti PJ, Allaman I. Lactate in the brain: from metabolic end-product to signalling molecule. *Nat Rev Neurosci*. (2018) 19:235–49. doi: 10.1038/nrn.2018.19

171. Brooks GA. Lactate as a fulcrum of metabolism. *Redox Biol.* (2020) 35:101454. doi: 10.1016/j.redox.2020.101454
172. Fernandez-Marcos PJ, Auwerx J. Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis. *Am J Clin Nutr.* (2011) 93:884S–90S. doi: 10.3945/ajcn.110.001917
173. Gureev AP, Shaforostova EA, Popov VN. Regulation of mitochondrial biogenesis as a way for active longevity: interaction between the Nrf2 and PGC-1alpha signaling pathways. *Front Genet.* (2019) 10:435. doi: 10.3389/fgene.2019.00435
174. Galardo MaN, Regueira M, Riera MaF, Pellizzari EH, Cigorraga SB, Meroni SB. Lactate regulates rat male germ cell function through reactive oxygen species. *PLoS ONE.* (2014) 9:e88024. doi: 10.1371/journal.pone.0088024
175. Zelenka J, Dvorak A, Alan L. L-lactate protects skin fibroblasts against aging-associated mitochondrial dysfunction via mitohormesis. *Oxid Med Cell Longev.* (2015) 2015:351698. doi: 10.1155/2015/351698
176. Bari LD, Valenti D, Atlante A, Passarella S. L-Lactate generates hydrogen peroxide in purified rat liver mitochondria due to the putative L-lactate oxidase localized in the intermembrane space. *FEBS Letters.* (2010) 584:2285–90. doi: 10.1016/j.febslet.2010.03.038
177. Ge H, Weiszmann J, Reagan JD, Gupte J, Baribault H, Gyuris T, et al. Elucidation of signaling and functional activities of an orphan GPCR, GPR81. *J Lipid Res.* (2008) 49:797–803. doi: 10.1194/jlr.M700513-JLR200
178. Ahmed K, Tunaru S, Tang C, Muller M, Gille A, Sassmann A, et al. An autocrine lactate loop mediates insulin-dependent inhibition of lipolysis through GPR81. *Cell Metab.* (2010) 11:311–19. doi: 10.1016/j.cmet.2010.02.012
179. Brooks GA. Role of the heart in lactate shuttling. *Front Nutr.* (2021) 8:663560. doi: 10.3389/fnut.2021.663560
180. Li G, Wang HQ, Wang LH, Chen RP, Liu JP. Distinct pathways of ERK1/2 activation by hydroxycarboxylic acid receptor-1. *PLoS ONE.* (2014) 9:e93041. doi: 10.1371/journal.pone.0093041
181. Ohno Y, Oyama A, Kaneko H, Egawa T, Yokoyama S, Sugiura T, et al. Lactate increases myotube diameter via activation of MEK/ERK pathway in C2C12 cells. *Acta Physiol.* (2018) 223:e13042. doi: 10.1111/apha.13042
182. Wu Y, Wang M, Zhang K, Li Y, Xu M, Tang S, et al. Lactate enhanced the effect of parathyroid hormone on osteoblast differentiation via GPR81-PKC-Akt signaling. *Biochem Biophys Res Commun.* (2018) 503:737–43. doi: 10.1016/j.bbrc.2018.06.069
183. Hoque R, Farooq A, Ghani A, Gorelick F, Mehal WZ. Lactate reduces liver and pancreatic injury in Toll-like receptor- and inflammasome-mediated inflammation via GPR81-mediated suppression of innate immunity. *Gastroenterology.* (2014) 146:1763–74. doi: 10.1053/j.gastro.2014.03.014
184. Harun-Or-Rashid M, Inman DM. Reduced AMPK activation and increased HCAR activation drive anti-inflammatory response and neuroprotection in glaucoma. *J Neuroinflammation.* (2018) 15:313. doi: 10.1186/s12974-018-1346-7
185. Jeninga EH, Bugge A, Nielsen R, Kersten S, Hamers N, Dani C, et al. Peroxisome proliferator-activated receptor gamma regulates expression of the anti-lipolytic G-protein-coupled receptor 81 (GPR81/Gpr81). *J Biol Chem.* (2009) 284:26385–93. doi: 10.1074/jbc.M109.040741
186. Wanders D, Graff EC, Judd RL. Effects of high fat diet on GPR109A and GPR81 gene expression. *Biochem Biophys Res Commun.* (2012) 425:278–83. doi: 10.1016/j.bbrc.2012.07.082
187. Madaan A, Chaudhari P, Nadeau-Vallée M, Hamel D, Zhu T, Mitchell G, et al. Müller cell–localized G-protein–coupled receptor 81 (hydroxycarboxylic acid receptor 1) regulates inner retinal vasculature via Norrin/Wnt pathways. *Am J Pathol.* (2019) 189:1878–96. doi: 10.1016/j.ajpath.2019.05.016
188. Lee Y-S, Kim T-Y, Kim Y, Lee S-H, Kim S, Kang SW, et al. Microbiota-derived lactate accelerates intestinal stem-cell-mediated epithelial development. *Cell Host Microbe.* (2018) 24:833–46.e6. doi: 10.1016/j.chom.2018.11.002
189. Cai TQ, Ren N, Jin L, Cheng K, Kash S, Chen R, et al. Role of GPR81 in lactate-mediated reduction of adipose lipolysis. *Biochem Biophys Res Commun.* (2008) 377:987–91. doi: 10.1016/j.bbrc.2008.10.088
190. Liu C, Wu J, Zhu J, Kuei C, Yu J, Shelton J, et al. Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81. *J Biol Chem.* (2009) 284:2811–22. doi: 10.1074/jbc.M806409200
191. Alarcon P, Manosalva C, Carretta MD, Hidalgo AI, Figueroa CD, Taubert A, et al. Fatty and hydroxycarboxylic acid receptors: The missing link of immune response and metabolism in cattle. *Vet Immunol Immunopathol.* (2018) 201:77–87. doi: 10.1016/j.vetimm.2018.05.009
192. Morland C, Lauritzen KH, Puchades M, Holm-Hansen S, Andersson K, Gjedde A, et al. The lactate receptor, G-protein-coupled receptor 81/hydroxycarboxylic acid receptor 1: expression and action in brain. *J. Neurosci Res.* (2015) 93:1045–55. doi: 10.1002/jnr.23593
193. Huckabee WE. Relationship of pyruvate and lactate during anaerobic metabolism. V coronary adequacy. *Am J Physiol Physiol.* (1961) 200:1169. doi: 10.1152/ajplegacy.1961.200.6.1169
194. Osnes JB, Hermansen L. Acid-base balance after maximal exercise of short duration. *J Appl Physiol.* (1972) 32:59–63. doi: 10.1152/jappl.1972.32.1.59
195. Kreisberg RA. Lactate homeostasis and lactic acidosis. *Ann Intern Med.* (1980) 92:227. doi: 10.7326/0003-4819-92-2-227
196. Vardjan N, Chowdhury HH, Horvat A, Velebit J, Malnar M, Muhic M, et al. Enhancement of astroglial aerobic glycolysis by extracellular lactate-mediated increase in cAMP. *Front Mol Neurosci.* (2018) 11:148. doi: 10.3389/fnmol.2018.00148
197. Martin SJ, Grimwood PD, Morris RG. Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci.* (2000) 23:649–711. doi: 10.1146/annurev.neuro.23.1.649
198. Sun S, Li H, Chen J, Qian Q. Lactic acid: no longer an inert and end-product of glycolysis. *Physiology.* (2017) 32:453–63. doi: 10.1152/physiol.00016.2017

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Cai, Wang, Song, Yang, Wang, Xue, Sun and Hu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 02 May 2022
doi: 10.3389/fnut.2022.837066

Higher Consumption of Fruit and Vegetables Is Associated With Lower Worries, Tension and Lack of Joy Across the Lifespan

Simone Radavelli-Bagatini[1]*, *Marc Sim*[1,2], *Lauren C. Blekkenhorst*[1,2], *Nicola P. Bondonno*[1,3], *Catherine P. Bondonno*[1,2], *Richard Woodman*[4], *Joanne M. Dickson*[1,5], *Craig Harms*[6], *Dianna J. Magliano*[7,8], *Jonathan E. Shaw*[7,9], *Robin M. Daly*[10], *Jonathan M. Hodgson*[1,2] *and Joshua R. Lewis*[1,2,11]

[1] Nutrition & Health Innovation Research Institute, School of Medical and Health Sciences, Edith Cowan University, Perth, WA, Australia, [2] Medical School, The University of Western Australia, Perth, WA, Australia, [3] Danish Cancer Society Research Centre (DCRC), Copenhagen, Denmark, [4] Flinders Centre for Epidemiology and Biostatistics, Flinders University, Adelaide, SA, Australia, [5] School of Arts and Humanities (Psychology), Edith Cowan University, Perth, WA, Australia, [6] School of Arts and Humanities, Psychology and Criminology, Edith Cowan University, Perth, WA, Australia, [7] Diabetes and Population Health, Baker Heart and Diabetes Institute, Melbourne, VIC, Australia, [8] Clinical Diabetes and Epidemiology, Baker Heart and Diabetes Institute, Melbourne, VIC, Australia, [9] School of Public Health and Preventive Medicine, Monash University, Melbourne, VIC, Australia, [10] School of Exercise and Nutrition Science, Institute for Physical Activity and Nutrition, Deakin University, Geelong, VIC, Australia, [11] Centre for Kidney Research, School of Public Health, Sydney Medical School, Children's Hospital at Westmead, The University of Sydney, Sydney, NSW, Australia

OPEN ACCESS

Edited by:
Andrew Scholey, Swinburne University of Technology, Australia

Reviewed by:
Katie Barfoot, University of Reading, United Kingdom
Lauren M. Young, Deakin University, Australia
Chong Chen, Yamaguchi University Graduate School of Medicine, Japan

***Correspondence:**
Simone Radavelli-Bagatini
s.radavellibagatini@ecu.edu.au

Specialty section:
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

Received: *16 December 2021*
Accepted: *11 April 2022*
Published: *02 May 2022*

Citation:
Radavelli-Bagatini S, Sim M, Blekkenhorst LC, Bondonno NP, Bondonno CP, Woodman R, Dickson JM, Harms C, Magliano DJ, Shaw JE, Daly RM, Hodgson JM and Lewis JR (2022) Higher Consumption of Fruit and Vegetables Is Associated With Lower Worries, Tension and Lack of Joy Across the Lifespan. Front. Nutr. 9:837066. doi: 10.3389/fnut.2022.837066

Background and Aims: Higher total fruit and vegetable (FV) intakes have been associated with lower perceived stress. However, the relationship of FV intake with domains of perceived stress is unclear. The aim of this cross-sectional study was to explore the relationship between consumption of FV and four perceived stress domains (worries, tension, lack of joy and demands) in a population-based cohort of Australian adults.

Methods: Participants (n = 8,640) were men and women aged ≥25 years from the Australian Diabetes, Obesity and Lifestyle (AusDiab) Study. Dietary intake was assessed using a 74-item validated Food Frequency Questionnaire. Perceived stress domains were determined using a validated 20-item version of the Perceived Stress Questionnaire, with higher scores representing higher perceived stress. Cut-offs for high perceived stress domains were obtained from the highest quartiles of each domain for each sex. Multivariable-adjusted logistic regression was performed to investigate cross-sectional associations.

Results: The mean age of participants (50.1% females) was 47.8 (SD 15) years. Those with higher intakes of FV, combined and separately, had a significantly lower odds (16–36%) for higher worries, tension and lack of joy, independent of other lifestyle factors.

Conclusion: In Australian adults, higher consumption of FV was associated with lower odds of worries, tension and lack of joy. Following the dietary guidelines for the

recommended intake of FV may help improve feelings of worries, tension and lack of joy, which are linked to mental health problems.

Keywords: AusDiab, fruit and vegetable consumption, worries, tension, joy, stress

INTRODUCTION

Prolonged exposure to stress can be a risk factor for chronic illnesses (1), having been linked to mental health issues such as anxiety and depression (2, 3), as well as physical health issues (4), including cardiovascular disease (CVD) (5, 6). The number of people affected by stress as well as mental health issues continue to grow globally, with depression reported to affect more than 300 million people worldwide (7).

Increasing evidence shows that fruit and vegetable-rich diets may have a protective role on stress, with consumption of whole fruit and vegetables (8), as well as some specific types (such as citrus fruits and leafy green vegetables) being linked with less stress (9). Moreover, healthy lifestyle factors, such as being active and adhering to a healthy diet (10, 11), particularly a fruit and vegetable-rich diet, can help alleviate mental health issues (10, 12–14) and improve mental well-being (15). Therefore, greater intakes of FV could help reduce the estimated 7.8 million premature deaths worldwide, which have been associated with low intakes of fruit and vegetables (16), and consequently decrease the additional burden of chronic diseases and deaths caused by mental health worldwide (7).

It is noteworthy that this relationship is likely to be bidirectional, as stress can also lead to overeating (17, 18) and poor dietary choices, such as increased intake of sugar and fat-rich foods (19, 20). Growing evidence suggests that adherence to a healthy diet (e.g., rich in FV and low in sugar and fat) may improve well-being (21). Bioactive compounds found in FV, such as vitamins (e.g., B and C vitamins), minerals (e.g., zinc, magnesium) and fiber seem to modulate neurochemicals (e.g., endorphin, dopamine, serotonin, and norepinephrine) in the brain that promote feelings of happiness (22). Similarly, polyphenols and carotenoids found in FV can reduce oxidative stress (23) and inflammation (24) that have also been linked with improved well-being. Thus, studies investigating the relationship between diet and stress may contribute to a healthier lifestyle and help reduce stress-related conditions. Exploring the association of FV intake with specific stress domains (25), such as "worries," "tension," "joy," and "demands" (stressor) (25) will allow for more precise understanding of this relationship. By definition, "worry" is "a feeling of being unhappy and frightened about something" (26), it is the repetitive feeling of potential future harmful life events (27). This domain covers "worries, anxious concern for the future, and feelings of desperation and frustration" (25). "Tension" is "a feeling of nervousness before an important or difficult event" (26). This domain explores "tense disquietude, exhaustion and the lack of relaxation" (25). "Joy" is defined as a "feeling of great pleasure and happiness" (26). This domain includes "positive feelings of challenges, joy, energy, and security" (25). "Demands" is defined as "the difficult things that you have to do" (26). This domain covers "perceived environmental demands, such as lack of time, pressure, and overload," and it is considered an environmental stressor, rather than a stress reaction (25).

Therefore, the aim of this study was to examine the relationship between consumption of FV and four perceived stress domains (worries, tension, joy, and demands) in a population-based cohort of Australian adults.

MATERIALS AND METHODS

Study Population

This study includes participants from the Australian Diabetes, Obesity, and Lifestyle (AusDiab) study, a large prospective study which aimed to determine the prevalence of diabetes, and other cardiovascular risk factors, including obesity, hypertension, and dyslipidaemia in a cohort representative of the general Australian population. The AusDiab study collected data from 42 randomly selected census collector districts across Australia in 1999–2000, as previously reported (28). A stratified cluster sampling method was used to recruit Australian men and women from six states and the Northern Territory (29). A total of 11,247 participants aged ≥25 years attended a clinical examination (5,049 men; 6,198 women) and were enrolled in the study (28). More details have been previously reported (30).

A flow diagram for participants included in this study is shown in **Supplementary Figure 1**. We excluded participants with missing data for perceived stress ($n = 1{,}697$) and dietary intake ($n = 148$), as well as those missing data for confounding factors ($n = 436$). We also excluded participants with extremely low or high energy intake (<3,300 or >17,500 kJ in men and <2,500 kJ or >14,500 kJ in women) ($n = 275$) (31), and pregnant women ($n = 51$), as per previous studies (8, 14). After exclusions ($n = 2{,}607$), 8,640 were deemed eligible for the study.

Assessment of Perceived Stress Domains

Perceived stress was assessed over the previous 12 months using the original perceived stress questionnaire (PSQ) developed by Levenstein et al. (32). Different stress domains were calculated using the perceived stress domains 20-item reduced PSQ, validated by Fliege and colleagues (25), in which the authors selected the statements that showed the highest item-scale-correlation. This resulted in four stress domains ("worries," "tension," "joy" [the answers were reversed, therefore referred to as "lack of joy"], and "demands"), each one containing five items (25). "Worries" comprise feelings of anxiety in relation to the future, desperation, and frustration. "Tension" includes tense agitation, exhaustion, and the lack of relaxation. "Joy" relates to positive feelings of challenge, energy, and security. Finally, "demands" covers perceived environmental demands, such as lack of time, pressure, and overload (25). All items were scored on a four-point scale ranging from one ("almost never") to four

("usually"), with positive questions (Questions 1, 7, 10, 13, 17, 21, 25, 29), being reverse scored (four ["almost never"] to one ["usually"]). The scores for each domain were derived from five specific questions (25), as shown in **Supplementary Table 1**. A total score was calculated for each domain, ranging from a minimum of 5 to a maximum of 20 (25), with higher scores indicating higher perceived stress levels (33). This questionnaire showed high internal consistency (Cronbach's $\alpha = 0.85$) (25). Specific cut-points for each domain were calculated based on the highest quartile of raw scores for men and women separately (**Supplementary Table 2**), since perceived stress is usually higher in women compared to men (34, 35). As there are no pre-defined cut-offs for the perceived stress domains, we assume the 25% in the highest quartiles presented higher perceived stress. These specific cut-point for worries, tension, lack of joy, and demands were equal to or higher than 9, 11, 11, 12 for men; and equal to or higher than 10, 12, 12, 13 for women, respectively.

Dietary Intake

A validated food frequency questionnaire (FFQ) from the Cancer Council of Victoria (36) was used to assess dietary intake at baseline (1999–2000). This questionnaire comprises 74 food items and recalls usual food intake in the previous 12 months. Total food consumption was computed based on intakes of each food (i.e., bread, milk, FV) in grams per day, calculated according to season, to estimate total energy intake (Kj/d) (36). Nutrient intakes were assessed by using the NUTTAB95 food composition database. Alcohol intake was assessed based on alcoholic beverages frequency of consumption (varying from "never" to "every day") (36).

Intake of Fruit and Vegetables

Intake of fruit and vegetables were assessed using the FFQ and estimated based on the frequency of consumption in the previous 12 months, as previously reported (8). Total fruit included 11 different types **(Supplementary Table 3)**, excluding fruit juice and tinned fruits, due to their high sugar content. The recommendations of the Australian Dietary Guidelines is to consume fresh fruit, with consumption of fruit juice, tinned and dried fruits being recommended as substitutes for fresh fruit only occasionally (37). Total vegetable intake included 24 types (**Supplementary Table 3**), excluding potatoes roasted and fried—including hot chips, as these are not recommended as part of a healthy diet (37). The daily intake was estimated in grams per day (g/d). Serves of fruit and vegetables were calculated by dividing the total intake in grams by the recommended serving size (37): for fruit, one serve equals 150 g/day; and for vegetables one serve equals 75 g/day (37).

Demographic and Clinical Assessment

The following demographic variables were collected during household interviews: date of birth, sex (male/female), relationship status (married, single, de facto, separated, divorced, widowed), and education status (never to some high school, completed University or equivalent). Relationship status (38) and level of education (39) have been added as potential confounding factors in our study as they can affect stress and mental health. Furthermore, higher educational level (40) and being married (41) have also been associated with greater FV intakes. The socio-economic index for areas (SEIFA), which assesses the socio-economic status per geographic area according to the 5-yearly census (42) was used to estimate socio-economic status. SEIFA is an Index of Relative Socio-economic Disadvantage (IRSD) (43). Anthropometric measurements were performed at the clinic visits (28, 44) and included: height, measured using a stadiometer to the nearest 0.5 cm without shoes; and weight, measured without shoes and excess clothing, using a mechanical beam balance to the nearest 0.1 kg (45). Body mass index (BMI) was calculated as weight (kg) divided by height in meters squared.

Physical activity in the previous week was estimated using the Active Australia Survey Questionnaire (46). Total walking time (if continuous for $\geq$ 10 min) and/or moderate-intensity physical activity, were summed with the time performing vigorous-intensity activity (multiplied by two) (46) to obtain "total physical activity" (min/week). Levels of physical activity were classified as sufficient (>150 min per week across at least five sessions), insufficient (<150 min per week and/or fewer than five sessions per week), or no physical activity (46).

Smoking status was assessed using an interviewer-administered questionnaire (28), and classified as: current smokers (smoked at least daily), ex-smokers (smoked less than daily for at least the last 3 months), or never smokers (smoked <100 cigarettes during life) (47).

Plasma glucose levels was assessed and categorized as normal glucose levels, impaired fasting glucose, impaired glucose tolerance, newly diagnosed diabetes mellitus (DM), or known DM. Prior cardiovascular disease (CVD) was self-reported by answering yes/no (48). Variables indicating diabetes and CVD status were added to the multivariable adjusted model as potential confounding factors, as those participants may have received dietary advice promoting a healthier lifestyle. Average weekly income was estimated based on six categories: $0–199, $200–399, $400–599, $600–799, $800–1,499, and >$1,500.

The Dietary Guideline Index (DGI) comprised of 15 items—including fruit and vegetable intakes (49), based on the Australian Dietary Guidelines (50, 51), was used as a marker of diet quality. Items were scored from 0 to 10 (lowest to optimal intake) and total scores ranged from 0 to 150 (higher scores represent better diet quality and adherence to dietary guidelines) (49).

Life events scale comprised 13 potential life events (i.e., marriage breakdown, financial hardship, and death of a family member/friend in the 12 months (52). Participants were asked how much those life events affected them in the previous 12 months (with responses being: 0 = no event, 1 = had minimum or no effect, 2 = affected a little, 3 = affected somewhat, and 4 = affected a great deal). Participants were given a score of "0" for the items they selected "no life event"; or a score of "1" to each item their response was $\geq$1. Items were summed with scores ranging from 0 to 13.

A physical component score (PCS) assessed using the SF-36 (36-item short form survey health-related quality of life scale) (53) was added to the multivariable adjusted model, to control

for physical issues. SF36 is composed of 8 domains comprising two summary measures: physical (four domains: physical-functioning, role-physical, bodily pain, and general health) and mental health (four domains: vitality, social functioning, role-emotional, and mental health). In the present study, only the physical health score was used (since the mental health measure would have been an overadjustment). Internal consistency (Cronbach's alpha) for the eight domains was 0.83–0.93, 0.94 for the physical health summary score, and 0.89 for the mental health summary score (54).

Statistical Analysis

Statistical analyses were performed in Stata (Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC) (55) using commands for complex survey designs when adopting logistic regression. The survey commands provide population-level estimates, by adjusting standard errors and incorporating stratification weights (28). Main exposures for this study were fruit and vegetable intake, combined and separately. Primary outcomes included the four domains of perceived stress (worries, tension, joy and demands). The association between the exposures and the odds of being in the highest quartile of each specific perceived stress domains was assessed using logistic regression. Statistical significance was set at $p \leq 0.05$ (two-tailed) for all tests. Two models were considered: unadjusted and the multivariable adjusted for age, sex, BMI, energy intake, relationship status, physical activity levels, level of education, SEIFA, smoking status, self-reported history of CVD, and diabetes, as these covariates could affect both the exposure and outcomes of the study.

To further examine whether a healthier diet in general could confound the observed associations, we adjusted for diet quality (49). Income was used as a further adjustment for socio-economic status at the level of the individual. The life event scale was added to the multivariable adjusted model to adjust for the influence of major life events (e.g., death of a family member/friend) on the results. Finally, a physical health score was also added to the adjusted model to account for physical issues.

Additional Analyses

Generalized linear models adopting restricted cubic splines in R (56) were also used to examine the relationship between FV intake and each of the four perceived stress domains on a continuous scale.

Interaction terms between fruit and vegetable intake, separately, and age (<45 y, 45–65 y, ≥65 y), sex and physical activity were used to investigate potential effect modification domains where a significant overall relationship was observed.

RESULTS

Baseline Characteristics

Baseline characteristics of all 8,640 participants is shown in **Table 1**. Participants' mean ± SD age was 47.8 ±15 years, with 50.1% being female. Median (IQR) scores for the perceived stress domains (maximum score of 20) including worries, tension, lack of joy and demands were 8 (6–10), 9 (7–12), 9 (7–11), and 10 (7–10), respectively. Participants who were excluded from the study presented similar characteristics (**Supplementary Table 4**).

TABLE 1 | Clinical and demographic characteristics of all participants[a].

Characteristics	All
Sample size (*n*)	**8,640**
Age (years), mean ± SD	47.8 ± 15.0
Sex (women), *n* (%)	4,747 (50.1)
BMI (kg/m^2), mean ± SD	26.6 ± 4.8
Underweight, n (%)	79 (1%)
Normal weight, n (%)	3,152 (39%)
Overweight, n (%)	3,483 (40%)
Obese, n (%)	1,926 (20%)
Energy intake (kcal/d), mean ± SD	2,042 ± 697
Physical activity (min/week), mean (IQR)	277 (258–295)
Insufficient physical activity, n (%)	4,142 (48%)
Sufficient physical activity, n (%)	4,498 (52%)
Relationship status, *n* (%)	
Married	6,268 (72.8)
De facto	414 (4.4)
Separated	217 (2.4)
Divorced	515 (5.1)
Widowed	528 (5.2)
Single	698 (10.0)
SEIFA	1,025 ± 87
Level of education, *n* (%)	
Never to some high school	3,445 (35.7)
Completed university or equivalent	5,195 (64.3)
Smoking status, *n* (%)	
Current	1,338 (16.3)
Ex-smoker	2,537 (25.9)
Non-smoker	4,765 (57.8)
Prior CVD, *n* (%)	685 (6.7)
Diabetes status, *n* (%)	
Normal glucose levels	6,387 (77.1)
Impaired fasting glucose	512 (5.8)
Impaired glucose tolerance	1,043 (10.3)
Newly diagnosed diabetes mellitus	350 (3.5)
Known diabetes mellitus	348 (3.3)
Score for worries, median (IQR)	8 (6–10)
Score for tension, median (IQR)	9 (7–12)
Score for lack of joy, median (IQR)	9 (7–11)
Score for demands, median (IQR)	10 (7–13)

[a]*Estimated using the survey command to apply the necessary weighting for selection bias. Worries, tension lack of joy and demands represent Q4. BMI, Body Mass Index; CVD, Cardiovascular Disease; SEIFA, Socio-Economical Index For Areas.*

Associations of Fruit and Vegetable Intake With Perceived Stress Domains

When we examined the associations of the highest vs. lowest intakes of FV in those with higher perceived stress domains (top 25%), compared to participants with the lowest FV intake, those

TABLE 2 | Odds ratios (OR) for worries, tension, lack of joy and demands by quartiles of FV intake.

	Fruit and vegetable (FV) intake quartiles			
	Q1	**Q2**	**Q3**	**Q4**
FV intake	*n = 2,161*	*n = 2,161*	*n = 2,158*	*n = 2,160*
Median (IQR) (g/day)	193 (155–221)	293 (269–318)	400 (371–436)	575 (518–676)
Worries, *n* (%)	860 (43%)	756 (38%)	641 (33%)	664 (32%)
Unadjusted	Ref.	0.80 (0.64, 1.01)	0.65 (0.54, 0.78)#	0.63 (0.52, 0.75)#
Multivariable adjusted	Ref.	0.82 (0.66, 1.02)	0.71 (0.58, 0.88)#	0.68 (0.55, 0.85)#
Multivariable adjusted plus income	Ref.	0.83 (0.67, 1.03)	0.72 (0.59, 0.89)#	0.69 (0.55, 0.86)#
Multivariable adjusted plus life events	Ref.	0.87 (0.67, 1.11)	0.74 (0.58, 0.94)*	0.73 (0.57, 0.93)*
Multivariable adjusted plus physical health score	Ref.	0.82 (0.66, 1.02)	0.72 (0.59, 0.89)#	0.70 (0.56, 0.89)#
Tension, *n* (%)	751 (37%)	643 (32%)	547 (30%)	542 (26%)
Unadjusted	Ref.	0.79 (0.64, 0.98)*	0.72 (0.57, 0.90)#	0.58 (0.49, 0.70) #
Multivariable adjusted	Ref.	0.81 (0.65, 1.00)	0.78 (0.62, 0.98)*	0.64 (0.52, 0.79)#
Multivariable adjusted plus income	Ref.	0.81 (0.66, 1.00)	0.78 (0.62, 0.99)*	0.64 (0.52, 0.79)#
Multivariable adjusted plus life events	Ref.	0.82 (0.65, 1.03)	0.83 (0.64, 1.07)	0.67 (0.54, 0.84)#
Multivariable adjusted plus physical health score	Ref.	0.84 (0.69, 1.02)	0.79 (0.63, 0.99)*	0.66 (0.54, 0.82)#
Lack of joy, *n* (%)	744 (35%)	646 (32%)	561 (27%)	525 (27%)
Unadjusted	Ref.	0.86 (0.68, 1.08)	0.67 (0.54, 0.83)#	0.68 (0.57, 0.80)#
Multivariable adjusted	Ref.	0.89 (0.71, 1.13)	0.76 (0.60, 0.95)*	0.77 (0.64, 0.93)#
Multivariable adjusted plus income	Ref.	0.90 (0.72, 1.14)	0.76 (0.60, 0.96)*	0.78 (0.64, 0.94)#
Multivariable adjusted plus life events	Ref.	0.93 (0.74, 1.16)	0.79 (0.62, 1.00)*	0.81 (0.68, 0.97)*
Multivariable adjusted plus physical health score	Ref.	0.92 (0.74, 1.14)	0.77 (0.61, 0.97)*	0.78 (0.64, 0.96)*
Demands, *n* (%)	711 (37%)	671 (34%)	567 (31%)	565 (30%)
Unadjusted	Ref.	0.90 (0.71, 1.14)	0.79 (0.65, 0.95)*	0.74 (0.60, 0.91)#
Multivariable adjusted	Ref.	0.92 (0.68, 1.23)	0.88 (0.72, 1.08)	0.88 (0.69, 1.13)
Multivariable adjusted plus income	Ref.	0.89 (0.66, 1.22)	0.87 (0.71, 1.07)	0.87 (0.67, 1.12)
Multivariable adjusted plus life events	Ref.	0.93 (0.68, 1.28)	0.91 (0.73, 1.13)	0.94 (0.72, 1.22)
Multivariable adjusted plus physical health score	Ref.	0.94 (0.71, 1.24)	0.89 (0.73, 1.08)	0.89 (0.69, 1.15)

*Odds ratios (ORs) and 95% confidence intervals obtained using the survey command for logistic regression. Cut-points for worries, tension, lack of joy and demands were ≥9, ≥11, ≥11, ≥12 for men; and ≥10, ≥12, ≥12, ≥13 for women, respectively. Analyses were unadjusted and multivariable adjusted for age, sex, BMI [body mass index], energy intake, relationship status, physical activity, level of education, SEIFA [Socio-economical index for areas], smoking status, diabetes and prior cardiovascular disease). *Indicates a statistically significant difference ($p < 0.05$) from Q1; #indicates a statistically significant difference ($p < 0.013$) from Q1 based on Bonferroni-corrections.*

with the highest FV intake (Q4) had 32, 36, and 23% lower odds of worries, tension, and lack of joy, respectively (**Table 2**). There was, however, no association between FV intake and the demands domain of perceived stress.

The associations between fruit intake alone and each domain of perceived stress are shown in **Table 3**. Compared to participants with the lowest fruit intake, those with the highest fruit intake had 25, 27, and 16% lower odds for worries, tension, and lack of joy, respectively. No relationship was observed for the demands domain.

The associations between vegetable consumption alone and domains of perceived stress are shown in **Table 4**. Compared to individuals with lower intake, those with the highest vegetables intake (Q4) had a statistically significant 29, 28, and 33% lower odds for worries, tension, and lack of joy, respectively. No relationship was observed for the demand domain.

The addition of a marker of diet quality into the multivariable adjusted model only slightly affected our results; specifically, the association of higher intake of fruit, and FV combined, with lower lack of joy was attenuated (Q4 for fruit, OR [95%CI] 0.93 [0.77, 1.13]; for FV, 0.87 [0.70, 1.07]), perhaps because the diet quality index includes fruit and vegetable in its calculation. This relationship for vegetable intake remained similar to the primary analysis (OR [95% CI]: 0.72 [0.55, 0.95]). Similar relationships were observed for higher FV intake with lower worries (Q4 for FV, 0.69 [0.54, 0.89]; for fruit, 0.77 [0.64, 0.93]; for vegetable, 0.73 [0.58, 0.94]), compared to Q4. Likewise, the relationship for higher FV intake and lower odds for tension (Q4 for FV, 0.70 [0.56, 0.87]; fruit, 0.80 [0.65, 0.98]; vegetable, 0.77 [0.61, 0.98]), compared to lower intake (Q1) remained similar.

Adding income, life events and a physical health score as further covariates to the multivariable adjusted model did not change the associations of FV intake (**Table 2**) with the domains of perceived stress, as well as with vegetable intake (**Table 4**). The association of fruit intake with worries and tension remained similar after further adjustments for the aforementioned covariates. However, the association of fruit intake with lack of joy did not remain significant after further adjusting for income, life events and physical health score (**Table 3**).

TABLE 3 | Odds ratios (OR) for worries, tension, lack of joy and demands by quartiles of fruit intake.

	Fruit intake quartiles			
	Q1	**Q2**	**Q3**	**Q4**
Fruit intake	*n = 2,160*	*n = 2,160*	*n = 2,161*	*n = 2,159*
Median (IQR) (g/day)	62 (52–72)	119 (106–133)	225 (193–250)	372 (322–448)
Worries, *n* (%)	803 (40%)	711 (36%)	733 (36%)	674 (33%)
Unadjusted	Ref.	0.69 (0.58, 0.82)#	0.58 (0.44, 0.75)#	0.61 (0.52, 0.71)#
Multivariable adjusted	Ref.	0.80 (0.67, 0.96)*	0.74 (0.58, 0.95)*	0.75 (0.62, 0.89)#
Multivariable adjusted plus income	Ref.	0.80 (0.67, 0.95)*	0.75 (0.59, 0.95)*	0.75 (0.63, 0.90)#
Multivariable adjusted plus life events	Ref.	0.84 (0.67, 1.06)	0.77 (0.55, 1.09)	0.80 (0.65, 0.98)*
Multivariable adjusted plus physical health score	Ref.	0.82 (0.69, 0.97)*	0.75 (0.59, 0.95)*	0.77 (0.63, 0.94)#
Tension, *n* (%)	765 (37%)	604 (32%)	577 (29%)	537 (27%)
Unadjusted	Ref.	0.80 (0.66, 0.97)*	0.68 (0.57, 0.82)#	0.63 (0.54, 0.74)#
Multivariable adjusted	Ref.	0.85 (0.71, 1.02)	0.79 (0.65, 0.96)*	0.73 (0.62, 0.86)#
Multivariable adjusted plus income	Ref.	0.85 (0.71, 1.01)	0.79 (0.65, 0.96)*	0.73 (0.62, 0.86)#
Multivariable adjusted plus life events	Ref.	0.88 (0.69, 1.12)	0.84 (0.65, 1.07)	0.77 (0.63, 0.93)#
Multivariable adjusted plus physical health score	Ref.	0.85 (0.72, 1.00)	0.78 (0.64, 0.96)*	0.74 (0.62, 0.87)#
Lack of joy, *n* (%)	778 (37%)	590 (29%)	581 (27%)	527 (28%)
Unadjusted	Ref.	0.71 (0.62, 0.81)#	0.63 (0.51, 0.77)#	0.66 (0.56, 0.77)#
Multivariable adjusted	Ref.	0.84 (0.73, 0.96)#	0.80 (0.64, 1.01)	0.84 (0.71, 0.99)*
Multivariable adjusted plus income	Ref.	0.84 (0.73, 0.96)#	0.81 (0.65, 1.01)	0.84 (0.71, 1.00)
Multivariable adjusted plus life events	Ref.	0.88 (0.75, 1.03)	0.85 (0.68, 1.07)	0.89 (0.73, 1.08)
Multivariable adjusted plus physical health score	Ref.	0.85 (0.74, 0.96)*	0.79 (0.63, 0.99)*	0.86 (0.70, 1.04)
Demands, *n* (%)	715 (37%)	635 (34%)	601 (30%)	563 (31%)
Unadjusted	Ref.	0.86 (0.72, 1.03)	0.73 (0.58, 0.92)#	0.78 (0.65, 0.93)#
Multivariable adjusted	Ref.	0.93 (0.74, 1.16)	0.89 (0.67, 1.18)	0.97 (0.79, 1.18)
Multivariable adjusted plus income	Ref.	0.93 (0.73, 1.18)	0.87 (0.65, 1.17)	0.95 (0.77, 1.18)
Multivariable adjusted plus life events	Ref.	0.97 (0.74, 1.26)	0.92 (0.65, 1.29)	1.02 (0.81, 1.30)
Multivariable adjusted plus physical health score	Ref.	0.91 (0.72, 1.16)	0.88 (0.66, 1.17)	0.95 (0.77, 1.18)

*Odds ratios (ORs) and 95% confidence intervals obtained using the survey command for logistic regression. Cut-points for worries, tension, lack of joy, and demands were ≥9, ≥11, ≥11, ≥12 for men; and ≥10, ≥12, ≥12, ≥13 for women, respectively. Analyses were unadjusted and multivariable adjusted for age, sex, BMI [body mass index], energy intake, relationship status, physical activity, level of education, SEIFA [Socio-economical index for areas], smoking status, diabetes and prior cardiovascular disease). *Indicates a statistically significant difference ($p < 0.05$) from Q1; #indicates a statistically significant difference ($p < 0.013$) from Q1 based on Bonferroni-corrections.*

Excluding participants with diabetes ($n = 698$, 6.8%) did not alter the results of the study. Among participants without diabetes ($n = 7{,}942$), those with higher consumption of FV had 36, 40, and 27% lower odds of worries (0.64 [0.50, 0.80]), tension (0.60 [0.49, 0.73]), and lack of joy (0.73 [0.60, 0.89]), respectively. This was similar for the associations of fruit intake with worries (0.71 [0.59, 0.84]), tension (0.68 [0.59, 0.81]), and lack of joy (0.80 [0.66, 0.97]), and for vegetables with worries (0.69 [0.56, 1.11]), tension (0.71 [0.57, 0.88]), and lack of joy (0.67 [0.51, 0.87]).

Additional Analyses

In multivariable adjusted general linear models, FV intake was non-linearly associated with worries, tension and lack of joy (all p for non-linearity <0.01) and linearly associated with demands (p for non-linearity $= 0.59$). **Supplementary Figure 2** demonstrates the relationship of FV intake (g/day) with (a) worries, (b) tension, (c) lack of joy, and (d) demands (scores ranging from 5 to 20, lowest to highest; all $p < 0.01$).

In the interaction testing, sex and age did not influence the relationship of fruit and vegetables with worries, tension and lack of joy (all $p_{interaction} > 0.1$). We observed a significant interaction between physical activity and intake of vegetables for tension ($p_{interaction} = 0.002$). In those with sufficient weekly amount of physical activity (≥150 min/week) there was 19% lower odds (0.81 [0.71, 0.93]) for higher tension, for each serve increase in vegetable intake, but this was not statistically significant (0.95 [0.85, 1.07]) in those with insufficient physical activity (<150 min/week).

All four perceived stress domains were significantly intercorrelated (worries vs. tension: $r = 0.71$, worries vs. joy: $r = 0.53$, worries vs. demands: $r = 0.50$, tension vs. joy: $r = 0.63$, tension vs. demands: $r = 0.57$: all $p < 0.001$), with the correlation between joy and demands being the weakest ($r = 0.33$).

DISCUSSION

We have previously reported an inverse association between FV intake, combined and separately, with ∼10% lower levels of perceived stress (8). We now extend our work and have examined

TABLE 4 | Odds ratios (OR) for worries, tension, lack of joy and demands by quartiles of vegetable intake.

	Vegetable intake quartiles			
	Q1	**Q2**	**Q3**	**Q4**
Vegetable intake	*n = 2,160*	*n = 2,163*	*n = 2,157*	*n = 2,160*
Median (IQR) (g/day)	89 (65–105)	142 (130–153)	188 (175–202)	264 (238–308)
Worries, *n* (%)	888 (45%)	705 (36%)	669 (32%)	659 (33%)
Unadjusted	Ref.	0.84 (0.69, 1.01)	0.85 (0.66, 1.10)	0.73 (0.58, 0.94)*
Multivariable adjusted	Ref.	0.95 (0.82, 1.11)	0.88 (0.67, 1.14)	0.71 (0.56, 0.90)#
Multivariable adjusted plus income	Ref.	0.96 (0.84, 1.11)	0.88 (0.67, 1.15)	0.72 (0.58, 0.90)#
Multivariable adjusted plus life events	Ref.	0.95 (0.75, 1.20)	0.89 (0.65, 1.21)	0.70 (0.52, 0.95)*
Multivariable adjusted plus physical health score	Ref.	0.96 (0.83, 1.11)	0.88 (0.67, 1.14)	0.72 (0.58, 0.91)#
Tension, *n* (%)	706 (36%)	608 (32%)	608 (30%)	561 (28%)
Unadjusted	Ref.	0.84 (0.65, 1.08)	0.76 (0.56, 1.02)	0.71 (0.57, 0.87)#
Multivariable adjusted	Ref.	0.91 (0.70, 1.16)	0.78 (0.57, 1.08)	0.72 (0.57, 0.90)#
Multivariable adjusted plus income	Ref.	0.91 (0.71, 1.17)	0.78 (0.57, 1.08)	0.72 (0.57, 0.90)#
Multivariable adjusted plus life events	Ref.	0.92 (0.69, 1.22)	0.79 (0.55, 1.12)	0.72 (0.55, 0.94)*
Multivariable adjusted plus physical health score	Ref.	0.93 (0.73, 1.18)	0.80 (0.59, 1.08)	0.74 (0.59, 0.92)#
Lack of joy, *n* (%)	727 (36%)	587 (29%)	588 (29%)	574 (28%)
Unadjusted	Ref.	0.72 (0.59, 0.89)#	0.71 (0.51, 1.00)	0.69 (0.52, 0.90)#
Multivariable adjusted	Ref.	0.81 (0.65, 1.01)	0.72 (0.51, 1.02)	0.67 (0.51, 0.89)#
Multivariable adjusted plus income	Ref.	0.81 (0.65, 1.01)	0.72 (0.51, 1.02)	0.68 (0.52, 0.89)#
Multivariable adjusted plus life events	Ref.	0.82 (0.66, 1.02)	0.72 (0.50, 1.05)	0.67 (0.49, 0.93)*
Multivariable adjusted plus physical health score	Ref.	0.83 (0.69, 1.00)	0.74 (0.54, 1.02)	0.69 (0.52, 0.90)#
Demands, *n* (%)	687 (36%)	642 (35%)	620 (30%)	565 (31%)
Unadjusted	Ref.	0.93 (0.78, 1.11)	0.75 (0.57, 0.98)*	0.78 (0.63, 0.98)*
Multivariable adjusted	Ref.	1.07 (0.88, 1.30)	0.82 (0.60, 1.11)	0.84 (0.67, 1.05)
Multivariable adjusted plus income	Ref.	1.05 (0.86, 1.29)	0.81 (0.60, 1.10)	0.82 (0.67, 1.02)
Multivariable adjusted plus life events	Ref.	1.08 (0.87, 1.33)	0.82 (0.59, 1.14)	0.86 (0.68, 1.08)
Multivariable adjusted plus physical health score	Ref.	1.08 (0.89, 1.31)	0.84 (0.62, 1.14)	0.85 (0.68, 1.06)

*Odds ratios (ORs) and 95% confidence intervals obtained using the survey command for logistic regression. Cut-points for worries, tension, lack of joy and demands were ≥9, ≥11, ≥11, ≥12 for men; and ≥10, ≥12, ≥12, ≥13 for women, respectively. Analyses were unadjusted and multivariable adjusted for age, sex, BMI [body mass index], energy intake, relationship status, physical activity, level of education, SEIFA [Socio-economical index for areas], smoking status, diabetes and prior of cardiovascular disease). *Indicates a statistically significant difference ($p < 0.05$) from Q1; #indicates a statistically significant difference ($p < 0.013$) from Q1 based on Bonferroni-corrections.*

the relationship of FV intake with four perceived stress domains (worries, tension, lack of joy and demands) for the first time in adults across all ages. When considering the four domains of perceived stress, we demonstrate that higher intakes of FV, combined and separately, were associated with lower odds for worries, tension, and lack of joy, independent of other lifestyle and socioeconomic factors. No relationship was observed for the environmental stressor "demands" domain.

It is noteworthy that fruit and vegetable data in this study was collected in 1999–2000. In 2017, according to the Australian Bureau of Statistics (57), the average fruit and vegetables consumption of adults (≥18 years) was approximately 1.7 serves of fruit (∼250 g/d) and 2.4 serves of vegetables (∼180 g/d) per day, respectively. Such findings are comparable to the average fruit and vegetable intake of 200 and 170 g/d, respectively, in the AusDiab cohort in 1999–2000. Although there was a slight increase in FV intake in the last 20 years, consumption of FV remains similar and below the recommendations.

A positive relationship of FV intake with lower odds of worries was seen in our study. This concurs with a previous study including ∼8,500 year-5 Canadian students, where an inverse association between Diet Quality Index-International ([DQI-I]—consisting of four components: (1) variety [including FV groups], (2) adequacy [including FV], (3) moderation [i.e., sodium, total and saturated fats], and 4) balance [macronutrients and fatty acids ration]) (58) and feelings of worry (OR [95% CI]: 0.90 (0.85–0.97) was observed (59).

In our study, a higher intake of FV was associated with lower odds for tension. In support of our results, a previous study with 138 healthy men and women (60) observed lower scores of tension in those with a vegetarian diet, compared to omnivorous diets. Similarly, both a 14-week DASH (Dietary Approaches to Stop Hypertension) diet—including recommendation of ≥4 serves of fruit and ≥4 serves of vegetables, and a standard healthy diet—with recommendations of FV being ≥2 serves of fruit and 2–3 serves of vegetables, led to improvements in overall mood and other mood-related domains, including tension, in post-menopausal healthy women (61). All of the aforementioned diets are considered healthy diets and contain

high amounts of FV, suggesting a beneficial role of FV-rich diets for tension.

Similar to our results on the positive relationship between greater intake of FV and joy, after examining the food diaries of more than 12,000 adults, a national Australian survey reported that consumption of FV predicted improvements in life satisfaction, happiness and well-being (62). Consuming a range of FV with different colors has also been positively linked to improved mood and increased creativity, curiosity, and conduciveness to happiness in young adults (63).

Bioactive compounds found in a healthy diet, particularly in a FV-rich diet, such as vitamins (i.e., B and C vitamins), minerals (i.e., zinc, magnesium) and fiber (i.e., resistant starch), have been linked to health and well-being (21). These constituents appear to play a protective role on mental health conditions (11), modulating some of the neurotransmitters in the brain, which regulate mood (21) and could potentially increase joy and happiness (64). Some neurochemicals (e.g., endorphin, dopamine, serotonin, norepinephrine, and melatonin) have been shown to cause feelings of happiness (22). Similarly, polyphenols found in FV have been positively associated with mental well-being, potentially due to their anti-inflammatory, neuroprotective, and prebiotic properties (65). Bioactive compounds in FV seem to protect against oxidative stress (23), inflammation (24) and brain plasticity (66), which are impaired in those with mental health issues (67). Dysbiosis of the gut microbiota is another potential pathway, with evidence showing the involvement of the microbiome in the modulation of stress response (11) and diet being an important modulator of gut health (68).

In the present study, consumption of FV was not associated with "demands." We also observed that all four perceived stress domains were intercorrelated, with the "demands" domain showing the lowest correlation, as previously reported (25). This could be due to the nature of the "demands" domain, as demands is considered an external stressor, rather than a stress reaction (25). Also, feelings of demands have been reported to be stronger in middle-aged people, due to high career and social strains, in addition to caregiving responsibilities (69). By including people of all ages in the current study, potential demands perceived by participants may not have been as pronounced.

When we examined the relationship of FV intake as a continuous variable (g/day) with the scores of all four perceived stress domains, greater FV intake was significantly non-linearly associated with less worries, tension and lack of joy, and linearly associated with lower demands. Intake of FV >400 g/day appears to be more beneficial. A FV intake of approximately ≥400 g/day (median of Q3), was associated with lower odds of worries, tension and lack of joy. Such findings are supported by current recommendations by the World Health Organisation to consume a minimum of 400 g/day of FV for the prevention of non-communicable health conditions (70). When examining the relationship of fruit and vegetable separately, with perceived stress domains, we observed that consumption of ≥225 g/day (median in Q3) of fruit (≥1.5-serves per day), and at least 260 g/day of vegetables (at least 3.5 serves of vegetables) would provide the greatest benefits for perceived stress domains. Such findings also compliment Australian dietary guidelines (37) that recommend consuming 2 × 150 g serves of fruit and 5–6 × 75 g serves of vegetables each day as part of a healthy diet.

Another interesting finding of this study from the interaction analysis was that sex and age did not influence the relationship of FV with worries, tension and lack of joy. However, a significant interaction between physical activity and vegetable consumption for tension was observed. Upon further investigation, those with sufficient weekly amount of physical activity (≥150 min/week) had 19% lower odds for higher tension, for each serve increase in vegetable intake. Although the relationship between higher consumption of vegetable intake and lower tension (OR [95%CI]: 0.62 [0.41–0.93]) was significant in those with sufficient physical activity (≥150 min/week), despite a similar point estimate, this relationship was attenuated (0.83 [0.61–1.13]) in those with insufficient physical activity (0–150 min/week). Such findings highlight the importance of a high vegetable intake in combination with regular physical activity to minimize feelings of tension; findings also promoted previously (71). The importance of exercise for mental health disorders is well-established and likely to work in synergy with FV nutrients (71) to reduce tension. Although the mechanisms underlying the beneficial effects of exercise on well-being are not fully understood, some pathways have been suggested (72). Physical activity may improve psychological well-being by promoting self-efficacy (73). Physical activity has been associated with positive changes to insulin resistance, inflammation, and oxidative stress, which could potentially explain the beneficial effects of physical activity on cognitive function and brain-related diseases (74, 75). Evidence suggests that exercise (76), and a healthy diet (77) have a beneficial impact on gut microbiota diversity (76), which has been linked to better brain processes and behavior (78, 79). Current recommendations to increase FV intake for improved physical health may have positive benefits on mental health. Perhaps, a combination of the aforementioned lifestyle factors may work synergistically to further contribute toward positive mental health outcomes. For example, it is likely that the benefit of a healthy diet on perceived stress, specifically FV intake, is likely to be most beneficial when combined with regular exercise.

Overall, our results suggest a beneficial link between greater FV intake and lower worries, tension and lack of joy. However, the relationship between FV intake and stress is likely bidirectional, with an alternative interpretation of the associations being possible. Evidence shows that stress and mental health disorders can also lead to poor dietary habits, such as lower intake of FV and higher intake of energy-dense foods (13, 80).

Limitations and Strengths of the Study

Limitations of this work include the cross-sectional nature of the study; therefore, we cannot infer causality. Despite AusDiab being a national survey including participants from 42 randomly selected census collector districts across all states and territories in Australia, designed to reflect the Australian general population, we cannot rule out the risk of selection bias. On

the basis of the analysis, it's difficult to differentiate between a FV-rich diet leading to lower stress levels and improved well-being or vice versa. Based on previous evidence, it is likely that both co-exist. The mean BMI indicated our participants were slightly overweight (26.6 ± 4.8 kg/m^2). Overeating and increased intake of high-dense energy and fat foods may exacerbate stress levels (19, 20). Therefore, caution should be exercised when generalizing these results to populations with substantially different BMI (e.g., underweight, severely obese). Approximately 7% of the participants had diabetes in 2000, which may have influenced the results of our study. As such, diabetes status was added to the multivariable adjusted model as a potential confounding factor to minimize potential bias. Although the findings from our study rely on a valid tool for assessing perceived stress domains in healthy populations (such us the participants from the AusDiab cohort), it is possible that these results may differ between cohorts due to differences in demographics.

Strengths of this study include the investigations of the relationship between FV intake and four domains of perceived stress, for the first time. Additionally, the sample size was large, national, population-based and included adults across the lifespan. Although most information was self-reported, both perceived stress and dietary intake were assessed using validated questionnaires.

Future studies could aim to further understand the potential mechanisms for the negative association between FV intake and perceived stress domains. There is also need for longitudinal studies to investigate the impact of FV on perceived stress domains, and vice-versa. The relationship between diet and stress, and stress-related mental health issues is likely to be bidirectional, and understanding its associations may have critical implications in alleviating stress levels and improving mental well-being (81). Clinical trials exploring the effects of FV intake on perceived stress domains would enhance understanding on the impact of FV intake on perceived stress domains, and vice-versa.

CONCLUSION

Our data suggest that higher intakes of FV are inversely associated with overall stress perceived by adults, and specific stress domains, such as worries, tension and lack of joy. However, there is need for further research to examine the effects of overall and specific types of FV intake in a clinical trial setting.

DATA AVAILABILITY STATEMENT

The data presented in this article are not readily available because and belong to the Baker Heart and Diabetes Institute. Restrictions apply to the availability of these data, which were used under license for the current study. Raw data are not publicly available. However, data described in the manuscript, codebook, and analytic code will be made available upon request and approval of the AusDiab Steering Committee. Requests to access the datasets should be directed to jonathan.shaw@baker.edu.au.

AUTHOR CONTRIBUTIONS

SR-B, MS, and JL designed this cross-sectional study. DM, JS, and RD provided data collected in the original cohort study. SR-B and MS analyzed the data. SR-B drafted and had primary responsibility for final content of the manuscript. SR-B, MS, LB, NB, CB, RW, JD, CH, DM, JS, RD, JH, and JL interpreted the results, had significant input, reviewed, and approved the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The salary of MS is supported by a Royal Perth Hospital Research Foundation Career Advancement Fellowship (ID: CAF 130/2020) and an Emerging Leader Fellowship from the Western Australian Future Health Research and Innovation Fund. The salary of LB was supported by a National Health and Medical Research Council (NHMRC) of Australia Emerging Leadership Investigator Grant (ID: 1172987) and a National Heart Foundation of Australia Post-Doctoral Research Fellowship (ID: 102498). The salary of CB is supported by a Royal Perth Hospital Research Foundation 'Lawrie Beilin' Career Advancement Fellowship (ID: CAF 127/2020). The salary of JH was supported by a National Health and Medical Research Council of Australia Senior Research Fellowship (ID: 1116973). The salary of JS was supported by a National Health and Medical Research Council Investigator Grant (ID: 1173952). The salary of JL was supported by a National Heart Foundation of Australia Future Leader Fellowship (ID: 102817). None of the funding agencies had any role in the conduct of the study; collection, management, analysis, or interpretation of the data; or preparation, review, or approval of the manuscript.

ACKNOWLEDGMENTS

The AusDiab study, initiated and coordinated by the International Diabetes Institute, and subsequently coordinated by the Baker Heart and Diabetes Institute, gratefully acknowledges the support and assistance given by: A. Allman, B. Atkins, S. Bennett, S. Chadban, S. Colagiuri, M. de Courten, M. Dalton, M. D'Emden, T. Dwyer, D. Jolley, I. Kemp, P. Magnus, J. Mathews, D. McCarty, A. Meehan, K. O'Dea, P. Phillips, P. Popplewell, C. Reid, A. Stewart, R. Tapp, H. Taylor, T. Welborn, F. Wilson, and P. Zimmet. Also, for funding or logistical support, we are grateful to: The Commonwealth Dept of Health and Aged Care, Abbott Australasia Pty Ltd., Alphapharm Pty Ltd., AstraZeneca, Aventis Pharmaceutical, Bristol-Myers Squibb Pharmaceuticals, Eli Lilly (Aust) Pty Ltd., GlaxoSmithKline, Janssen-Cilag (Aust) Pty Ltd., Merck Lipha s.a., Merck Sharp & Dohme (Aust), Novartis Pharmaceutical (Aust) Pty Ltd., Novo Nordisk Pharmaceutical Pty Ltd., Pharmacia and Upjohn Pty Ltd., Pfizer Pty Ltd., Roche Diagnostics, Sanofi Synthelabo (Aust) Pty Ltd., Servier Laboratories (Aust) Pty Ltd., BioRad Laboratories Pty Ltd., HITECH Pathology Pty Ltd., the Australian Kidney Foundation, Diabetes Australia,

Diabetes Australia (Northern Territory), Queensland Health, South Australian Department of Human Services, Tasmanian Department of Health and Human Services, Territory Health Services, Victorian Department of Human Services, the Victorian OIS program and Health Department of Western Australia.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.837066/full#supplementary-material

REFERENCES

1. VanItallie TB. Stress: a risk factor for serious illness. *Metab Clin Exp.* (2002) 51:40–45. doi: 10.1053/meta.2002.33191
2. Hayward RD, Taylor WD, Smoski MJ, Steffens DC, Payne ME. Association of five-factor model personality domains and facets with presence, onset, and treatment outcomes of major depression in older adults. *Am J Geriatr Psychiatry.* (2013) 21:88–96. doi: 10.1016/j.jagp.2012.11.012
3. Schiavone S, Colaianna M, Curtis L. Impact of early life stress on the pathogenesis of mental disorders: relation to brain oxidative stress. *Curr Pharm Des.* (2015) 21:1404–12. doi: 10.2174/1381612821666150105143358
4. Rueggeberg R, Wrosch C, Miller GE. The different roles of perceived stress in the association between older adults' physical activity and physical health. *Health Psychol.* (2012) 31:164. doi: 10.1037/a0025242
5. Surtees PG, Wainwright NW, Luben RN, Wareham NJ, Bingham SA, Khaw T, et al. Depression and ischemic heart disease mortality: evidence from the EPIC-Norfolk United Kingdom prospective cohort study. *Am J Psychiatry.* (2008) 165:515–23. doi: 10.1176/appi.ajp.2007.07061018
6. Pope BS, Wood SK. Advances in understanding mechanisms and therapeutic targets to treat comorbid depression and cardiovascular disease. *Neurosci Biobehav Rev*. (2020) 116:337–49. doi: 10.1016/j.neubiorev.2020.06.031
7. WHO. *Depression: Let's Talk*. Geneva: World Health Organization (2017).
8. Radavelli-Bagatini S, Blekkenhorst LC, Sim M, Prince RL, Bondonno NP, Bondonno CP, et al. Fruit and vegetable intake is inversely associated with perceived stress across the adult lifespan. *Clin Nutr.* (2021) 40:2860–7. doi: 10.1016/j.clnu.2021.03.043
9. Baharzadeh E, Siassi F, Qorbani M, Koohdani F, Pak N, Sotoudeh G. Fruits and vegetables intake and its subgroups are related to depression: a cross-sectional study from a developing country. *Ann Gen Psychiatry.* (2018) 17:1–10. doi: 10.1186/s12991-018-0216-0
10. Jacka FN, O'Neil A, Opie R, Itsiopoulos C, Cotton S, Mohebbi M, et al. A randomised controlled trial of dietary improvement for adults with major depression (the 'SMILES'trial). *BMC Med.* (2017) 15:23. doi: 10.1186/s12916-017-0791-y
11. Marx W, Moseley G, Berk M, Jacka F. Nutritional psychiatry: the present state of the evidence. *Proc Nutr Soc.* (2017) 76:427–36. doi: 10.1017/S0029665117002026
12. Kingsbury M, Dupuis G, Jacka F, Roy-Gagnon, M.-H., McMartin SE, et al. Associations between fruit and vegetable consumption and depressive symptoms: evidence from a national Canadian longitudinal survey. *J Epidemiol Community Health.* (2016) 70:155–61. doi: 10.1136/jech-2015-205858
13. Głabska D, Guzek D, Groele B, Gutkowska K. Fruit and vegetable intake and mental health in adults: a systematic review. *Nutrients.* (2020) 12:115. doi: 10.3390/nu12010115
14. Radavelli-Bagatini S, Anokye R, Bondonno NP, Sim M, Bondonno CP, Stanley MJ, et al. Association of habitual intake of fruits and vegetables with depressive symptoms: the AusDiab study. *Eur J Nutr.* (2021) 60:3743–55. doi: 10.1007/s00394-021-02532-0
15. Saghafian F, Malmir H, Saneei P, Milajerdi A, Larijani B, Esmaillzadeh A. Fruit and vegetable consumption and risk of depression: accumulative evidence from an updated systematic review and meta-analysis of epidemiological studies. *Br J Nutr.* (2018) 119:1087–101. doi: 10.1017/S0007114518000697
16. Aune D, Giovannucci E, Boffetta P, Fadnes LT, Keum N, Norat T, et al. Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality—a systematic review and dose-response meta-analysis of prospective studies. *Int J Epidemiol.* (2017) 46:1029–56. doi: 10.1093/ije/dyw319
17. Oliver G, Wardle J, Gibson EL. Stress and food choice: a laboratory study. *Psychosom Med.* (2000) 62:853–65. doi: 10.1097/00006842-200011000-00016
18. Habhab S, Sheldon JP, Loeb RC. The relationship between stress, dietary restraint, and food preferences in women. *Appetite.* (2009) 52:437–44. doi: 10.1016/j.appet.2008.12.006
19. Torres SJ, Nowson CA. Relationship between stress, eating behavior, and obesity. *Nutrition.* (2007) 23:887–94. doi: 10.1016/j.nut.2007.08.008
20. Mikolajczyk RT, El Ansari W, Maxwell AE. Food consumption frequency and perceived stress and depressive symptoms among students in three European countries. *Nutr J.* (2009) 8:1–8. doi: 10.1186/1475-2891-8-31
21. AlAmmar WA, Albeesh FH, Khattab RY. Food and mood: the corresponsive effect. *Curr Nutr Rep.* (2020) 9:296–308. doi: 10.1007/s13668-020-00331-3
22. Dfarhud D, Malmir M, Khanahmadi M. Happiness and health: the biological factors-systematic review article. *Iran J Public Health.* (2014) 43:1468–77.
23. Moylan S, Berk M, Dean OM, Samuni Y, Williams LJ, O'Neil A, et al. Oxidative and nitrosative stress in depression: why so much stress? *Neurosci Biobehav Rev.* (2014) 45:46–62. doi: 10.1016/j.neubiorev.2014.05.007
24. Berk M, Williams LJ, Jacka FN, O'Neil A, Pasco JA, Moylan S, et al. So depression is an inflammatory disease, but where does the inflammation come from? *BMC Med.* (2013) 11:1–16. doi: 10.1186/1741-7015-11-200
25. Fliege H, Rose M, Arck P, Walter OB, Kocalevent, R.-D., et al. The Perceived Stress Questionnaire (PSQ) reconsidered: validation and reference values from different clinical and healthy adult samples. *Psychosom Med.* (2005) 67:78–88. doi: 10.1097/01.psy.0000151491.80178.78
26. Cambridge. *Cambridge Dictionary.* Cambridge University Press (2021).
27. Makovac E, Smallwood J, Watson DR, Meeten F, Critchley HD, Ottaviani C. The verbal nature of worry in generalized anxiety: insights from the brain. *Neuroimage Clin.* (2018) 17:882–92. doi: 10.1016/j.nicl.2017.12.014
28. Dunstan DW, Zimmet PZ, Welborn TA, Cameron AJ, Shaw J, de Courten M, et al. The Australian Diabetes, Obesity and Lifestyle Study (AusDiab)—methods and response rates. *Diabetes Res Clin Pract.* (2002) 57:119–29. doi: 10.1016/S0168-8227(02)00025-6
29. AusDiab. *Diabesity and Associated Disorders in Australia, 2000: The Accelerating Epidemic.* Melbourne, VIC: The International Diabetes Institute (AusDiab Steering Committee) (2001).
30. Cameron AJ, Zimmet PZ, Dunstan DW, Dalton M, Shaw JE, Welborn TA, et al. Overweight and obesity in Australia: the 1999–2000 Australian diabetes, obesity and lifestyle study (AusDiab). *Med J Australia.* (2003) 178:427–32. doi: 10.5694/j.1326-5377.2003.tb05283.x
31. Subar AF, Thompson FE, Kipnis V, Midthune D, Hurwitz P, McNutt S, et al. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires: the Eating at America's Table Study. *Am J Epidemiol.* (2001) 154:1089–99. doi: 10.1093/aje/154.12.1089
32. Levenstein S, Prantera C, Varvo V, Scribano ML, Berto E, Luzi C, et al. Development of the Perceived Stress Questionnaire: a new tool for psychosomatic research. *J Psychosom Res.* (1993) 37:19–32. doi: 10.1016/0022-3999(93)90120-5
33. Leppert B, Junge KM, Röder S, Borte M, Stangl GI, Wright RJ, et al. Early maternal perceived stress and children's BMI: longitudinal impact and influencing factors. *BMC Public Health.* (2018) 18:1–10. doi: 10.1186/s12889-018-6110-5
34. Bergdahl J, Bergdahl M. Perceived stress in adults: prevalence and association of depression, anxiety and medication in a Swedish population. *Stress Health.* (2002) 18:235–41. doi: 10.1002/smi.946
35. Liu RT, Alloy LB. Stress generation in depression: a systematic review of the empirical literature and recommendations for future study. *Clin Psychol Rev.* (2010) 30:582–93. doi: 10.1016/j.cpr.2010.04.010
36. Ireland P, Jolley D, Giles G, O'Dea K, Powles J, Rutishauser I, et al. Development of the Melbourne FFQ: a food frequency questionnaire for use

in an Australian prospective study involving an ethnically diverse cohort. *Asia Pac J Clin Nutr.* (1994) 3:19–31.
37. NHMRC. *Australian Dietary Guidelines.* Canberra, ACT: Australian Government, N.H.a.M.R. Council (2013).
38. Braithwaite S, Holt-Lunstad J. Romantic relationships and mental health. *Curr Opin Psychol.* (2017) 13:120–5. doi: 10.1016/j.copsyc.2016.04.001
39. Niemeyer H, Bieda A, Michalak J, Schneider S, Margraf J. Education and mental health: do psychosocial resources matter? *SSM Popul Health.* (2019) 7:100392. doi: 10.1016/j.ssmph.2019.100392
40. Fard NA, Morales GDF, Mejova Y, Schifanella R. On the interplay between educational attainment and nutrition: a spatially-aware perspective. *EPJ Data Science.* (2021) 10:18. doi: 10.1140/epjds/s13688-021-00273-y
41. Conklin AI, Forouhi NG, Surtees P, Khaw, K.-T., Wareham NJ, et al. Social relationships and healthful dietary behaviour: evidence from over-50s in the EPIC cohort, UK. *Soc Sci Med.* (2014) 100:167–75. doi: 10.1016/j.socscimed.2013.08.018
42. Pink B. *Socio-Economic Indexes for Areas (SEIFA)*. Canberra, ACT: Australian Bureau of Statistics (2011).
43. ABO. *2033.0. 55.001—Census of Population and Housing: Socio-Economic Indexes for Areas (seifa)*. Canberra, ACT: Australian Bureau of Statistics (2016).
44. Dalton M, Cameron AJ, Zimmet PZ, Shaw JE, Jolley D, Dunstan DW, et al. Waist circumference, waist–hip ratio and body mass index and their correlation with cardiovascular disease risk factors in Australian adults. *J Intern Med.* (2003) 254:555–63. doi: 10.1111/j.1365-2796.2003.01229.x
45. Barr ELM, Magliano DJ, Zimmet PZ, Polkinghorne KR, Atkins RC, Dunstan DW, et al. *AusDiab 2005: The Australian Diabetes, Obesity and Lifestyle Study - Tracking the Accelerating Epidemic: Its Causes and Outcomes*. International Diabetes Institute, Melbourne, VIC (2006). Available online at: https://www.baker.edu.au/Assets/Files/AUSDIAB_REPORT_2005.pdf
46. Armstrong T, Bauman AE, Davies J. *Physical Activity Patterns of Australian Adults: Results of the 1999 National Physical Activity Survey.* Australian Institute of Health and Welfare (2000).
47. Diabetes A, Dunstan DW. *Diabesity and Associated Disorders in Australia-2000: The Accelerating Epidemic.* International Diabetes Institute (2001).
48. Dunstan DW, Zimmet PZ, Welborn TA, Cameron AJ, Shaw J, De Courten M, et al. The Australian diabetes, obesity and lifestyle study (AusDiab)—methods and response rates. *Diabetes Res Clin Pract.* (2002) 57:119–29. doi,: 10.1016/S.0168-8227(02)00025-6
49. McNaughton SA, Ball K, Crawford D, Mishra GD. An index of diet and eating patterns is a valid measure of diet quality in an Australian population. *J Nutr.* (2008) 138:86–93. doi: 10.1093/jn/138.1.86
50. AGHE. *Australian Guide to Healthy Eating | Eat for Health* (2015).
51. NHMRC. *Australian Dietary Guidelines* (2020).
52. Caldwell JR, Theisen V, Kaunisto CA, Reddy PJ, Smythe PS, Smith DW. Psychosocial factors influence control of moderate and severe hypertension. *Soc Sci Med.* (1983) 17:773–82. doi: 10.1016/0277-9536(83)90028-X
53. Framework IC. The MOS 36-item short-form health survey (SF-36). *Med Care.* (1992) 30:473–83. doi: 10.1097/00005650-199206000-00002
54. Gandek B, Sinclair SJ, Kosinski M, Ware JE Jr. Psychometric evaluation of the SF-36® health survey in medicare managed care. *Health Care Financ Rev.* (2004) 25:5–25.
55. StataCorp L. *Stata Statistical Software (Version Release 14)*. College Station, TX: StataCorp L (2015).
56. Team RC. *R: A Language and Environment for Statistical Computing* (2013).
57. ABO. *Dietary Behaviour*. Australian Bureau of Statistics (2017).
58. Kim S, Haines PS, Siega-Riz AM, Popkin BM. The Diet Quality Index-International (DQI-I) provides an effective tool for cross-national comparison of diet quality as illustrated by China and the United States. *J Nutr.* (2003) 133:3476–84. doi: 10.1093/jn/133.11.3476
59. McMartin SE, Willows ND, Colman I, Ohinmaa A, Storey K, Veugelers PJ. Diet quality and feelings of worry, sadness or unhappiness in Canadian children. *Can J Public Health.* (2013) 104:e322–6. doi: 10.17269/cjph.104.3845
60. Beezhold BL, Johnston CS, Daigle DR. Vegetarian diets are associated with healthy mood states: a cross-sectional study in seventh day adventist adults. *Nutrition J.* (2010) 9:1–7. doi: 10.1186/1475-2891-9-26
61. Torres SJ, Nowson CA. A moderate-sodium DASH-type diet improves mood in postmenopausal women. *Nutrition.* (2012) 28:896–900. doi: 10.1016/j.nut.2011.11.029
62. Mujcic R, Oswald AJ. Evolution of well-being and happiness after increases in consumption of fruit and vegetables. *Am J Public Health.* (2016) 106:1504–10. doi: 10.2105/AJPH.2016.303260
63. Conner TS, Brookie KL, Richardson AC, Polak MA. On carrots and curiosity: eating fruit and vegetables is associated with greater flourishing in daily life. *Br J Health Psychol.* (2015) 20:413–27. doi: 10.1111/bjhp.12113
64. Briguglio M, Dell'Osso B, Panzica G, Malgaroli A, Banfi G, Zanaboni Dina C, et al. Dietary neurotransmitters: a narrative review on current knowledge. *Nutrients.* (2018) 10:591. doi: 10.3390/nu10050591
65. González R, Ballester I, López-Posadas R, Suárez M, Zarzuelo A, Martinez-Augustin O, et al. Effects of flavonoids and other polyphenols on inflammation. *Crit Rev Food Sci Nutr.* (2011) 51:331–62. doi: 10.1080/10408390903584094
66. Jacka FN, Cherbuin N, Anstey KJ, Sachdev P, Butterworth P. Western diet is associated with a smaller hippocampus: a longitudinal investigation. *BMC Med.* (2015) 13:1–8. doi: 10.1186/s12916-015-0461-x
67. Morris G, Walder K, McGee SL, Dean OM, Tye SJ, Maes M, et al. A model of the mitochondrial basis of bipolar disorder. *Neurosci Biobehav Rev.* (2017) 74:1–20. doi: 10.1016/j.neubiorev.2017.01.014
68. Jacka FN. Nutritional psychiatry: where to next? *EBioMedicine.* (2017) 17:24–9. doi: 10.1016/j.ebiom.2017.02.020
69. Epel ES, Crosswell AD, Mayer SE, Prather AA, Slavich GM, Puterman E, et al. More than a feeling: a unified view of stress measurement for population science. *Front Neuroendocrinol.* (2018) 49:146–69. doi: 10.1016/j.yfrne.2018.03.001
70. WHO. *European Food and Nutrition Action Plan 2015–2020.* World Health Organization (2015).
71. Martinsen EW. Physical activity in the prevention and treatment of anxiety and depression. *Nord J Psychiatry.* (2008) 62:25–9. doi: 10.1080/08039480802315640
72. Chan JS, Liu G, Liang D, Deng K, Wu J, Yan JH. Special issue–therapeutic benefits of physical activity for mood: a systematic review on the effects of exercise intensity, duration, and modality. *J Psychol.* (2019) 153:102–25. doi: 10.1080/00223980.2018.1470487
73. Kwan BM, Bryan AD. Affective response to exercise as a component of exercise motivation: attitudes, norms, self-efficacy, and temporal stability of intentions. *Psychol Sport Exerc.* (2010) 11:71–9. doi: 10.1016/j.psychsport.2009.05.010
74. Jackson PA, Pialoux V, Corbett D, Drogos L, Erickson KI, Eskes GA, et al. Promoting brain health through exercise and diet in older adults: a physiological perspective. *J Physiol.* (2016) 594:4485–98. doi: 10.1113/JP271270
75. Guure CB, Ibrahim NA, Adam MB, Said SM. Impact of physical activity on cognitive decline, dementia, and its subtypes: meta-analysis of prospective studies. *Biomed Res Int.* (2017) 2017:9016924. doi: 10.1155/2017/9016924
76. Clarke SF, Murphy EF, O'Sullivan O, Lucey AJ, Humphreys M, Hogan A, et al. Exercise and associated dietary extremes impact on gut microbial diversity. *Gut.* (2014) 63:1913–20. doi: 10.1136/gutjnl-2013-306541
77. Claesson MJ, Jeffery IB, Conde S, Power SE, O'connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. *Nature.* (2012) 488:178–84. doi: 10.1038/nature11319
78. Cryan JF, O'Riordan KJ, Cowan CS, Sandhu KV, Bastiaanssen TF, Boehme M, et al. The microbiota-gut-brain axis. *Physiol Rev.* (2019) 99:1877–2013. doi: 10.1152/physrev.00018.2018
79. Berding K, Vlckova K, Marx W, Schellekens H, Stanton C, Clarke G, et al. Diet and the microbiota–gut–brain axis: sowing the seeds of good mental health. *Adv Nutr.* (2021) 12:1239–85. doi: 10.1093/advances/nmaa181
80. Konttinen H, Männistö S, Sarlio-Lähteenkorva S, Silventoinen K, Haukkala A. Emotional eating, depressive symptoms and self-reported food consumption. A population-based study. *Appetite.* (2010) 54:473–9. doi: 10.1016/j.appet.2010.01.014
81. Bremner JD, Moazzami K, Wittbrodt MT, Nye JA, Lima BB, Gillespie CF, et al. Diet, stress and mental health. *Nutrients.* (2020) 12:2428. doi: 10.3390/nu12082428

Conflict of Interest: CB and JH report grants from FruitWest, and from Department of Agriculture and Food WA, outside the submitted work. DM

and JS report grants from Abbott Australasia Pty Ltd., Alphapharm Pty Ltd., AstraZeneca, Bristol-Myers Squibb, Eli Lilly Australia, GlaxoSmithKline, Janssen-Cilag, Merck Sharp and Dohme, Novartis Pharmaceuticals, Novo Nordisk Pharmaceuticals, Roche Diagnostics Australia, Sanofi Aventis and Sanofi-synthelabo, during the conduct of the study. RD received a Primary Growth Partnership grant via the Ministry of Primary Industries in New Zealand with Fonterra Co-operative Group Ltd., outside the submitted work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LY declared a shared affiliation with the author RD to the handling editor at the time of review.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Radavelli-Bagatini, Sim, Blekkenhorst, Bondonno, Bondonno, Woodman, Dickson, Harms, Magliano, Shaw, Daly, Hodgson and Lewis. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 02 June 2022
doi: 10.3389/fnut.2022.873623

Segmental Bioimpedance Variables in Association With Mild Cognitive Impairment

*Dieu Ni Thi Doan[1,2], Boncho Ku[1], Kahye Kim[1], Minho Jun[1], Kyu Yeong Choi[3], Kun Ho Lee[3,4,5] and Jaeuk U. Kim[1,2]**

[1] Department of Digital Health Research, Korea Institute of Oriental Medicine, Daejeon, South Korea, [2] Korean Convergence Medicine, University of Science and Technology, Daejeon, South Korea, [3] Gwangju Alzheimer's Disease and Related Dementias (GARD) Cohort Research Center, Chosun University, Gwangju, South Korea, [4] Department of Biomedical Science, Chosun University, Gwangju, South Korea, [5] Dementia Research Group, Korea Brain Research Institute, Daegu, South Korea

Objective: To examine the changes in body composition, water compartment, and bioimpedance in mild cognitive impairment (MCI) individuals.

Methods: We obtained seven whole-body composition variables and seven pairs of segmental body composition, water compartment, and impedance variables for the upper and lower extremities from the segmental multi-frequency bioelectrical impedance analysis (BIA) of 939 elderly participants, including 673 cognitively normal (CN) people and 266 individuals with MCI. Participants' characteristics, anthropometric information, and the selected BIA variables were described and statistically compared between the CN participants and those with MCI. The correlations between the selected BIA variables and neuropsychological tests such as the Korean version of the Mini-Mental State Examination and Seoul Neuropsychological Screening Battery – Second Edition were also examined before and after controlling for age and sex. Univariate and multivariate logistic regression analyses with estimated odds ratios (ORs) were conducted to investigate the associations between these BIA variables and MCI prevalence for different sexes.

Results: Participants with MCI were slightly older, more depressive, and had significantly poorer cognitive abilities when compared with the CN individuals. The partial correlations between the selected BIA variables and neuropsychological tests upon controlling for age and sex were not greatly significant. However, after accounting for age, sex, and the significant comorbidities, segmental lean mass, water volume, resistance, and reactance in the lower extremities were positively associated with MCI, with ORs [95% confidence interval (CI)] of 1.33 (1.02–1.71), 1.33 (1.03–1.72), 0.76 (0.62–0.92), and 0.79 (0.67–0.93), respectively; with presumably a shift of water from the intracellular area to extracellular space. After stratifying by sex, resistance and reactance in lower extremities remained significant only in the women group.

Conclusion: An increase in segmental water along with segmental lean mass and a decrease in body cell strength due to an abnormal cellular water distribution

OPEN ACCESS

Edited by:
Andrew Scholey, Swinburne University of Technology, Australia

Reviewed by:
Takeshi Hashimoto, Ritsumeikan University, Japan
Lenka Lhotská, Czech Technical University in Prague, Czechia
Paula Chiarello, University of São Paulo, Ribeirão Preto, Brazil

***Correspondence:**
Jaeuk U. Kim
jaeukkim@kiom.re.kr

Specialty section:
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

Received: *11 February 2022*
Accepted: *04 May 2022*
Published: *02 June 2022*

Citation:
Doan DNT, Ku B, Kim K, Jun M, Choi KY, Lee KH and Kim JU (2022) Segmental Bioimpedance Variables in Association With Mild Cognitive Impairment. Front. Nutr. 9:873623. doi: 10.3389/fnut.2022.873623

demonstrated by reductions in resistance and reactance are associated with MCI prevalence, which are more pronounced in the lower extremities and in women. These characteristic changes in BIA variables may be considered as an early sign of cognitive impairment in the elderly population.

Keywords: bioelectrical impedance analysis (BIA), segmental analysis, mild cognitive impairment (MCI), Alzheimer's disease (AD), body composition

INTRODUCTION

Changes in body composition commonly occur with aging in healthy individuals and in those with protein–energy malnutrition, poor physical conditions, or cognitive impairments such as Alzheimer's disease (AD). Body composition changes over the aging process are characterized by increasing body fat mass, particularly by the reformation of the body's fat distribution from appendicular fat to trunk fat, with decreasing lean mass and bone mineral density, especially in older adults (1–3). In middle-aged individuals, these changes increase both fat and lean mass, resulting in an increase in body weight (4, 5). Besides these changes, physiological changes in water metabolism and sodium balance occur, leading to alterations in plasma osmolality and changes in the volume within the body's fluid compartments (6–10).

Mild cognitive impairment (MCI) is an age-related disorder which describes the symptomatic transitional phase between normal cognition and dementia. Clinically, MCI is defined as a set of criteria, including evidence of a change in cognition and lower performance in one or more cognitive domains despite the preservation of independent daily functioning [diagnostic guidelines from National Institute on Aging and the Alzheimer's Association (11)]. Aside from age, several risk factors contribute to the occurrence and progression of MCI, such as negative lifestyle behaviors and poor dietary habits (12–14). These factors could also lead to chronic non-communicable diseases such as obesity, hypertension, cardiovascular disease, diabetes, and depression, which disrupt physiological homeostasis and hasten the progression of MCI (15, 16).

Recognizing and monitoring changes in body composition can reveal early signs of the body's vital status, thereby allowing the application of appropriate therapies or lifestyle changes to help reverse the risk of MCI and progression to dementia. Anthropometrical measurements such as weight, height, body mass index (BMI), body circumference, and waist to hip ratio (WHR) have been commonly used in clinical practice to indirectly estimate fat or muscle mass (17–19). However, these measurements can only provide a rough estimate of body mass distribution and cannot discreetly quantify fat or lean mass percentages or intracellular/extracellular water levels (18). Bioelectrical impedance analysis (BIA) is a simple, portable, and relatively accurate technique (20, 21) that can be used to repeatedly measure the body composition of many participants to recognize early changes in body composition status and prognosticate the likelihood of having MCI.

Most previous studies employed specific bioelectrical impedance vector analysis in the later stage of AD rather than during early cognitive impairment. They reported a lower body cell mass, indicated by a lower reactance and phase angle or a shifting to the lower right half of the ellipse, in the AD or dementia group than in individuals with normal cognition (22, 23). These patterns were more prevalent in women with worse psycho-functional status (24, 25). Higher impedance, higher resistance, and longer impedance vector were found in AD patients than in normal controls, suggesting a lower proportion of fat free mass (FFM) (23, 24, 26). Nevertheless, few studies have applied conventional BIA techniques to the assessment of body composition changes in patients with MCI. Some examples include findings of a lower phase angle in women (27) or a higher visceral fat area associated with a lower risk of non-amnestic MCI in older women {mean [standard deviation (SD)] age of 75.0 $\pm$ 5.18} (28), though the number of these studies is limited.

Sex is another aspect to be considered for the evaluation and comparison of body composition in individuals with cognitive decline. Sex has a strong relationship with body composition, where women present with a relatively higher fat mass and lower FFM than men (29, 30). Buffa et al. reported a lower body cell mass with dehydration in women with severe AD than in those with mild-moderate AD, but these findings were not evident in men. Regarding bioimpedance, Cova et al. described significantly higher height-normalized values of impedance and resistance in men with MCI than in those without MCI, though this was not observed among women with MCI (26).

Regarding segmental body analysis, few studies have reported changes in the composition of different body segments in individuals with cognitive decline. Mereu et al. demonstrated that bioimpedance results obtained for only the right arm would be similar to whole-body composition in patients with AD (31). Reduction of skeletal muscle mass or muscle strength in the lower limbs was associated with a higher MCI risk in women (32, 33), whereas loss of skeletal muscle mass in the upper and lower limbs was associated with a higher risk of MCI in men (32). Lower extremity skeletal muscle mass was associated with lower cognitive performance and higher cortical beta-amyloid burden (34, 35), whereas upper extremity skeletal muscle mass did not show any association (34). Similarly, lower extremity motor function was found to be prominent in patients with MCI and AD (36, 37).

Subsequently, there is a lack of information about the relationship between body composition, and bioimpedance for each body segment and cognitive decline. Differences by sex in these components between the upper and lower extremities are not well established, especially for individuals with MCI. Therefore, using a segmental multi-frequency BIA technique,

we aimed to examine how whole-body composition changes, segmental body fluids, and bioimpedance variables distinctly for the upper and lower extremities differed between cognitively normal (CN) people and those with MCI among both women and men. We hypothesized that lower extremity variables are more strongly linked to the incidence of MCI than upper extremity variables and that this pattern is more noticeable in women than in men. To test our hypotheses, linear logistic regression methods were conducted to examine odds ratios (ORs) before and after controlling for potential covariates, which was then repeated for the different sexes.

MATERIALS AND METHODS

Participants

In total, 1,153 individuals participated in the study. MCI patients were recruited at Chonnam University Hospital (Gwangju City, South Korea) alongside their family members or caregivers, who were recruited as participants with normal cognition. Written informed consent was obtained from each participant or their legal guardian prior to the study. Individuals were excluded from the study if they met one of the following criteria: obtained less than 3 years of education; had a medical history or ongoing acute or chronic illness that interfered with the intended study design, such as neurological diseases, infections, or mental health instability; excessive alcohol consumption; as a CN individual, had a baseline magnetic resonance imaging showing an abnormal pattern or atrophy; and showing clinical signs of hydration imbalance.

Mild cognitive impairment patients were defined as those who were not yet demented, had a Clinical Dementia Rating scale of 0.5, had a Seoul Neuropsychological Screening Battery – Second Edition (SNSB-II) z-score no less than −1.5 in at least one of the domains. In contrast, to be considered as CN, participants must have had no history or current symptoms of cognitive decline, a Clinical Dementia Rating of zero, and a SNSB-II z-score no less than −1.5 for all tests. All participants aged between 55 and 90 years had adequate hearing and vision on neuropsychological examination. The included participants then underwent a more precise clinical assessment to obtain demographic information and a medical history alongside a general medical examination, neuropsychological tests, and neurophysiological tests. In the data preprocessing, participants who had non-random missing data (n = 4), extreme results for any parameters (values above or below three times the interquartile are considered as extreme points) (n = 61), and were diagnosed with dementia or other causes of cognitive decline (n = 61) were excluded from the analysis. According to the principle of multi-frequency BIA, correct impedance measurements should have lower values for higher frequencies (i.e., impedance at 1 kHz is greater than impedance at 5 kHz); therefore, observations that did not follow this pattern were excluded (n = 88). There was a total of 939 participants included in the final analysis, including 673 CN and 266 MCI individuals, as illustrated in **Figure 1**. The study protocol was approved by the Institutional Review Board of the Chonnam National University Hospital (CNUH; approval number: CNUH-2019-279). The study was performed in agreement with the Declaration of Helsinki.

Neuropsychological Assessment

The Korean version of the Mini-Mental State Examination (K-MMSE) and SNSB-II were used as neuropsychological assessments in this study.

Seoul Neuropsychological Screening Battery – Second Edition is a comprehensive neuropsychological test that is widely used in Korea for evaluating cognitive performance in people with brain injuries and neurological disorders such as dementia (38, 39). It is composed of five cognitive domains including attention, memory, language, visuospatial function, and frontal/executive function. The attention domain includes the Digit Span Test, vigilance test, and letter cancellation. The memory domain contains the Seoul Verbal Learning Test and the Rey Complex Figure Test. The domain of language and related functions consists of the Boston Naming Test and comprehension, repetition, reading, and writing tests. The visuospatial domain is tested by the Rey Complex Figure Test-Copy and the Clock Drawing Test. The frontal and executive function domain encompasses motor regulation, the Stroop Test, the Controlled Oral Word Association Test, Digit Symbol Coding, and the Trail Making Test. The SNSB-II test and domain scores were normalized by age, sex, and educational levels according to a population 1,067 elderly Korean participants aged 45–90 years who represented normal development (40). The validity and reliability of the SNSB-II has been demonstrated by many previous studies, especially for MCI over other cognitive tests (38).

The K-MMSE is a commonly used screening test for dementia. It tests the five cognitive domains of time and spatial orientation, memory, attention and calculation, language, and visuospatial configuration with a maximum of 30 points.

Anthropometry and Bioimpedance Measurement

Anthropometric parameters such as height (cm) and weight (kg) were automatically measured from the multi-frequency BIA segmental analyzer Inbody S10 Korea (41) to the nearest 1 mm and 100 g, respectively. BMI was then also auto-calculated (kg/m^2). Body composition parameters including WHR, FFM, percent body fat (PBF), percent body cell mass (PBCM), total body water per fat free mass (TBW_FFM), and basal metabolic rate (BMR) were estimated from impedance variables obtained from the same device. This device uses a tetrapolar 8-point tactile electrode to measure impedance at six electrical frequencies: 1, 5, 50, 250, 500, and 1,000 kHz and reactance and phase angle at three frequencies: 5, 50, and 250 kHz. Notably, the tetrapolar BIA method was validated as it gives similar results to reference methods such as dual energy X-ray absorptiometry or magnetic resonance imaging techniques (42, 43). The impedance was measured separately for each body segment, including the two arms, two legs, and trunk, producing an estimated segmental body composition and water compartment. Participants were instructed to fast overnight

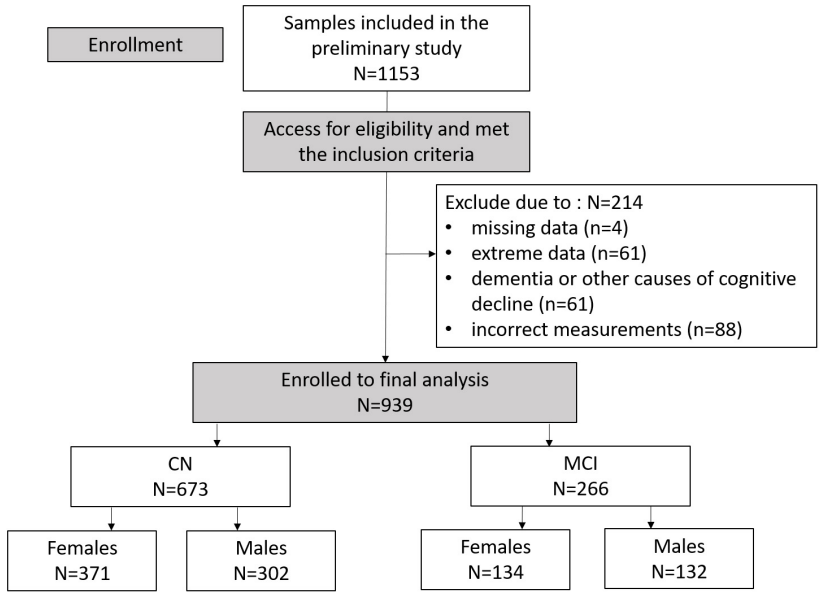


FIGURE 1 | Consolidated Standards of Reporting Trials (CONSORT) diagram illustrating enrollment and exclusion criteria for this study.

before the examination. Although there are various impedance parameters at several frequencies, this study focused on the impedance variables obtained at 50 kHz. All measurements were carried out by well-trained staff with the Inbody S10 (41) instructions for reference.

Statistical Analysis

The characteristics of the two groups of participants (CN and MCI) were compared. Demographic information, neuropsychological test results, and body composition characteristics were summarized as means and SDs for continuous variables and as frequencies and proportions for categorical variables. Regarding hypothesizing differences by continuous variables, a univariate independent two-sample *t*-test or Mann–Whitney–Wilcoxon rank sum test was used after confirming distribution normality by the Shapiro–Wilk test. Cohen's *d* was applied to test the effect size of the difference. To check the independence of each categorical variable with cognitive status, Pearson's Chi-squared test or Fisher's exact test was used. Pearson correlation coefficients were employed to examine the bivariate correlations between age, BMI, and neuropsychological test (SNSB-II and K-MMSE) scores with body composition, segmental water, and impedance-related variables. Pearson correlation coefficients were also used to inspect the partial correlations between those variables with conditioning on age and sex, the two factors that are known to be associated with cognition and body composition (44, 45). Univariate and multiple regression analyses were then carried out with cognitive group as the dependent variable and the selected BIA variables as the independent variables, before and after adjusting for the potential confounders. Lastly, we examined the association between MCI and each selected BIA variable after stratifying groups according to sex. A *p*-value of less than 0.05 was considered statistically significant. R version 4.1.2 (The R Project for Statistical Computing[1]) was used for all statistical analyses.

Covariates

Considering the role of age in the relationship between MCI and changes in body composition and water compartment, we used age as one of the potential confounders in the multivariate regression analysis models in the **Tables 3** and **4**. As body composition differs between women and men, sex was included as another covariate. Moreover, any significant difference in participants' basic characteristics that could affect the associations between MCI and body composition variables were incorporated as covariates, including Geriatric Depression Scale (GDS) score, hyperlipidemia, diabetes, and central nervous system disorder.

Data Selection

Considering that all participants appeared to have a symmetrical body structure upon the in-person screening phase, their left-side was strongly positively correlated with the corresponding variable on the right side of the body for most of the segmental BIA variables (**Supplementary Figure 1**). The average values between the right and left arm and leg were computed for the upper and lower extremity variables (**Supplementary Table 1**). The difference between the right and its corresponding left variables produced statistically insignificant results in CN and MCI groups (**Supplementary Table 2**). In this study, seven whole-body composition variables were employed in the analysis, including BMI, FFM, PBF, PBCM, WHR,

[1]http://www.r-project.org/

TBW_FFM, and BMR. Additionally, seven pairs of segmental body composition, water compartment and bioimpedance variables for the upper and lower extremities were included, consist of segmental water (SW_upper, SW_lower), segmental lean mass (SL_upper, SL_lower), relative water volume to lean mass (Water_Lean_upper, Water_Lean_lower), extracellular to intracellular water ratio (ECW_ICW_upper, ECW_ICW_lower), resistance (R_upper, R_lower), reactance (Xc_upper, Xc_lower), and phase angle (PA_upper, PA_lower). A frequency of 50 kHz was used for all impedance results.

TABLE 1 | Demographic information and neuropsychological test results of MCI and CN subjects.

Variables	Total (*n* = 939)[1]	NC (*n* = 673)[1]	MCI (*n* = 266)[1]	*p*-value[2]
Age (year)				**0.002**
Mean (SD)	72.1 (6.3)	71.7 (6.2)	73.1 (6.5)	
Sex, *n* (%)				0.188
Female	505 (53.8%)	371 (55.1%)	134 (50.4%)	
Male	434 (46.2%)	302 (44.9%)	132 (49.6%)	
Education level (year)				0.295
Mean (SD)	12.1 (4.6)	12.0 (4.6)	12.3 (4.4)	
Systolic BP (mmHg)				0.634
Mean (SD)	124 (15)	125 (14)	124 (16)	
Diastolic BP (mmHg)				0.736
Mean (SD)	71 (10)	71 (10)	71 (10)	
GDS score				**0.042**
Mean (SD)	8.3 (6.9)	8.0 (6.8)	9.0 (7.0)	
K-MMSE score				**<0.001**
Mean (SD)	27.2 (2.2)	27.6 (1.9)	26.2 (2.5)	
Attention				**<0.001**
Mean (SD)	9.3 (2.2)	9.7 (2.2)	8.5 (1.8)	
Language				**<0.001**
Mean (SD)	0.2 (0.3)	0.2 (0.3)	0.0 (0.4)	
Visuospatial				**<0.001**
Mean (SD)	0.5 (0.4)	0.5 (0.4)	0.3 (0.6)	
Memory				**<0.001**
Mean (SD)	0.1 (0.7)	0.3 (0.6)	−0.4 (0.6)	
Frontal				**<0.001**
Mean (SD)	0.1 (0.6)	0.2 (0.6)	−0.2 (0.7)	
Diabetes, *n* (%)				**0.011**
Yes	168 (17.9%)	107 (15.9%)	61 (22.9%)	
No	771 (82.1%)	566 (84.1%)	205 (77.1%)	
Hypertension, *n* (%)				0.574
Yes	403 (42.9%)	285 (42.3%)	118 (44.4%)	
No	536 (57.1%)	388 (57.7%)	148 (55.6%)	
Hyperlipidemia, *n* (%)				**0.019**
Yes	183 (19.5%)	144 (21.4%)	39 (14.7%)	
No	756 (80.5%)	529 (78.6%)	227 (85.3%)	
Thyroid, *n* (%)				0.692
Yes	32 (3.4%)	22 (3.3%)	10 (3.8%)	
No	904 (96.6%)	650 (96.7%)	254 (96.2%)	
Mental disorder, *n* (%)				0.627
Yes	21 (2.2%)	14 (2.1%)	7 (2.6%)	
No	917 (97.8%)	658 (97.9%)	259 (97.4%)	
Circulatory disorder, *n* (%)				0.525
Yes	151 (16.1%)	105 (15.6%)	46 (17.3%)	
No	788 (83.9%)	568 (84.4%)	220 (82.7%)	
CNS disorder, *n* (%)				**0.024**
Yes	5 (0.5%)	1 (0.1%)	4 (1.5%)	
No	932 (99.5%)	671 (99.9%)	261 (98.5%)	

(Continued)

TABLE 1 | (Continued)

Variables	Total (n = 939)[1]	NC (n = 673)[1]	MCI (n = 266)[1]	p-value[2]
Cohabitation, *n* (%)				0.624
Single	126 (13.4%)	88 (13.1%)	38 (14.3%)	
With family	813 (86.6%)	585 (86.9%)	228 (85.7%)	
Dominant hand, *n* (%)				**0.049**
Righty	911 (97.0%)	655 (97.3%)	256 (96.2%)	
Lefty	7 (0.7%)	2 (0.3%)	5 (1.9%)	
Both	21 (2.2%)	16 (2.4%)	5 (1.9%)	

[1] *The values represent mean (SD) for continuous variables, and n (%) for categorical variables. The p-values for the continuous variables were obtained from an independent two sample t-test for normally distributed variables or Mann–Whitney–Wilcoxon rank sum test for non-normally distributed variables. For the categorical variables, the p-values were derived from the Chi-squared test statistics or Fisher-exact test. CNS, central nervous system.*

[2] *Wilcoxon rank sum test; Pearson's Chi-squared test; two sample t-test; Fisher's exact test. The bold fonts indicate a p-value lower than 0.05.*

RESULTS

Participants' Characteristics

The demographic information, neuropsychological test scores, and comorbidities for the CN and MCI groups are described in **Table 1**. Between the CN and MCI groups, there were indifferences in sex, educational years, and blood pressure. Individuals with MCI had a slightly higher mean age than CN participants with the mean (SD) of 73.1 (6.5) and 71.7 (6.2), respectively. The MCI group exhibited poorer cognitive performance with significantly lower SNSB-II and K-MMSE scores and higher levels of depression with an increased GDS score than the CN group. The prevalence of comorbidities including hypertension, thyroid disorders, mental disorders, and circulatory disorders between the groups were equivalent. However, the prevalence of diabetes and CNS disorder were higher and the prevalence of hyperlipidemia was lower in the MCI group when compared with the CN group. For cohabitation, more than 85% of our participants lived with family, and there were no significant differences between the groups.

Anthropometric and Whole-Body Composition Results

Anthropometric information and the whole-body composition results were described in **Table 2**. In terms of anthropometric indices, the CN and MCI groups had comparable height, weight, and therefore BMI. However, MCI participants had a slightly lower body fat indices as indicated by lower PBF with a mean (SD) of 32.1 (7.6) and 33.3 (7.9) and smaller WHR with a mean (SD) of 0.879 (0.063) and 0.887 (0.055) compared with CN individuals, respectively. Similarly, MCI participants had a slightly higher PBCM with respect to CN individuals with a mean (SD) of 43.5 (5.0) and 42.9 (5.2) and greater TWB_FFM with those of 73.92 (0.29) and 73.88 (0.27), respectively.

Descriptions of the Segmental Body Composition and Bioimpedance Variables

Figure 2 visualizes the data of the selected segmental variables and comparative statistics between the two groups. Among the segmental body composition and water compartment variables, the MCI group exhibited a tendency of increasing relative water volume and extra- to intracellular water ratios compared to CN group indicated by significant effect size of approximately 0.20 for Water_Lean_lower, ECW_ICW_upper, and ECW_ICW_lower. For the segmental bioimpedance variables, resistance, reactance, and phase angle displayed a similar pattern of decreasing in the MCI group when compared with the CN group, although resistance and phase angle showed significant differences between the two groups only for the lower extremity (R_lower and PA_lower). The effect sizes in these impedance comparisons ranged from −0.18 to −0.31.

Correlations Between Age, Neuropsychological Test Scores, Body Composition, Water Compartment, and Bioimpedance Variables

Figure 3 illustrates the correlations between age and the cognitive tests with the selected BIA variables, as well as partial correlations after controlling for age and sex.

Bivariate Correlations

Age

Age displayed positive correlations with the water-related variables but negative correlations with the impedance-related variables. Age demonstrated relatively stronger correlations with the lower extremity variables such as Water_Lean_lower, ECW_ICW_lower, Xc_lower, and PA_lower than the corresponding upper extremity variables. With aging, relative water volume (Water_Lean_lower, r = 0.46) and cellular water ratio (ECW_ICW_lower, r = 0.47) variables in the lower extremity tended to increase. The reactance and phase angle variables in the lower extremity tended to decrease with aging (Xc_lower, r = −0.49; PA_lower, r = −0.41). Whole-body water hydration status indicated by the ratio of TBW_FFM was positively correlated with age (r = 0.39), whereas other parameters such as PBF, PBCM, and BMR did not show significant correlations.

Neuropsychological Tests

All BIA variables had weak or no correlations with the five domains of the SNSB-II and the K-MMSE. The highest coefficients were found between the SNSB-II frontal domain and

TABLE 2 | Anthropometry and whole-body composition results.

Variables	Total (n = 939)[1]	CN (n = 673)[1]	MCI (n = 266)[1]	p-value[2]
Height (cm)				0.162
Mean (SD)	159.4 (8.4)	159.2 (8.3)	159.9 (8.5)	
Weight (kg)				0.977
Mean (SD)	62.7 (9.5)	62.7 (9.3)	62.7 (10.1)	
BMI (kg/m^2)				0.111
Mean (SD)	24.6 (2.9)	24.7 (2.8)	24.4 (3.0)	
FFM (kg)				0.228
Mean (SD)	42.0 (7.9)	41.8 (7.9)	42.4 (8.0)	
PBF (%)				**0.018**
Mean (SD)	33.0 (7.8)	33.3 (7.9)	32.1 (7.6)	
PBCM (%)				**0.044**
Mean (SD)	43.1 (5.2)	42.9 (5.2)	43.5 (5.0)	
WHR				**0.028**
Mean (SD)	0.885 (0.057)	0.887 (0.055)	0.879 (0.063)	
TBW_FFM				**0.038**
Mean (SD)	73.89 (0.28)	73.88 (0.27)	73.92 (0.29)	
BMR (kcal)				0.226
Mean (SD)	1,277 (171)	1,273 (171)	1,287 (172)	

[1] The values represent mean (SD) for continuous variables, and n (%) for categorical variables. The p-values for the continuous variables were obtained from an independent two sample t-test for normally distributed variables or Mann–Whitney–Wilcoxon rank sum test for non-normally distributed variables. For the categorical variables, the p-values were derived from the Chi-squared test statistics or Fisher-exact test.

[2] Wilcoxon rank sum test. The bold fonts indicate a p-value lower than 0.05.

Water_Lean_lower, ICW_ECW_lower, Xc_lower, and PA_lower in the lower extremity, with a maximum coefficient of 0.34 (absolute value). K-MMSE had the highest positive correlation with PA_lower, with a coefficient of 0.21. Overall, the correlations were more observable with lower extremity variables than with those of the upper extremity.

Partial Correlations

After adjusting for age and sex, most of the significant correlations between the BIA variables and the neuropsychological test scores were diminished. The highest coefficients were obtained between the SNSB-II visuospatial and frontal domains and PA_lower with $r = 0.14$ and TBW_FFM with $r = -0.16$ and $r = -0.17$, respectively. These results determined the role of these covariates in the association between BIA variables and cognitive tests.

Relationship Between the Bioelectrical Impedance Analysis Variables and Mild Cognitive Impairment

Table 3 presents the relationship between each of the BIA variables and MCI prevalence by ORs obtained from univariate and multiple logistic regression before and after adjusting for the potential covariates.

Before controlling for the covariates, several BIA variables showed significant associations with MCI, such as the whole-body composition variables PBF, WHR, and TBW_FFM or the segmental variables Water_Lean_lower, ECW_ICW_upper, ECW_ICW_lower, R_lower, Xc_upper, Xc_lower, and PA_lower, with significant ORs and their 95% confidence intervals (CIs) away from 1. Regarding whole-body composition, PBF and WHR exhibited negative associations with MCI, whereas TBW_FFM had a positive association with MCI with ORs (95% CI) of 0.86 (0.75–0.99), 0.86 (0.74–0.99), and 1.17 (1.02–1.35), respectively. With respect to segmental variables, there was a positive association between the water-related variables and MCI, and a negative association between the impedance-related variables and MCI. A unit increase in Water_Lean_lower, ECW_ICW_upper, or ECW_ICW_lower was associated with a nearly 22–26% increase in the odds of having MCI, whereas one unit decrease in R_lower, Xc_upper, Xc_lower, or PA_lower was associated with an approximate 17–27% increase in the odds of having MCI.

After adjusting for age, sex, GDS score, and the comorbidities, PBF, WHR, and TBW_FFM were no longer associated with MCI according to their p-values. R_lower and Xc_lower remained associated with MCI prevalence, with ORs and 95% CIs of 0.76 (0.62–0.92) and 0.79 (0.67–0.93), respectively; decreasing one unit in one of these variables was associated with increasing the odds of MCI by 21–24%. Noticeably, SW_lower and SL_lower which did not associate with MCI in the crude model became significantly associated with MCI after adjusting for the covariates, with the OR (95% CIs) of 1.32 (1.02–1.71) and 1.33 (1.03–1.72), respectively. Cellular water distribution between extra- and intra-spaces indicated by ECW_ICW_upper and ECW_ICW_lower variables exhibited a tendency of negatively associated with MCI which was demonstrated by an adjoining statistical significance threshold of the ORs (approximate p-value = 0.07). Relative water volume in both upper and lower extremities (Water_Lean_upper, Water_Lean_lower) did not show significant associations with the incidence of MCI.

Relationship Between the Bioelectrical Impedance Analysis Variables and Mild Cognitive Impairment for Each Sex

Table 4 separately examined the relationship between the BIA variables and MCI for women and men. After stratifying by sex, R_lower and Xc_lower were significantly associated with MCI in women, with ORs (95% CI) of 0.74 (0.57–0.95) and 0.75 (0.60–0.94), respectively. There were no significant associations between MCI and any of the BIA variables in the men group.

DISCUSSION

In this study, we aimed to examine the association between the BIA variables with the incidence of MCI, including seven whole-body composition variables and seven pairs of segmental BIA variables. The MCI group was shown to be slightly older, more depressive, and had significantly poorer cognitive abilities than CN group. The CN and MCI groups had approximately equivalent body anthropometric indices such as height, weight, and BMI. However, individuals with MCI had a slightly lower fat mass as indicated by lower WHR and PBF and higher PBCM and TBW_FFM than the CN participants (**Table 2**).

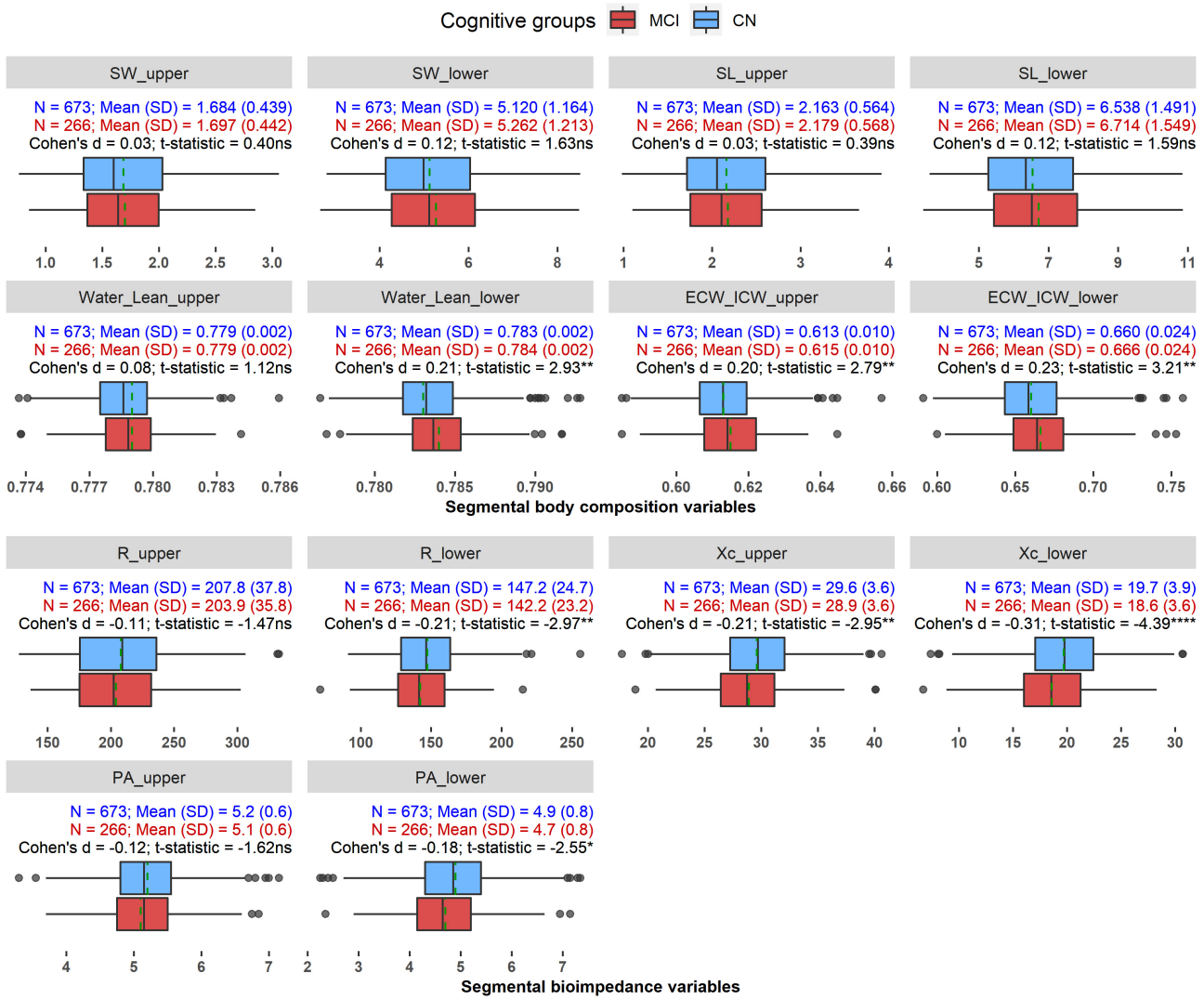


FIGURE 2 | The distributions of the segmental BIA variables in CN and MCI groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$, ns: Not significant. Dashed green line indicates mean value of each variable in each group.

Segmental analysis revealed that MCI individuals had a higher amount of relative water volumes and extra- to intracellular water ratios, but lower resistance, reactance, and phase angle in the upper and/or lower extremities when compared with CN individuals (**Figure 2**). After accounting for age, sex, GDS score, and the comorbidities, although the whole-body composition variables failed to differentiate CN and MCI, segmental analysis found that segmental water, segmental lean mass, resistance and reactance variables of the lower extremity were significantly associated with MCI (**Table 3**).

First of all, the segmental water and segmental lean mass in the lower extremities were proportionally associated with MCI, but their ratio indicated by the Water_Lean_lower variable was not significantly associated with MCI (**Table 3**). This result suggests that the increase in segmental water was directly proportional to the increase in segmental lean mass in the lower extremity, reflecting an increase in segmental lean mass but equal water volume per lean mass in the cognitive decline subjects. Despite the water volume normality, the water distribution between extra- and intracellular spaces showed the tendency of a shifting of water from the intracellular space to the extracellular areas, demonstrating by the ORs of ECW_ICW_lower closely adjoin to the statistical significance threshold (**Table 3**). This tendency suggests an abnormal water distribution and cellular dehydration status in individuals with cognitive decline, independently of age and the other covariates.

Secondly, the reduction in lower extremity reactance was found to be associated with the higher prevalence of MCI, independent of age, sex, and the other covariates. Lower reactance indicates a lower cell membrane capacitance due to a reduction in body cell mass or a decrease in cell strength due to malnutrition (20, 46). Cova et al. reported a lower nutritional status as

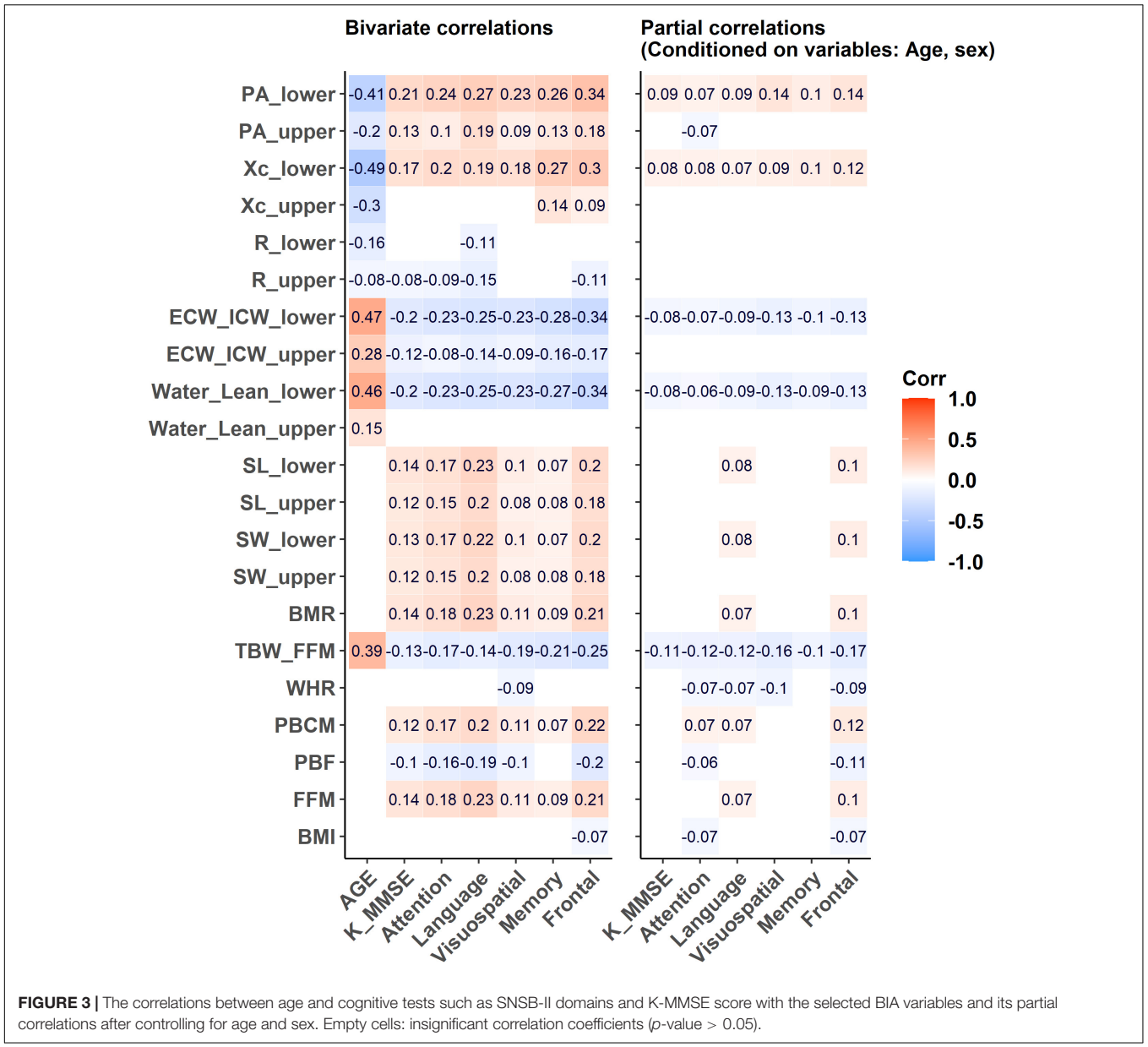


FIGURE 3 | The correlations between age and cognitive tests such as SNSB-II domains and K-MMSE score with the selected BIA variables and its partial correlations after controlling for age and sex. Empty cells: insignificant correlation coefficients (p-value > 0.05).

indicated by a lower Mini Nutritional Assessment score and a decrease in body cell mass as demonstrated by a shifting of the RXc graph on the right side in individuals with MCI when compared with the reference (26). In studies of dementia, lower reactance was found in women with severe AD than in patients with mild-to-moderate AD, as it was more evident in women with worse psycho-functional status (24, 25) and in men with dementia (23). Our finding of a reactance reduction in the MCI group is in alignment with these publications as we found a negative association between reactance and MCI, demonstrating a decrease in body cell mass or cell strength in the lower extremity in the individuals with cognitive decline. However, considering the positive association between SL_lower and MCI which implies an increase of segmental lean mass in lower extremity, the decrease in body cell mass might be due to the reduction in the volume of individual cell rather than decrease in total cell quantity. Most of living cells contain a large amount of water (47), so that ICW accounts for approximately 70% of total cell mass (48). The decrease in body cell mass or presumably reduced volume of individual cell therefore might be due to the reduction of ICW content in the MCI individuals.

Furthermore, resistance in lower extremity was also found to be linked with the higher prevalence of MCI, regardless of age and the other covariates. Resistance is a measure of resistivity and is inversely proportional to the relative amount of total body water, decrease in resistance indicates a higher proportion of water volume (20, 49). Saragat et al. reported a higher height-normalized resistance in both sexes with AD, but a preserved value in patients with lower psychological status (MMSE $<$ 20.7 compared with MMSE $\geq$ 20.7 in CN

TABLE 3 | Estimated odds ratios and 95% confidence intervals derived from the two logistic regression models.

	Crude model			Adjusted model		
Variables	**OR[1]**	**95% CI[1]**	***p*-value[2]**	**OR[1]**	**95% CI[1]**	***p*-value[2]**
BMI	0.91	0.79, 1.05	0.196	0.94	0.81, 1.09	0.430
FFM	1.08	0.94, 1.24	0.282	1.20	0.92, 1.57	0.173
PBF	0.86	0.75, 0.99	**0.041**	0.84	0.70, 1.02	0.072
PBCM	1.13	0.98, 1.31	0.088	1.17	0.96, 1.42	0.111
WHR	0.86	0.74, 0.99	**0.033**	0.87	0.75, 1.01	0.067
TBW_FFM	1.17	1.02, 1.35	**0.028**	1.09	0.92, 1.27	0.319
BMR	1.08	0.94, 1.24	0.282	1.20	0.92, 1.57	0.174
SL_upper	1.03	0.89, 1.18	0.699	1.02	0.79, 1.32	0.856
SL_lower	1.12	0.97, 1.29	0.108	1.32	1.02, 1.71	**0.033**
SW_upper	1.03	0.89, 1.19	0.690	1.03	0.79, 1.32	0.847
SW_lower	1.13	0.98, 1.30	0.098	1.33	1.03, 1.72	**0.031**
Water_Lean_upper	1.09	0.94, 1.25	0.254	1.04	0.90, 1.20	0.599
Water_Lean_lower	1.23	1.07, 1.42	**0.004**	1.14	0.96, 1.35	0.140
ECW_ICW_upper	1.22	1.06, 1.41	**0.006**	1.15	0.99, 1.35	0.068
ECW_ICW_lower	1.26	1.09, 1.45	**0.001**	1.17	0.99, 1.39	0.067
R_upper	0.90	0.78, 1.04	0.149	0.84	0.65, 1.08	0.163
R_lower	0.81	0.70, 0.93	**0.004**	0.76	0.62, 0.92	**0.005**
Xc_upper	0.81	0.70, 0.93	**0.003**	0.86	0.73, 1.01	0.064
Xc_lower	0.73	0.63, 0.85	**<0.001**	0.79	0.67, 0.93	**0.005**
PA_upper	0.89	0.77, 1.03	0.109	0.88	0.73, 1.06	0.193
PA_lower	0.83	0.72, 0.96	**0.012**	0.89	0.74, 1.06	0.182

[1]OR, odds ratio; CI, confidence interval.

[2]p-value obtains from Wald test. The models adjusted for age, sex, GDS score, hyperlipidemia, diabetes, and CNS disorder. The bold fonts indicate a p-value lower than 0.05.

individuals), suggesting a relative increase in fat in individuals with AD than that in CN individuals (24). The same team reported dehydration in women with severe AD as showed by a lengthening of the impedance vector on the RXc graph compared to the control (25). In this study, our MCI group did not have an increase in fat mass percentage or dehydration. In contrast, they had a higher segmental lean mass and a tendency of higher extracellular water compared with CN individuals (**Figure 2** and **Table 3**). Consequently, the increase in segmental lean mass and ECW volume might cause the R_lower reduction in the MCI individuals.

In this study, those with MCI were in the early stage of cognitive decline, with a mean (SD) K-MMSE score of 26.2 (2.5) that is somewhat closer to the mean (SD) of 27.6 (1.9) seen in the CN group than those in the aforementioned studies. Similarly, our MCI participants had better cognitive test scores than the cognitively impaired individuals in the previous studies (24, 25). Therefore, they might not yet have substantial changes in physical activity or nourishment and thus did not experience solid changes in body composition. Furthermore, age was reported to be an important factor to influence the fluctuation of cellular water distribution, such that ECW to ICW ratio increases with age and might be due to the steeper decrease in the ICW content than in the ECW compartment (9, 50). After controlling for age and other covariates, most body composition variables became insignificant; only segmental BIA variables such as resistance and reactance in the lower extremities and the related body composition/water compartment variables such as SL_lower and SW_lower remained to be significantly associated with the incidence of MCI.

After stratifying by sex, we found that women with MCI showed a significant reduction in terms of reactance and resistance of the lower extremity, whereas this pattern did not emerge in men (**Table 4**). The body composition differences between men and women were diversely reported in previous BIA publications (22, 24, 28). Sex hormones have been known to strongly influence body fat distribution, particularly estrogen and progesterone in women during menopause (49, 51). A common finding is that there is a higher possibility of obesity with predominant abdominal fat accumulation in aging women with a decrease in estrogen secretion (52, 53). However, Collelueri et al. described a U-shaped relationship between estradiol (a major estrogen in females) and body fat mass, such that total body fat mass was found to be decreased when estradiol levels were between 14.0 and 17.4 pg/ml and increased elsewhere (54). The complex relationship between hormonal changes during aging and body composition might regulate the relationship between impedance variables and cognitive decline. Nonetheless, our findings suggest that women with MCI experience more dramatic changes in their body composition and water compartment than men with MCI.

In this study, whole-body composition measurements, such as FFM or PBCM, were investigated as indicators of muscle volume in relation to MCI. Although individuals with MCI

TABLE 4 | Estimated odds ratios and 95% confidence intervals derived from the logistic regression models in women and men.

	Women			Men		
Variables	**OR[1]**	**95% CI[1]**	***p*-value[2]**	**OR[1]**	**95% CI[1]**	***p*-value[2]**
BMI	0.92	0.75, 1.11	0.387	1.01	0.80, 1.28	0.906
FFM	1.25	0.80, 1.96	0.320	1.24	0.88, 1.74	0.217
PBF	0.78	0.61, 1.01	0.062	0.92	0.70, 1.22	0.572
PBCM	1.26	0.97, 1.65	0.088	1.07	0.80, 1.42	0.654
WHR	0.85	0.69, 1.05	0.139	0.89	0.72, 1.09	0.270
TBW_FFM	1.10	0.88, 1.37	0.389	1.06	0.83, 1.35	0.650
BMR	1.26	0.80, 1.97	0.313	1.23	0.88, 1.73	0.221
SL_upper	1.05	0.68, 1.60	0.831	1.06	0.76, 1.48	0.714
SL_lower	1.32	0.86, 2.04	0.199	1.36	0.98, 1.89	0.066
SW_upper	1.05	0.69, 1.60	0.827	1.07	0.77, 1.48	0.706
SW_lower	1.33	0.87, 2.05	0.189	1.36	0.98, 1.88	0.064
Water_Lean_upper	1.01	0.84, 1.22	0.910	1.08	0.86, 1.36	0.517
Water_Lean_lower	1.21	0.95, 1.55	0.116	1.07	0.84, 1.36	0.594
ECW_ICW_upper	1.19	0.94, 1.51	0.140	1.12	0.91, 1.38	0.277
ECW_ICW_lower	1.23	0.96, 1.57	0.100	1.13	0.89, 1.43	0.326
R_upper	0.79	0.57, 1.08	0.146	0.87	0.56, 1.32	0.508
R_lower	0.74	0.57, 0.95	**0.020**	0.75	0.55, 1.02	0.071
Xc_upper	0.81	0.64, 1.01	0.061	0.91	0.71, 1.15	0.412
Xc_lower	0.75	0.60, 0.94	0.014	0.81	0.63, 1.04	0.101
PA_upper	0.88	0.67, 1.16	0.380	0.91	0.70, 1.18	0.481
PA_lower	0.86	0.66, 1.12	0.255	0.92	0.71, 1.17	0.487

[1]*OR, odds ratio; CI, confidence interval.*
[2]*p-value obtains from Wald test. The models adjusted for age, GDS score, hyperlipidemia, diabetes, and CNS disorder. The bold fonts indicate a p-value lower than 0.05.*

showed slightly more muscle volume due to larger FFM and PBCM compared with CN individuals (**Table 2**), these variables were insignificantly linked with MCI when taking into account age, sex, GDS score, and comorbidities (**Table 3**). However, segmental reactance in the lower extremity, which is associated with segmental cell mass or segmental muscle volume was significantly associated with the incidence of MCI, especially in women (**Tables 3**, **4**). Individuals with cognitive impairment are likely to experience protein–energy malnutrition and/or decreased physical activity, thus having a decrease in body lean mass or muscle volume. Myokines, which are synthesized and secreted from muscle mass during muscular contractions, regulate the metabolism in the muscle itself (autocrine) as well as in other tissues and organs, such as the brain, through their receptors (para/endocrine) (55). A lower muscle volume reduces myokine secretion and therefore lowers the amount of myokines crossing the blood–brain barrier. This imbalance in the myokine level upregulates pro-inflammatory cytokine production and impairs glucose and lipid metabolism in the brain (56). As a result, individuals with lower muscle volume or muscle atrophy are susceptible to neuronal damage which induces abnormal cognitive ability (57). In this study, a significant reduction in lower extremity reactance indicating a lower body cell mass or lower muscle volume and therefore a decrease in myokine secretion, might be one of the causes of cognitive impairment in the individuals with MCI (58). This finding revealed the importance of segmental body cell mass or muscle volume when examining the association with cognitive decline, especially in women (59).

In terms of muscle quality, phase angle and ECW/ICW have been recommended as indicators of muscle quality (60, 61). Phase angle is a major parameter previously investigated as a predictor of morbidity and mortality for several pathological conditions or critical illnesses, such as mortality in intensive care or in postoperative patients (62–64), sarcopenia, frailty (65), and Human Immunodeficiency Virus infection (66). It is also suggested as a potential indicator for diabetes (67), hyperlipidemia (68), and hypertension (69), which are known risk factors of cognitive impairment. The phase angle cutoff threshold varies across samples of different ages, sexes, and morbidities (65, 70, 71). In this study, MCI patients of both sexes had just a slightly lower phase angle than the normal controls, and this difference disappeared after controlling for covariates in both women and men; the muscle quality expressed by phase angle might not be a sensitive marker of cognitive impairment in its early stage. Moreover, ECW/ICW is another sensitive parameter that is positively correlated with age as well as cognitive function such as executive function, inhibition, or attention ability in elderly people (9, 50). In this study, after controlling for age, sex and the comorbidities, ECW/ICW in upper and lower extremities showed a tendency of associations with MCI prevalence. Further investigations with individuals in the early cognitive

impairment stages are needed to confirm the relation between ECW/ICW and MCI.

In the correlation analysis, age was the primary factor in the correlations between cognitive tests (SNSB-II and K-MMSE) and the BIA variables (**Figure 3**). After adjusting for age and sex, the cognitive tests showed only weak correlations with some BIA variables; resistance and reactance variables did not or only weakly correlated with the cognitive test scores. However, it was still able to produce significant associations with the incidence of MCI, regardless of age and the other covariates. Since the cognitive impairment in the older individuals is a degenerative symptom, it seems to be accompanied by overall decline in physiological functions with an early change of cellular water volume especially in lower extremity.

Daily activities involve physical strengths in both arms and both legs. In this study, bioimpedance changes in the lower extremity were observed and were more significantly associated with MCI than those in the upper extremity. A reduction in physical function in the lower extremity is likely to be related to a higher risk of cognitive decline (32, 33); it suggests that more focus should be placed on impedance variable changes in the lower extremity in relation to the early stage of cognitive impairment.

In this study, among multiple frequencies of 5, 50, and 250 kHz, the frequency of 50 kHz was selected to simplify the data analysis and be representative of the impedance variables. Indeed, the bioimpedance variables at other frequencies, such as 5 and 250 kHz, behave similarly as those at 50 kHz; lower extremity resistance and reactance variables at those frequencies were associated with MCI prevalence as well (**Supplementary Table 4**).

Compared to precedent publications (22–27, 36), our study contained a sufficient sample size of MCI and CN participants and thus can provide additional insight into body composition/impedance changes occurring in the early stage of cognitive decline. Regarding the transition from CN to dementia, MCI individuals represented a closer-to-normal stage based on cognition test scores. Despite this limitation, segmental bioimpedance variables such as resistance and reactance in the lower extremity and the related water compartment/body composition components were still effective in recognizing and distinguishing MCI patients from CN individuals. These lower extremity features might be used as an early sign of cognitive decline.

There are some limitations in this study. First, confounding factors, such as daily activity hours and nutritional states, may affect body composition and bioimpedance characteristics (72, 73). Second, segmental impedance variables were derived directly from BIA measurements, whereas body composition and segmental water compartments were estimated based on these impedance variables; therefore, accuracy may vary between ethnicity, age, sex, and manufacturer. Third, longitudinal studies are needed to monitor and confirm the changes in body composition, water compartment and bioimpedance features even during the earlier stages of cognitive decline (e.g., from CN or subjective memory impairment).

CONCLUSION

The selected BIA variables, which reflect the changes in body composition, water compartment and bioimpedance for upper and lower extremities, have weak correlations with the K-MMSE score as well as with the SNSB-II domains, especially when taking age and sex into consideration. Nevertheless, multiple logistic regression analysis reveals significant associations between the lower extremity BIA variables with MCI. Specifically, we found an increase in segmental lean mass and a decrease in body cell volume due to an abnormal cellular water distribution in the lower extremities with MCI, which were demonstrated by the reductions in both resistance and reactance. These characteristic changes of BIA variables may be considered as an early sign accompanied by cognitive impairment in the older population.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Chonnam National University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DD analyzed the data and wrote the manuscript. BK assisted in the data analysis. KK handled the Institutional Review Board approval and managed the data. MJ participated in writing the manuscript. KC and KL took care of the data collection and curation. JK designed the study and wrote the manuscript. All authors revised and approved the contents of the manuscript, contributed to the article, and approved the submitted version.

FUNDING

This study was supported by the Korea Institute of Oriental Medicine (KIOM; Grant No. KSN2022130) funded by the Korean Government.

ACKNOWLEDGMENTS

We thank all the contributors of this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.873623/full#supplementary-material

REFERENCES

1. Baumgartner RN, Stauber PM, McHugh D, Koehler KM, Garry PJ. Cross-sectional age differences in body composition in persons 60 + years of age. *J Gerontol A Biol Sci Med Sci.* (1995) 50A:M307–16. doi: 10.1093/gerona/50A.6.M307
2. Seidell JC, Visscher TL. Body weight and weight change and their health implications for the elderly. *Eur J Clin Nutr.* (2000) 54(Suppl. 3):S33–9. doi: 10.1038/sj.ejcn.1601023
3. Ponti F, Santoro A, Mercatelli D, Gasperini C, Conte M, Martucci M, et al. Aging and imaging assessment of body composition: from fat to facts. *Front Endocrinol.* (2020) 10:861. doi: 10.3389/fendo.2019.00861
4. Drøyvold WB, Nilsen TI, Krüger O, Holmen TL, Krokstad S, Midthjell K, et al. Change in height, weight and body mass index: longitudinal data from the HUNT Study in Norway. *Int J Obes (Lond).* (2006) 30:935–9. doi: 10.1038/sj.ijo.0803178
5. Alley DE, Ferrucci L, Barbagallo M, Studenski SA, Harris TB. A research agenda: the changing relationship between body weight and health in aging. *J Gerontol A Biol Sci Med Sci.* (2008) 63:1257–9. doi: 10.1093/gerona/63.11.1257
6. Cowen LE, Hodak SP, Verbalis JG. Age-associated abnormalities of water homeostasis. *Endocrinol Metab Clin North Am.* (2013) 42:349–70. doi: 10.1016/j.ecl.2013.02.005
7. Ritz P. Body water spaces and cellular hydration during healthy aging. *Ann N Y Acad Sci.* (2000) 904:474–83. doi: 10.1111/j.1749-6632.2000.tb06502.x
8. Schoeller DA. Changes in total body water with age. *Am J Clin Nutr.* (1989) 50(Suppl. 5):1176–81. doi: 10.1093/ajcn/50.5.1176
9. Ohashi Y, Joki N, Yamazaki K, Kawamura T, Tai R, Oguchi H, et al. Changes in the fluid volume balance between intra- and extracellular water in a sample of japanese adults aged 15–88 yr old: a cross-sectional study. *Am J Physiol Renal Physiol.* (2018) 314:F614–22. doi: 10.1152/ajprenal.00477.2017
10. Serra-Prat M, Lorenzo I, Palomera E, Ramírez S, Yébenes JC. Total body water and intracellular water relationships with muscle strength, frailty and functional performance in an elderly population. *J Nutr Health Aging.* (2019) 23:96–101. doi: 10.1007/s12603-018-1129-y
11. Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, et al. The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the national institute on Aging-Alzheimer's association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement.* (2011) 7:270–9. doi: 10.1016/j.jalz.2011.03.008
12. Tervo S, Kivipelto M, Hänninen T, Vanhanen M, Hallikainen M, Mannermaa A, et al. Incidence and risk factors for mild cognitive impairment: a population-based three-year follow-up study of cognitively healthy elderly subjects. *Dement Geriatr Cogn Disord.* (2004) 17:196–203. doi: 10.1159/000076356
13. Hai S, Dong B, Liu Y, Zou Y. Occurrence and risk factors of mild cognitive impairment in the older Chinese population: a 3-year follow-up study. *Int J Geriatr Psychiatry.* (2012) 27:703–8. doi: 10.1002/gps.2768
14. Chen PH, Cheng SJ, Lin HC, Lee CY, Chou CH. Risk factors for the progression of mild cognitive impairment in different types of neurodegenerative disorders. *Behav Neurol.* (2018) 2018:6929732. doi: 10.1155/2018/6929732
15. Lu Y, An Y, Guo J, Zhang X, Wang H, Rong H, et al. Dietary intake of nutrients and lifestyle affect the risk of mild cognitive impairment in the chinese elderly population: a cross-sectional study. *Front Behav Neurosci.* (2016) 10:229. doi: 10.3389/fnbeh.2016.00229
16. Xu Z, Zhang D, Sit RWS, Wong C, Tiu JYS, Chan DCC, et al. Incidence of and risk factors for mild cognitive impairment in Chinese older adults with multimorbidity in Hong Kong. *Sci Rep.* (2020) 10:4137. doi: 10.1038/s41598-020-60901-x
17. Casadei K, Kiel J. *Anthropometric Measurement: StatPearls*. Treasure Island FL: StatPearls Publishing (2021).
18. Scafoglieri A, Clarys JP, Cattrysse E, Bautmans I. Use of anthropometry for the prediction of regional body tissue distribution in adults: benefits and limitations in clinical practice. *Aging Dis.* (2013) 5:373–93. doi: 10.14366/AD.2014.0500373
19. Sebo P, Herrmann FR, Haller DM. Accuracy of anthropometric measurements by general practitioners in overweight and obese patients. *BMC Obes.* (2017) 4:23. doi: 10.1186/s40608-017-0158-0
20. Kyle UG, Bosaeus I, De Lorenzo AD, Deurenberg P, Elia M, Gómez JM, et al. Bioelectrical impedance analysis–part I: review of principles and methods. *Clin Nutr.* (2004) 23:1226–43. doi: 10.1016/j.clnu.2004.06.004
21. Lee SY, Gallagher D. Assessment methods in human body composition. *Curr Opin Clin Nutr Metab Care.* (2008) 11:566–72. doi: 10.1097/MCO.0b013e32830b5f23
22. Buffa R, Mereu RM, Putzu PF, Floris G, Marini E. Bioelectrical impedance vector analysis detects low body cell mass and dehydration in patients with Alzheimer's disease. *J Nutr Health Aging.* (2010) 14:823–7. doi: 10.1007/s12603-010-0115-9
23. Camina Martín MA, de Mateo Silleras B, Nescolarde Selva L, Barrera Ortega S, Domínguez Rodríguez L, Redondo del Río MP. Bioimpedance vector analysis and conventional bioimpedance to assess body composition in older adults with dementia. *Nutrition.* (2015) 31:155–9. doi: 10.1016/j.nut.2014.06.006
24. Saragat B, Buffa R, Mereu E, Succa V, Cabras S, Mereu RM, et al. Nutritional and psycho-functional status in elderly patients with Alzheimer's disease. *J Nutr Health Aging.* (2012) 16:231–6. doi: 10.1007/s12603-011-0347-3
25. Buffa R, Mereu E, Comandini O, Ibanez ME, Marini E. Bioelectrical impedance vector analysis (BIVA) for the assessment of two-compartment body composition. *Eur J Clin Nutr.* (2014) 68:1234–40. doi: 10.1038/ejcn.2014.170
26. Cova I, Pomati S, Maggiore L, Forcella M, Cucumo V, Ghiretti R, et al. Nutritional status and body composition by bioelectrical impedance vector analysis: a cross sectional study in mild cognitive impairment and Alzheimer's disease. *PLoS One.* (2017) 12:e0171331. doi: 10.1371/journal.pone.0171331
27. Willers J, Hahn A, Köbe T, Gellert S, Witte V, Tesky V, et al. Poor body composition in patients with mild cognitive impairment compared to healthy older controls. *J Aging Res Lifestyle.* (2018) 7:37–41. doi: 10.14283/jarcp.2018.8
28. Chiba I, Lee S, Bae S, Makino K, Shinkai Y, Shimada H. Visceral fat accumulation is associated with mild cognitive impairment in community-dwelling older Japanese women. *J Nutr Health Aging.* (2020) 24:352–7. doi: 10.1007/s12603-020-1330-7
29. Blaak E. Gender differences in fat metabolism. *Curr Opin Clin Nutr Metab Care.* (2001) 4:499–502. doi: 10.1097/00075197-200111000-00006
30. Karastergiou K, Smith SR, Greenberg AS, Fried SK. Sex differences in human adipose tissues – the biology of pear shape. *Biol Sex Differ.* (2012) 3:13. doi: 10.1186/2042-6410-3-13
31. Mereu E, Succa V, Buffa R, Sanna C, Mereu RM, Catte O, et al. Total body and arm bioimpedance in patients with Alzheimer's disease. *Exp Gerontol.* (2018) 102:145–8. doi: 10.1016/j.exger.2017.11.011
32. Bae S, Shimada H, Park H, Lee S, Makizako H, Doi T, et al. Association between body composition parameters and risk of mild cognitive impairment in older Japanese adults. *Geriatr Gerontol Int.* (2017) 17:2053–9. doi: 10.1111/ggi.13018
33. Caldas EC, Rezende LA, Oliveira KS, Pinheiro HA, Melo GF, Vilaça KHC. Muscle strength, lower extremity functional performance and body composition in elderly women with mild cognitive impairment. *Rev Bras Fisioter.* (2017) 30:753–9. doi: 10.1590/1980-5918.030.004.AO10
34. Low S, Ng TP, Lim CL, Moh A, Ang SF, Wang J, et al. Association between lower extremity skeletal muscle mass and impaired cognitive function in type 2 diabetes. *Sci Rep.* (2020) 10:2956. doi: 10.1038/s41598-020-59914-3
35. Tian Q, Resnick SM, Bilgel M, Wong DF, Ferrucci L, Studenski SA. β-Amyloid burden predicts lower extremity performance decline in cognitively unimpaired older adults. *J Gerontol A Biol Sci Med Sci.* (2017) 72:716–23. doi: 10.1093/gerona/glw183
36. Boyle PA, Wilson RS, Buchman AS, Aggarwal NT, Tang Y, Arvanitakis Z, et al. Lower extremity motor function and disability in mild cognitive impairment. *Exp Aging Res.* (2007) 33:355–71. doi: 10.1080/03610730701319210
37. Eggermont LH, Gavett BE, Volkers KM, Blankevoort CG, Scherder EJ, Jefferson AL, et al. Lower-extremity function in cognitively healthy aging, mild cognitive impairment, and Alzheimer's disease. *Arch Phys Med Rehabil.* (2010) 91:584–8. doi: 10.1016/j.apmr.2009.11.020
38. Ahn HJ, Chin J, Park A, Lee BH, Suh MK, Seo SW, et al. Seoul neuropsychological screening battery-dementia version (SNSB-D): a useful tool for assessing and monitoring cognitive impairments in dementia patients. *J Korean Med Sci.* (2010) 25:1071–6. doi: 10.3346/jkms.2010.25.7.1071
39. Lee AY, Lee J, Oh E, Yoon SJ, Yoom B, Yu SD. Clinical utility of seoul neuropsychological screening battery-core for dementia management project

in the community. *J Korean Neurol Assoc.* (2019) 37:277–83. doi: 10.17340/jkna.2019.3.5
40. Kang YW, Jahng SM, Na DR. *Seoul Neuropsychological Screening Battery (SNSB-II)*. 2nd ed. Incheon: Human Brain Research & Consulting (2012).
41. InBody S10. *InBody370 USER'S MANUAL*. (1996). Available online at:https://nl.inbody.com/wp-content/uploads/2019/01/InBodyS10_CDmanual_Eng_E.pdf (accessed March 15, 2020).
42. Bosy-Westphal A, Later W, Hitze B, Later W, Hitze B, Sato T, et al. Accuracy of bioelectrical impedance consumer devices for measurement of body composition in comparison to whole-body magnetic resonance imaging and dual X-ray absorptiometry. *Obes Facts.* (2008) 1:319–24. doi: 10.1159/000176061
43. Xu L, Cheng X, Wang J, Cao Q, Sato T, Wang M, et al. Comparisons of body-composition prediction accuracy: a study of 2 bioelectric impedance consumer devices in healthy Chinese persons using DXA and MRI as criteria methods. *J Clin Densitom.* (2011) 14:458–64. doi: 10.1016/j.jocd.2011.04.001
44. Joo SH, Yun SH, Kang DW, Hahn CT, Lim HK, Lee CU. Body mass index in mild cognitive impairment according to age, sex, cognitive intervention, and hypertension and risk of progression to Alzheimer's disease. *Front Psychiatry.* (2018) 9:142. doi: 10.3389/fpsyt.2018.00142
45. Kang SY, Kim YJ, Jang W, Son KY, Park HS, Kim YS. Body mass index trajectories and the risk for Alzheimer's disease among older adults. *Sci Rep.* (2021) 11:3087. doi: 10.1038/s41598-021-82593-7
46. Khalil SF, Mohktar MS, Ibrahim F. The theory and fundamentals of bioimpedance analysis in clinical status monitoring and diagnosis of diseases. *Sensors.* (2014) 14:10895–928. doi: 10.3390/s140610895
47. Tobias A, Ballard BD, Mohiuddin SS. *Physiology, Water Balance: StatPearls [Internet]*. Treasure Island, FL: StatPearls Publishing (2022).
48. Delpire E, Gagnon KB. Water homeostasis and cell volume maintenance and regulation. *Curr Top Membr.* (2018) 81:3–52. doi: 10.1016/bs.ctm.2018.08.001
49. Lizcano F, Guzmán G. Estrogen deficiency and the origin of obesity during menopause. *Biomed Res Int.* (2014) 2014:757461. doi: 10.1155/2014/757461
50. Lee J, Shields RK. Extracellular to intracellular body water and cognitive function among healthy older and younger adults. *J Funct Morphol Kinesiol.* (2022) 7:18. doi: 10.3390/jfmk7010018
51. Kozakowski J, Gietka-Czernel M, Leszczyńska D, Majos A. Obesity in menopause – our negligence or an unfortunate inevitability? *Prz Menopauzalny.* (2017) 16:61–5. doi: 10.5114/pm.2017.68594
52. Davis SR, Castelo-Branco C, Chedraui P, Lumsden MA, Nappi RE, Shah D, et al. Understanding weight gain at menopause. *Climacteric.* (2012) 15:419–29. doi: 10.3109/13697137.2012.707385
53. Greendale GA, Sternfeld B, Huang M, Han W, Karvonen-Gutierrez C, Ruppert K, et al. Changes in body composition and weight during the menopause transition. *JCI Insight.* (2019) 4:e124865. doi: 10.1172/jci.insight.124865
54. Colleluori G, Chen R, Napoli N, Aguirre LE, Qualls C, Villareal DT, et al. Fat mass follows a u-shaped distribution based on estradiol levels in postmenopausal women. *Front Endocrinol (Lausanne).* (2018) 9:315. doi: 10.3389/fendo.2018.00315
55. Lee JH, Jun HS. Role of myokines in regulating skeletal muscle mass and function. *Front Physiol.* (2019) 10:42. doi: 10.3389/fphys.2019.00042
56. Pedersen BK. Physical activity and muscle-brain crosstalk. *Nat Rev Endocrinol.* (2019) 15:383–92. doi: 10.1038/s41574-019-0174-x
57. Scisciola L, Fontanella RA, Surina, Cataldo V, Paolisso G, Barbieri M. Sarcopenia and cognitive function: role of myokines in muscle brain cross-talk. *Life (Basel).* (2021) 11:173. doi: 10.3390/life11020173
58. Jo D, Yoon G, Kim OY, Song J. A new paradigm in sarcopenia: cognitive impairment caused by imbalanced myokine secretion and vascular dysfunction. *Biomed Pharmacother.* (2022) 147:112636. doi: 10.1016/j.biopha.2022.112636
59. Canon ME, Crimmins EM. Sex differences in the association between muscle quality, inflammatory markers, and cognitive decline. *J Nutr Health Aging.* (2011) 15:695–8. doi: 10.1007/s12603-011-0340-x
60. Yamada Y, Yoshida T, Yokoyama K, Watanabe Y, Miyake M, Yamagata E, et al. The extracellular to intracellular water ratio in upper legs is negatively associated with skeletal muscle strength and gait speed in older people. *J Gerontol A Biol Sci Med Sci.* (2017) 72:293–8. doi: 10.1093/gerona/glw125
61. Akamatsu Y, Kusakabe T, Arai H, Yamamoto Y, Nakao K, Ikeue K, et al. Phase angle from bioelectrical impedance analysis is a useful indicator of muscle quality. *J Cachexia Sarcopenia Muscle.* (2022) 13:180–9. doi: 10.1002/jcsm.12860
62. Stapel SN, Looijaard WGPM, Dekker IM, Girbes ARJ, Weijs PJM, Oudemans-van Straaten HM. Bioelectrical impedance analysis-derived phase angle at admission as a predictor of 90-day mortality in intensive care patients. *Eur J Clin Nutr.* (2018) 72:1019–25. doi: 10.1038/s41430-018-0167-1
63. Mullie L, Obrand A, Bendayan M, Trnkus A, Ouimet MC, Moss E, et al. Phase angle as a biomarker for frailty and postoperative mortality: the BICS study. *J Am Heart Assoc.* (2018) 7:e008721. doi: 10.1161/JAHA.118.008721
64. Yu B, Park KB, Park JY, Lee SS, Kwon OK, Chung HY. Bioelectrical impedance analysis for prediction of early complications after gastrectomy in elderly patients with gastric cancer: the phase angle measured using bioelectrical impedance analysis. *J Gastric Cancer.* (2019) 19:278–89. doi: 10.5230/jgc.2019.19.e22
65. Rosas-Carrasco O, Ruiz-Valenzuela RE, López-Teros MT. Phase angle cut-off points and their association with sarcopenia and frailty in adults of 50-64 years old and older adults in Mexico City. *Front Med (Lausanne).* (2021) 8:617126. doi: 10.3389/fmed.2021.617126
66. Schwenk A, Beisenherz A, Römer K, Kremer G, Salzberger B, Elia M. Phase angle from bioelectrical impedance analysis remains an independent predictive marker in HIV-infected patients in the era of highly active antiretroviral treatment. *Am J Clin Nutr.* (2000) 72:496–501. doi: 10.1093/ajcn/72.2.496
67. Jun MH, Kim S, Ku B, Cho J, Kim K, Yoo HR, et al. Glucose-independent segmental phase angles from multi-frequency bioimpedance analysis to discriminate diabetes mellitus. *Sci Rep.* (2018) 8:648. doi: 10.1038/s41598-017-18913-7
68. Chen G, Lv Y, Ni W, Shi Q, Xiang X, Li S, et al. Associations between phase angle values obtained by bioelectrical impedance analysis and nonalcoholic fatty liver disease in an overweight population. *Can J Gastroenterol Hepatol.* (2020) 2020:8888405. doi: 10.1155/2020/8888405
69. Saad MA, Jorge AJ, de Andrade Martins W, Cardoso GP, Dos Santos MM, Rosa ML, et al. Phase angle measured by electrical bioimpedance and global cardiovascular risk in older adults. *Geriatr Gerontol Int.* (2018) 18:732–7. doi: 10.1111/ggi.13241
70. Norman K, Stobäus N, Zocher D, Bosy-Westphal A, Szramek A, Scheufele R, et al. Cutoff percentiles of bioelectrical phase angle predict functionality, quality of life, and mortality in patients with cancer. *Am J Clin Nutr.* (2010) 92:612–9. doi: 10.3945/ajcn.2010.29215
71. Di Vincenzo O, Marra M, Di Gregorio A, Pasanisi F, Scalfi L. Bioelectrical impedance analysis (BIA) -derived phase angle in sarcopenia: a systematic review. *Clin Nutr.* (2021) 40:3052–61. doi: 10.1016/j.clnu.2020.10.048
72. Khadilkar A, Chiplonkar S, Kajale N, Ekbote VH, Parathasarathi L, Padidela R, et al. Impact of dietary nutrient intake and physical activity on body composition and growth in Indian children. *Pediatr Res.* (2018) 83:843–50. doi: 10.1038/pr.2017.32
73. Zou Q, Su C, Du W, Ouyang Y, Wang H, Wang Z, et al. The association between physical activity and body fat percentage with adjustment for body mass index among middle-aged adults: China health and nutrition survey in 2015. *BMC Public Health.* (2020) 20:732. doi: 10.1186/s12889-020-08832-0

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Doan, Ku, Kim, Jun, Choi, Lee and Kim. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 23 June 2022
doi: 10.3389/fnut.2022.900887

Association of Dietary Vitamin K Intake With Cognition in the Elderly

Anni Wang[1†], Meng Zhao[2†], Jia Luo[1], Tianhao Zhang[1] and Dongfeng Zhang[1]*

[1] Department of Epidemiology and Health Statistics, School of Public Health, Qingdao University, Qingdao, China, [2] School of Nursing, Qingdao University Medical College, Qingdao, China

Several previous studies discussed the association between vitamin K (VK) status and cognition. But the association between dietary VK consumption and cognitive performance in the elderly was not well understood. Therefore, we investigated the correlation between dietary VK intake and the cognition of the elderly. Our research used the data of the National Health and Nutrition Examination Survey (NHANES) from 2011 to 2014. The dietary intake of VK was assessed by two 24-h dietary recalls. The cognitive function was measured in the survey of NHANES, including the Consortium to Establish a Registry for Alzheimer's disease Word Learning subtest (CERAD W-L), Animal Fluency Test (AFT), and Digit Symbol Substitution Test (DSST). Logistic regression and restricted cubic spline models were applied to assess the relationship between dietary VK intake and cognition. Compared with the lowest dietary VK intake group, the multivariate-adjusted odds ratio (OR) [95% confidence interval (95% CI)] of low CERAD W-L score for the highest intake group was 0.39 (0.26–0.60), the multivariate-adjusted OR (95% CI) of low AFT score was 0.59 (0.38–0.92), and the multivariate-adjusted OR (95% CI) of low DSST score was 0.44 (0.29–0.65), respectively. There was an L-shaped dose–response relationship between dietary VK intake and low CERAD W-L score. There was a linear dose–response relationship between dietary VK intake and low AFT score, and there was also a linear dose–response relationship for the low DSST score. In addition, we also found a negative association between VK from vegetables and the risk of low CERAD W-L scores. Dietary VK intake and VK intake from vegetables were inversely related to the risk of low cognitive performance of the elderly.

Keywords: cognition, vitamin K, dose–response, cross-sectional study, NHANES

OPEN ACCESS

Edited by:
Andrew Scholey, Swinburne University of Technology, Australia

Reviewed by:
Baodong Yao, Shanghai University of Traditional Chinese Medicine, China
Agnieszka Micek, Jagiellonian University, Poland

***Correspondence:**
Dongfeng Zhang
zhangdf1961@126.com

[†] These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

Received: *24 March 2022*
Accepted: *07 June 2022*
Published: *23 June 2022*

Citation:
Wang A, Zhao M, Luo J, Zhang T and Zhang D (2022) Association of Dietary Vitamin K Intake With Cognition in the Elderly. Front. Nutr. 9:900887. doi: 10.3389/fnut.2022.900887

INTRODUCTION

Cognitive function is associated with a variety of factors (1, 2). Many previous studies have discussed the association between nutritional factors and cognition, such as vitamin D (3, 4), zinc, copper, selenium (5), creatine (6), ω-3, and ω-6 fatty acids (7).

As a fat-soluble nutrient, vitamin K (VK) can regulate the synthesis of sphingolipids (8). As a main component of the myelin sheath and neuron membrane, sphingolipids participate in the proliferation and differentiation of neurons (9). The current studies find that VK has an important role in the nervous system (10, 11). Several previous studies have discussed the association between VK status and cognition.

For instance, a study of 31 patients with Alzheimer's disease and 31 healthy controls found that patients had lower VK intake (12). A study of 192 geriatric patients had reported that higher dietary VK intake had a better cognitive performance (13). The results of a study of 156

elderly Irish people showed that VK status was associated with cognition (14). But in the study of 599 participants (aged 55–65 years) from the Amsterdam longitudinal aging study, VK status was not associated with cognitive performance decline (15).

At present, the association between dietary VK consumption and cognitive performance in the elderly is not well understood. The dose–response relationship and the relationship between dietary intake of VK from different sources and cognitive function has not been explored.

Therefore, the National Health and Nutrition Examination Survey (NHANES) data was used to explore the relationship between dietary VK intake and cognition of the elderly in the United States. Our research explored the dose–response relationship and sex stratification results using a larger sample size. In addition, we also explored the relationship between dietary VK intake from different sources and cognitive function.

MATERIALS AND METHODS

Study Population

The data of dietary VK intake and cognitive function were obtained from two cycles (2011–2012 and 2013–2014) of the NHANES dataset. NHANES was an important plan belonging to the National Center for Health Statistics (NCHS) in the United States, and the relevant information of the survey can be obtained elsewhere (16). The 2 cycles of NHANES (2011–2012 and 2013–2014) included 19, 931 participants. We excluded participants who were under 60 years old, had incomplete cognitive test data, incomplete or unreliable dietary data. The "Dietary recall status code" in NHANES survey evaluated the quality and integrity of the dietary data. The reliable and unreliable dietary data were distinguished. Unreliable dietary data means that data on total nutrient intakes and the total foods reported in these cases were not available. These individuals had no records in the Individual Foods files (17). Finally, there were 2524 participants in our analysis, and the detailed flow chart was shown in **Figure 1**.

TABLE 1 | The cognition performance cut-off points of test score (CERAD W-L, AFT, and DSST), adjusted according to age (60–70 years, 70–80 years, and ≥80 years).

	CERAD W-L score	AFT score	DSST score
60–70 years	23	14	38
70–80 years	20	12	33
≥80 years	17	12	29

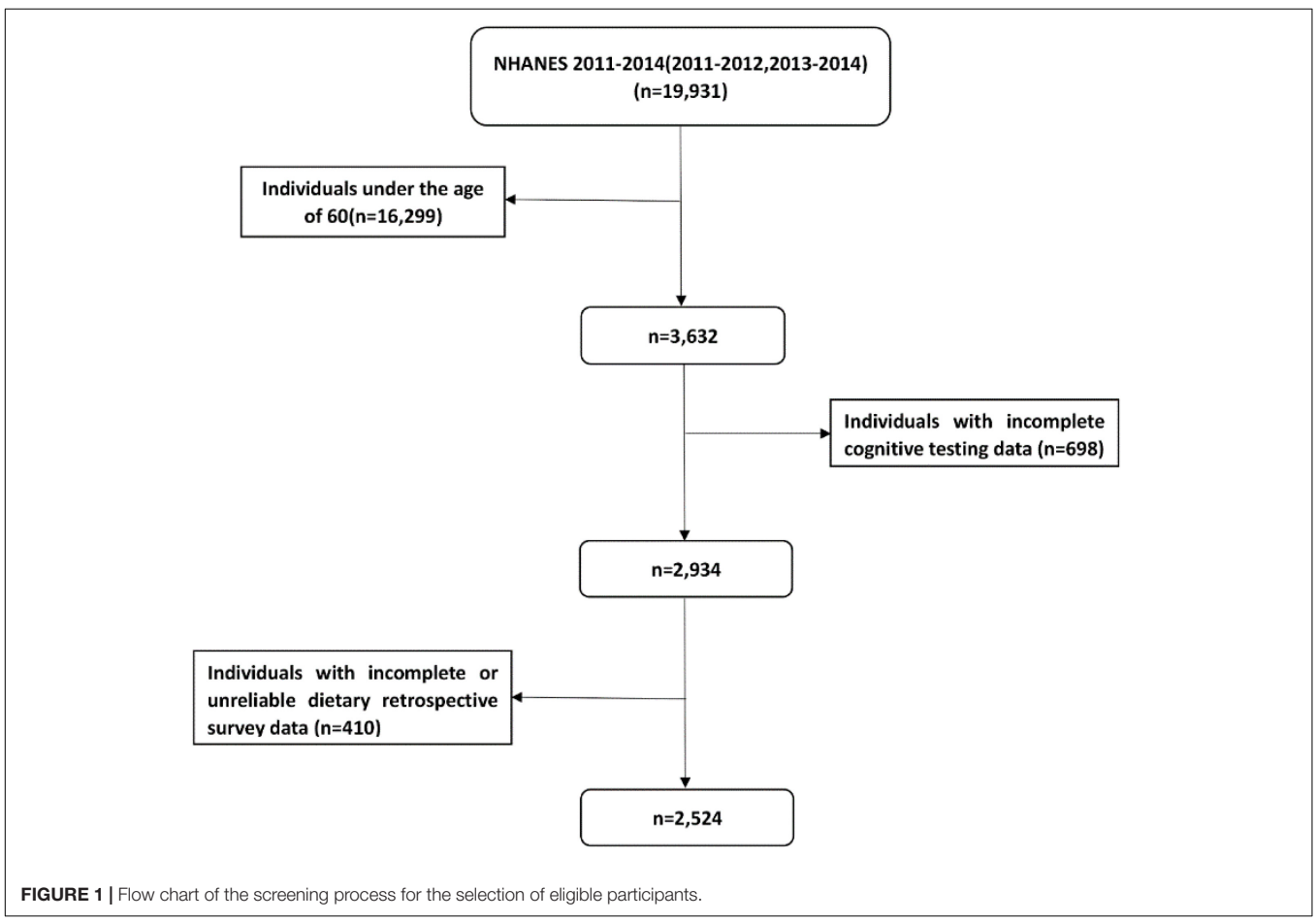


FIGURE 1 | Flow chart of the screening process for the selection of eligible participants.

TABLE 2 | The classifications of covariates.

Covariates	Classifications
Sex	Male; female
Age (year)	60–70; 70–80; ≥80
Educational level	Below high school; high school; above high school
Marital status	Married/living with partner; widowed/divorced/separated/never married
Poverty-income ratio	≤0.99; and ≥1
Race	Mexican American; other Hispanic; non-Hispanic White; non-Hispanic Black; other race
Body mass index (BMI)	≤25 kg/m^2; 25 to <30 kg/m^2; ≥30 kg/m^2
Smoked at least 100 cigarettes in life	No; yes
Had at least 12 alcohol drinks/year	No; yes
Occupational physical activity	Vigorous; moderate; other
Recreational physical activity	Vigorous; moderate; other
Hypertension[a]	No; yes
Diabetes[b]	No; yes
Stroke[c]	No; yes
Total energy intake (kcal/day)[d]	Continuous

[a]*Systolic blood pressure (SBP) ≥130 mmHg, or diastolic blood pressure (DBP) ≥80 mmHg, or ever been told by a doctor or other health professional that had hypertension, or currently taking antihypertensive drugs were hypertensive patients.*
[b]*Diabetes was defined as ever been told by a doctor or other health professional that had diabetes, or taking insulin, or fasting plasma glucose (FPG) level at least 126 mg/dL, or 2-h plasma glucose level above 200 mg/dL, or a glycated hemoglobin (HbA1c) level of 6.5% or greater.*
[c]*Stroke was defined as self-reported physician diagnosis of stroke.*
[d]*Total energy intake of each participant was the average of two 24-h dietary energy intake.*

Among the 2524 participants, 1712 participants had data on VK intake from vegetables and 1450 participants had data on VK intake from grains.

The Intake of Dietary Vitamin K

Dietary information was obtained through two 24-h dietary recall interview. Daily dietary VK intake was based on the average intake of two dietary retrospective surveys (18). A detailed description of dietary data collection can be found on the website of NHANES (19). Total VK intake data were obtained from "Total Nutrient Intakes Files," and different sources of VK intake data were obtained from "Individual Foods Files." Individual Foods files reported by each participant included detailed information about each food/beverage item (including the description, quantity, and nutritional ingredients). Different sources of VK intake can be identified by the Food/Individual component number (20). According to the food code of United States Department of Agriculture (USDA), we determined the intake of vegetable and grain sources VK from the dietary data (21). Referring to published articles (22), dietary VK intake was categorized into quartiles (Q1, Q2, Q3, and Q4 groups).

Cognitive Function

There were three cognitive function tests in NHANES surveys (23). The Consortium to Establish a Registry for Alzheimer's disease Word Learning subtest (CERAD W-L) included three consecutive learning trials and a delayed recall for new verbal information, which was used to evaluate immediate and delayed learning ability. The highest score on each test was 10. The Animal Fluency Test (AFT) was a component of executive function. To examine categorical verbal fluency, participants were asked to name as many animals as possible in 1 min, and each one scored one point. The Digit Symbol Substitution Test (DSST) assessed working memory, sustained attention, and processing speed. The score ranged from 0 to 133. In all three tests, higher scores indicated better cognitive performance. Although cognitive function tests cannot equate with clinical diagnosis, these tests proved to be good screening tools and used in research related to cognitive function (24–26).

According to the method used in the published literature (3, 5, 7, 22, 27), we further classified the scores according to three age stages. In each test, participants with scores below the minimum quartile for each age group were defined as having low poor cognitive performance. According to the cut-off points (shown in **Table 1**), participants were divided into low cognition performance and normal cognition performance groups.

Covariates

We included some covariates, according to the past literature on dietary factors and cognitive function (4, 27, 28). They mainly included socioeconomic status variables (such as age), health behavior variables (such as drinking status), and health factors (such as hypertension). In addition, we also adjusted the total energy intake. Detailed classification information of covariates was shown in **Table 2**.

Statistical Analysis

According to the analysis guide of NHANES, we calculated new sample weights (29). We used the *Kolmogorov–Smirnov normality test* to test the normality of continuous variables. To describe normal variables, we used mean ± standard deviation (SD). As for non-normally distributed variables, we used the median (interquartile range) to describe it and used the *Mann–Whitney U test* to compare it. For normal distribution variables, we used the *Student's t-test* to compare the factors between the low and normal cognition groups. The *Chi-square test* was used to compare the percentage of categorical variables.

The lowest dietary VK intake group (Q1) was taken as the reference group. Logistic regression analyses were used to explore the relationship between VK intake and cognition. We used odds ratio (OR) and 95% confidence interval (95% CI) to report results. Sex stratification analysis was also included in our study. Restricted cubic spline was used to explore the dose–response relationships between dietary VK and cognition. The three nodes were located at the 5th, 50th, and 95th percentile of dietary VK intake, respectively. We performed sensitivity analysis by excluding participants taking VK antagonists (warfarin or clopidogrel). In order to further excluded the influence of total energy intake on the results, we added a sensitivity analysis. The regression residual represented the difference between the actual VK intake for each participant and the predicted intake based on the total energy intake. The VK intake

TABLE 3 | Characteristics of the dietary VK and cognition study population, National Health and Nutrition Examination Survey (NHANES) 2011–2014 (*N* = 2524).

		CERAD W-L			AFT			DSST		
	Number of subjects (*N*)	**Normal cognitive performance**	**Low cognitive performance**	***p*-value**	**Normal cognitive performance**	**Low cognitive performance**	***p*-value**	**Normal cognitive performance**	**Low cognitive performance**	***p*-value**
Number of subjects (%)[a]		1939 (76.82)	585 (23.18)		1952 (77.34)	572 (22.66)		1913 (75.79)	611 (24.21)	
Age (%)[a]	2524			0.3261			0.0798			0.0028
60–70 years		1030 (58.09)	338 (52.41)		1040 (57.82)	323 (53.24)		1036 (58.53)	332 (47.35)	
70–80 years		589 (27.98)	160 (32.67)		602 (28.99)	146 (27.79)		567 (28.29)	182 (32.26)	
≥70 years		320 (13.93)	87 (14.92)		310 (13.19)	95 (18.97)		310 (13.18)	97 (20.39)	
Sex[a]	2524			0.0041			0.3511			0.5684
Male		862 (44.77)	355 (55.96)		944 (46.02)	273 (50.52)		875 (46.48)	342 (48.42)	
Female		1077 (55.23)	230 (44.04)		1008 (53.98)	299 (49.48)		1038 (53.52)	269 (51.58)	
Race[a]	2524			<0.01			<0.01			<0.01
Mexican American		139 (2.67)	72 (6.80)		164 (3.11)	47 (4.90)		125 (2.43)	86 (9.98)	
Other Hispanic		159 (2.97)	85 (8.61)		168 (3.29)	76 (7.51)		124 (2.41)	120 (14.45)	
Non-Hispanic White		1046 (81.32)	223 (67.77)		1106 (82.72)	163 (58.86)		1118 (83.71)	151 (46.55)	
Non-Hispanic Black		429 (8.01)	165 (11.44)		376 (6.68)	218 (18.87)		361 (6.20)	233 (24.98)	
Other races		166 (5.04)	40 (5.37)		138 (4.20)	68 (9.86)		185 (5.25)	21 (4.05)	
Educational level (%)[a]	2475			<0.01			<0.01			<0.01
Below high school		366 (12.51)	213 (27.79)		382 (13.32)	197 (25.15)		283 (11.05)	296 (43.42)	
High school		431 (20.23)	144 (24.57)		415 (19.58)	160 (28.49)		433 (20.65)	142 (23.29)	
Above high school		1103 (67.27)	218 (47.63)		1116 (67.10)	205 (46.36)		1161 (68.30)	160 (33.29)	
Material status (%)[a]	2514			0.0444			0.0848			<0.01
Married/living with partner		1156 (67.41)	328 (61.16)		1163 (67.34)	321 (60.82)		1170 (68.43)	314 (51.93)	
Widowers/divorced /separated/never married		776 (32.59)	254 (38.84)		782 (32.66)	248 (39.18)		736 (31.57)	294 (48.07)	
Poverty-income ratio (%)[a]	2524			<0.01			<0.01			<0.01
<1		248 (6.87)	125 (14.23)		240 (6.49)	133 (17.06)		196 (5.66)	177 (25.13)	
≥1		1691 (93.13)	460 (85.77)		1712 (93.51)	439 (82.94)		1717 (94.34)	434 (74.87)	
Body mass index (%)[a]	2492			0.3840			0.9928			0.6163
<25 kg/m^2		496 (27.24)	165 (28.02)		508 (27.42)	153 (27.15)		501 (27.05)	160 (29.64)	
25–30 kg/m^2		660 (34.36)	206 (38.09)		677 (35.04)	189 (34.90)		671 (35.53)	195 (31.54)	
≥30 kg/m^2		759 (38.40)	206 (33.89)		752 (37.54)	213 (37.95)		727 (37.42)	238 (38.82)	
Work activity (%)[a]	2521			<0.01			0.0857			<0.01
Vigorous		219 (14.04)	55 (9.30)		234 (13.88)	40 (9.65)		228 (13.97)	46 (8.06)	
Moderate		421 (24.46)	91 (16.19)		422 (24.07)	90 (17.31)		419 (24.09)	93 (15.66)	
Low		1297 (61.50)	438 (74.51)		1295 (62.05)	440 (73.04)		1263 (61.94)	472 (76.28)	
Recreational activity (%)[a]	2524			<0.01			<0.01			<0.01
Vigorous		209 (12.91)	34 (5.30)		210 (12.96)	33 (4.22)		229 (13.08)	14 (1.33)	
Moderate		647 (33.59)	177 (34.35)		675 (34.47)	149 (29.77)		654 (34.56)	170 (28.06)	
Low		1083 (53.49)	374 (60.36)		1067 (52.57)	390 (66.01)		1030 (52.36)	427 (70.61)	
Smoke at least 100 cigarettes in life (%)[a]	2522	971 (48.93)	304 (49.52)	0.8782	1002 (49.59)	273 (46.07)	0.3817	965 (48.59)	310 (52.01)	0.1803
Hypertension (%)[a]	2524	1553 (76.52)	472 (82.07)	0.0932	1540 (76.64)	485 (82.03)	0.0993	1508 (76.36)	517 (85.21)	0.0156
Diabetes (%)[a]	2524	585 (23.24)	217 (27.76)	0.0177	547 (22.05)	228 (34.54)	0.0013	536 (21.52)	266 (41.11)	<0.01
Stroke (%)[a]	2519	115 (5.42)	54 (8.52)	0.0524	114 (5.22)	55 (9.90)	0.0135	98 (4.97)	71 (12.72)	<0.01
Had at least 12 alcohol drinks/year (%)[a]	2506	1338 (74.12)	392 (68.12)	0.0332	1379 (75.03)	351 (62.69)	<0.01	1352 (75.04)	378 (59.66)	<0.01

(Continued)

TABLE 3 | (Continued)

		CERAD W-L			AFT			DSST		
	Number of subjects (*N*)	**Normal cognitive performance**	**Low cognitive performance**	***p*-value**	**Normal cognitive performance**	**Low cognitive performance**	***p*-value**	**Normal cognitive performance**	**Low cognitive performance**	***p*-value**
Total energy intake (kcal/day)[b]	2524	1751 (831.5)	1637.5 (975.5)	0.0003	1785.75 (821.5)	1547 (866.75)	<0.01	1789 (832)	1557.5 (869.5)	<0.01
Daily dietary VK intake (mcg/day)[b]	2524	86.25 (94.35)	67.7 (76)	<0.01	86.73 (94.53)	64.88 (73.95)	<0.01	87.95 (94.2)	60.45 (73.3)	<0.01
Daily dietary VK intake from vegetable source (mcg/day)[c]	1712	42 (87.15)	35.8 (65.95)	0.0163	42 (83.65)	36.15 (67.8)	0.1604	43.95 (87.35)	31.35 (62.45)	<0.01
Daily dietary VK intake from grain source (mcg/day)[c]	1450	16.2 (21)	15.8 (20.2)	0.6944	16.7 (21.15)	13.3 (21.425)	0.0048	16.7 (21.4)	14.3 (19.95)	0.0267

Data were the actual number of participants (weighted percentage) or medians (interquartile ranges).
[a]*Chi-square test was used to compare the difference between normal and low cognitive performance participants.*
[b]*Mann–Whitney U test was used to compare the difference between normal and low cognitive performance participants.*
[c]*Among 2524 individuals, 1712 had completed data on VK from vegetable source, 1450 had completed VK data from grain source.*

TABLE 4 | Weighted odds ratios (95% confidence intervals) for scores on CERAD W-L, AFT, DSST across dietary VK intake, NHANES 2011–2014 (*N* = 2524).

		Dietary VK intake (mcg/day)	**Q1 (≤47.55)**	**Q2 (47.55 to ≤80.95)**	**Q3 (80.95 to ≤136.6)**	**Q4 (>136.6)**
CERAD W-L	Case/participants		201/631	144/631	137/631	103/631
	Crude		1.00 (Ref.)	0.61 (0.45–0.82)*	0.48 (0.32–0.72)*	0.27 (0.17–0.42)*
	Model 1		1.00 (Ref.)	0.67 (0.49–0.91)*	0.63 (0.43–0.91)*	0.39 (0.26–0.60)*
AFT	Case/participants		197/631	157/631	120/631	98/631
	Crude		1.00 (Ref.)	1.06 (0.61–1.83)	0.53 (0.33–0.85)*	0.34 (0.23–0.52)*
	Model 1		1.00 (Ref.)	1.53 (0.81–2.90)	0.83 (0.50–1.37)	0.59 (0.38–0.92)*
DSST	Case/participants		233/631	152/631	131/631	95/631
	Crude		1.00 (Ref.)	0.36 (0.25–0.52)	0.41 (0.29–0.57)*	0.21 (0.14–0.30)*
	Model 1		1.00 (Ref.)	0.48 (0.30–0.74)*	0.72 (0.46–1.13)	0.44 (0.29–0.65)*

Model 1 adjusted for sex, race, educational level, marital status, BMI, work physical activity, recreational physical activity, poverty-income ratio, smoking status, alcohol consumption, energy (continuous), hypertension, diabetes, and stroke.
Ref. refers to the reference value (Q1).
**p < 0.05.*

of participants was recalculated, and then regression analysis was carried out. When the *p*-value < 0.05, the results were considered statistically significant. Stata 15.0 (Stata Corp., College Station, TX, United States) was used to perform the primary statistical analysis.

RESULTS

Participant's Characteristics

For all cognitive tests, the low cognitive performance group had a lower education level, poverty-income ratio, recreational activity, total energy intake, dietary VK intake, and a higher prevalence of diabetes. For the CERAD W-L and DSST, the low cognition group had lower work physical activity level. For DSST, the low cognitive function group had a high prevalence of hypertension. For the AFT and DSST, the low cognition group had a high prevalence of stroke. The characteristics of dietary VK intake participants classified by cognitive function were shown in **Table 3**.

Association Between Dietary Vitamin K and Cognition

In the crude model, compared with the lowest dietary VK intake group, the OR (95% CI) of low CERAD W-L score for the quartile two intake group was 0.61 (0.45–0.82), for the quartile three intake group was 0.48 (0.32–0.72), and for the highest intake group was 0.27 (0.17–0.42), respectively. Compared with the lowest dietary VK intake group, the OR (95% CI) of low AFT score for the quartile three intake group was 0.53 (0.33–0.85), and for the highest intake group was 0.34 (0.23–0.52). Compared with the lowest dietary VK intake group, the OR (95% CI) of low DSST score for the quartile three intake group was 0.41 (0.29–0.57), and for the highest intake group was 0.21 (0.14–0.30).

In multi-adjusted model (model 1), compared with the lowest dietary VK intake group, the OR (95% CI) of low CERAD W-L score for the quartile two intake group was 0.67 (0.49–0.91), for the quartile three intake group was 0.63 (0.43–0.91), and for the highest intake group was 0.39 (0.26–0.60), respectively.

TABLE 5 | Weighted odds ratios (95% confidence intervals) for scores on CERAD W-L, AFT and DSST across dietary VK intake, stratified by sex, NHANES 2011–2014 (*N* = 2524).

	CERAD W-L	Crude	Model 1	AFT	Crude	Model 1	DSST	Crude	Model 1
	Case/ participants			Case/ participants			Case/ participants		
Male	355/1217			273/1217			342/1217		
Q1 (≤47.55)		1.00 (Ref.)	1.00 (Ref.)		1.00 (Ref.)	1.00 (Ref.)		1.00 (Ref.)	1.00 (Ref.)
Q2 (47.55 to ≤80.95)		0.87 (0.61–1.23)	1.20 (0.79–1.81)		1.43 (0.75–2.74)	2.32 (0.96–5.60)		0.35 (0.22–0.56)*	0.62 (0.31–1.23)
Q3 (80.95 to ≤136.6)		0.56 (0.36–0.87)*	0.85 (0.52–1.39)		0.74 (0.37–1.50)	1.19 (0.56–2.51)		0.50 (0.30–0.83)*	1.23 (0.63–2.37)
Q4 (>136.6)		0.39 (0.22–0.70)*	0.68 (0.35–1.30)		0.28 (0.14–0.55)*	0.43 (0.19–0.96)*		0.22 (0.13–0.36)*	0.55 (0.25–1.22)
Female	230/1307			299/1307			269/1307		
Q1 (≤47.55)		1.00 (Ref.)	1.00 (Ref.)		1.00 (Ref.)	1.00 (Ref.)		1.00 (Ref.)	1.00 (Ref.)
Q2 (47.55 to ≤80.95)		0.35 (0.22–0.56)*	0.38 (0.23–0.63)*		0.76 (0.40–1.44)	1.04 (0.50–2.15)		0.36 (0.21–0.64)*	0.43 (0.23–0.81)*
Q3 (80.95 to ≤136.6)		0.40 (0.21–0.77)*	0.52 (0.31–0.89)*		0.38 (0.19–0.75)*	0.58 (0.30–1.13)		0.33 (0.18–0.58)*	0.48 (0.22–1.06)
Q4 (>136.6)		0.17 (0.10–0.30)*	0.27 (0.15–0.48)*		0.39 (0.22–0.69)*	0.78 (0.45–1.35)		0.20 (0.12–0.35)*	0.42 (0.23–0.76)*

Model 1 adjusted for race, educational level, marital status, BMI, work physical activity, recreational physical activity, poverty-income ratio, smoking status, alcohol consumption, energy (continuous), hypertension, diabetes and stroke.
Ref. refers to the reference value (Q1).
**p < 0.05.*

Compared with the lowest dietary VK intake group, the OR (95% CI) of low AFT score for the highest intake group was 0.59 (0.38–0.92). Compared with the lowest dietary VK intake group, the OR (95% CI) of low DSST score for the quartile two intake group was 0.48 (0.30–0.74), and for the highest intake group was 0.44 (0.29–0.65). The joint test of the effect for the multiple categorical variables was used, and the dietary VK intake was correlated with low CERAD W-L score, with an OR value of 0.995, $p = 0.000$. For low AFT score, the OR value was 0.996, $p = 0.001$, and for low DSST score, OR value was 0.997, $p = 0.006$. The results were shown in **Table 4**.

In sensitivity analysis, after excluding 218 participants who took VK antagonists, the association of dietary VK intake with low cognitive performance was still significant. Detailed information was shown in **Supplementary Table 1**. After readjusting VK intake according to total energy intake, dietary VK intake was negatively correlated with low CERAD W-L and AFT scores. Detailed information was shown in **Supplementary Table 2**.

In stratified analysis by sex, dietary VK intake was inversely associated with low cognitive function. In model 1, for females, compared with the lowest dietary VK intake group, the OR (95% CI) of low CERAD W-L score for the quartile two intake group was 0.38 (0.23–0.63), for the quartile three intake group was 0.52 (0.31–0.89), and for the highest intake group was 0.27 (0.15–0.48), respectively. For males, compared with the lowest dietary VK intake group, the OR (95% CI) of low AFT score for the highest intake group was 0.43 (0.19–0.96). For females, compared with the lowest dietary VK intake group, the OR (95% CI) of low DSST score for the quartile two intake group was 0.43 (0.23–0.81), and for the highest intake group was 0.42 (0.23–0.76). The results were shown in **Table 5**.

TABLE 6 | Association between VK intake derived from vegetables and cognitive performance.

Dietary VK intake derived from vegetables (mcg/day)	Odds ratio	95% CI	*p*-value
CERAD W-L			
Q1 (<16.70)	1	1	
Q2 (16.70–40.85)	1.033	0.63–1.70	0.896
Q3 (40.85–99.45)	1.000	0.56–1.79	0.999
Q4 (>99.45)	0.604	0.37–0.99	0.047
AFT			
Q1 (<16.70)	1	1	
Q2 (16.70–40.85)	0.629	0.32–1.24	0.174
Q3 (40.85–99.45)	0.710	0.35–1.42	0.323
Q4 (>99.45)	0.629	0.37–1.06	0.081
DSST			
Q1 (<16.70)	1	1	
Q2 (16.70–40.85)	1.248	0.73–2.12	0.403
Q3 (40.85–99.45)	1.269	0.68–2.38	0.447
Q4 (>99.45)	0.819	0.44–1.54	0.524

TABLE 7 | Association between VK intake derived from grain and cognitive performance.

Dietary VK intake derived from grain (mcg/day)	Odds ratio	95% CI	*p*-value
CERAD W-L			
Q1 (<7.90)	1	1	
Q2 (7.90–16.05)	1.581	0.94–2.66	0.082
Q3 (16.05–28.85)	1.112	0.50–2.46	0.788
Q4 (>28.85)	0.992	0.48–2.04	0.981
AFT			
Q1 (<7.90)	1	1	
Q2 (7.90–16.05)	0.707	0.42–1.18	0.178
Q3 (16.05–28.85)	0.752	0.49–1.14	0.176
Q4 (>28.85)	0.808	0.53–1.23	0.307
DSST			
Q1 (<7.90)	1	1	
Q2 (7.90–16.05)	0.939	0.56–1.58	0.809
Q3 (16.05–28.85)	0.591	0.31–1.12	0.103
Q4 (>28.85)	0.868	0.39–1.95	0.723

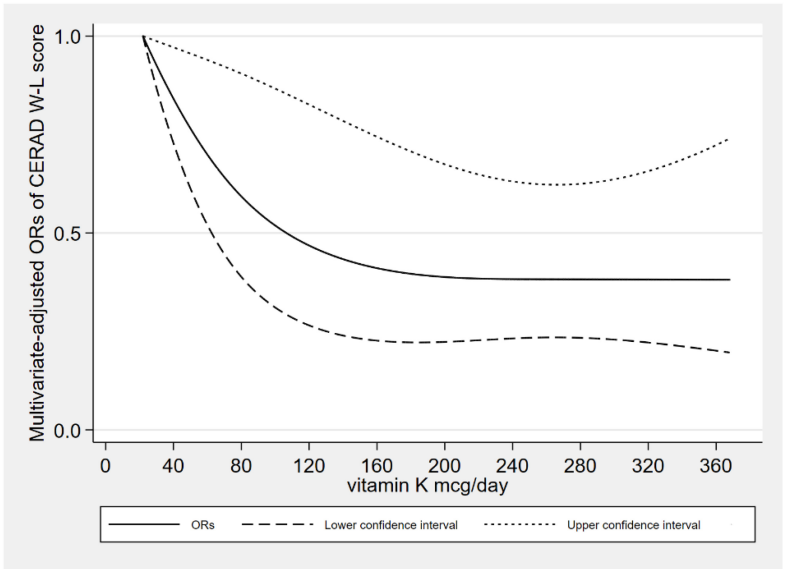


FIGURE 2 | Dose–response relationship between dietary VK intake and low CERAD W-L score.

Relationships Between Dietary Vitamin K Intake Derived From Different Sources (Vegetables, Grain) and the Risk of Low Cognitive Performance

The association of dietary VK intake derived from vegetables with low cognitive performance risk was shown in **Table 6**. In a multiple-adjusted model, dietary VK intake was correlated with low CERAD W-L score. Compared with the lowest dietary VK intake derived from vegetables group, the OR (95% CI) of low CERAD W-L score for the highest intake group was 0.60 (0.37–0.99). The joint test of the effect for the multiple categorical variables was used, with an OR value of 0.997, $p = 0.032$. We did not find the relationship between dietary VK intake from grains and the risk of low cognitive ability, and the results were shown in **Table 7**.

Dose–Response Relationships

Figure 2 showed the L-shaped dose–response relationship between dietary VK intake and low CERAD W-L scores ($p_{\text{fornon-linearity}} = 0.121$). It reached a plateau, when the dietary VK intake was higher than 215 mcg/day. As for dietary VK intake and low AFT scores, we found a linear dose–response relationship ($p_{\text{fornon-linearity}} = 0.664$). The relationship was significant when the dietary VK intake was above 110 mcg/day (**Figure 3**). For the low DSST score, we found a linear dose–response relationship ($p_{\text{fornon-linearity}} = 0.325$), and when dietary VK intake was lower than 163 mcg/day

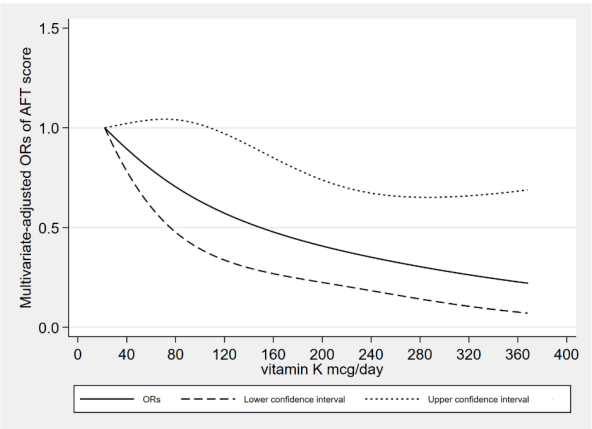


FIGURE 3 | Dose–response relationship between dietary VK intake and low AFT score.

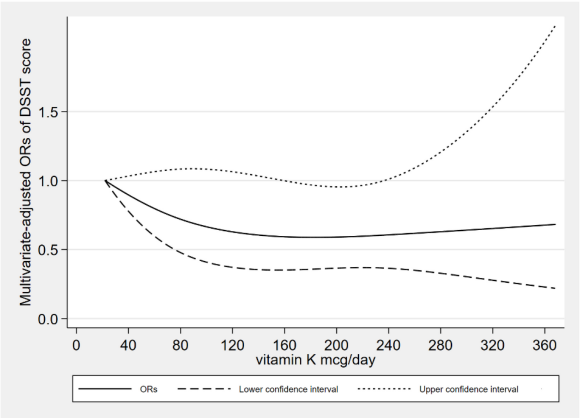


FIGURE 4 | Dose–response relationship between dietary VK intake and low DSST score.

or higher than 234 mcg/day, the relationship was no longer significant (**Figure 4**).

DISCUSSION

There was a negative correlation between the low cognitive risk and dietary VK intake. After adjusting for many confounding factors, the correlation was still significant. For the low CERAD W-L score, we found an L-shaped dose–response relationship. This dose–response relationship means that when the plateau is reached, even if the dietary intake of VK is increased, the protective effects on cognitive function will not be increased. As for low AFT and DSST scores, we found a linear dose–response relationship, which means the prevalence of low cognitive performance decreased with increasing dietary VK intake. According to the 2020–2025 American dietary guidelines (30), the adequate intake of VK was 120 mcg for elderly males and 90 mcg for females. Of the 2524 participants, only 947 (37.52%) met the criteria.

In stratified analysis by sex, it was significant in the AFT for men and was significant in the CERAD W-L and DSST among women. We also found that the dietary VK intake derived from vegetables was negatively correlated with the low CERAD W-L score. We did not find an association between VK intake from grains and the risk of low cognitive ability, possibly due to low VK content in grains or insufficient sample size. In the sensitivity analysis, after excluding the participants who took VK antagonists, the effect of VK antagonist on the results was ruled out, and the results were still significant. According to the total energy intake, we recalculated participants' VK intake and found that dietary VK intake was still negatively correlated with low CERAD W-L and AFT scores.

Previous studies partially explored the relationship between dietary VK intake and cognition. Some studies analyzed the clinical research evidence between VK and cognition (10). In addition, a survey conducted in France, the subjects were 160 hospitalized or seen in consultation patients (the average age was 82 years old), and the results showed that VK intake was positively correlated with the Memory Complaint Questionnaire scores (31). Another survey of 192 hospitalized or treated patients (the average age was 83 years old) in France also found higher dietary VK intake was related to better Mini-Mental State Examination scores of the elderly (13). A study of 156 elderly people (the average age was 78 years old) in Ireland showed that dietary VK was a predictor of good cognition (14). A Canadian study was conducted in 31 patients with Alzheimer's disease and 31 healthy controls (average 78 years old) found that the intake of VK in patients with Alzheimer's disease was lower (12). Although different dimensions of cognitive function had been evaluated, our study was consistent with previous results, and both of them found that there was an association between VK and cognitive function. A study among 599 participants (55–65 years old) showed that the association between VK status and cognition was not significant (15). This was inconsistent with our results, but we used different indicators of VK status, and our study participants were older. Such controversies may be due to the various age distribution, study design, geographical region, and methods of cognitive assessment.

There were several possible mechanisms of the relationship between dietary VK and cognitive ability (10, 32). VK played an important role in neurodegenerative diseases. VK participated in the synthesis of sphingolipids, which were the main components of myelin sheaths and were involved in the proliferation and differentiation of neurons (33). Studies showed that two VK-dependent proteins, named growth arrest-specific 6 (Gas6) and S protein, might affect the cognitive process (9). In addition, VK had anti-inflammatory activity and provided antioxidant stress protection (11).

This research had several advantages. Firstly, the dose–response relationship between dietary VK intake and cognitive performance was explored. Secondly, we used a large sample of representative American elderly data, and NHANES had a high-quality data collection method. Thirdly, we adjusted many confounding factors by referring to previous articles and made sensitivity analysis. Finally, we explored the dietary intake of VK from vegetable and grain sources.

However, this research also had some limitations. Firstly, due to the cross-sectional design, we were unable to determine a causal relationship between dietary VK intake and low cognitive risk. Second, the data obtained through 24-h dietary recall might have recall bias. Thirdly, we were unable to estimate endogenous VK production, considering only dietary VK. Finally, we can't rule out the possible influence of other unadjusted confounding factors on the observation results.

CONCLUSION

In the study, we explored the relationship between dietary VK intake and cognition. Dietary VK intake and VK intake from vegetables were inversely related to the risk of low cognitive performance of the elderly. We need further prospective studies to explore their relationship.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.cdc.gov/nchs/nhanes/index.htm.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Center for Health Statistics Research Ethics Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AW and MZ: conceptualization and data curation. MZ and TZ: methodology. AW and JL: writing – original draft. MZ and

DZ: writing – review and editing. DZ: supervision. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

Thanks to all individuals at the National Center for Health Statistics of the Centers for Disease Control and Prevention who collected data and making the datasets of NHANES available on the website.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.900887/full#supplementary-material

REFERENCES

1. McGrattan AM, McGuinness B, McKinley MC, Kee F, Passmore P, Woodside JV, et al. Diet and inflammation in cognitive ageing and Alzheimer's disease. *Curr Nutr Rep.* (2019) 8:53–65. doi: 10.1007/s13668-019-0271-4
2. Wang W, Li W, Jiang W, Lin H, Wu Y, Wen Y, et al. Genome-wide DNA methylation analysis of cognitive function in middle and old-aged Chinese monozygotic twins. *J Psychiatr Res.* (2021) 136:571–80. doi: 10.1016/j.jpsychires.2020.10.031
3. Wang R, Wang W, Hu P, Zhang R, Dong X, Zhang D. Association of dietary vitamin D intake, serum $25(OH)D_3$, $25(OH)D_2$ with cognitive performance in the elderly. *Nutrients.* (2021) 13:3089. doi: 10.3390/nu13093089
4. Peeri NC, Egan KM, Chai W, Tao MH. Association of magnesium intake and vitamin D status with cognitive function in older adults: an analysis of US national health and nutrition examination survey (NHANES) 2011 to 2014. *Eur J Nutr.* (2021) 60:465–74. doi: 10.1007/s00394-020-02267-4
5. Li S, Sun W, Zhang D. Association of zinc, iron, copper, and selenium intakes with low cognitive performance in older adults: a cross-sectional study from national health and nutrition examination survey (NHANES). *J Alzheimers Dis.* (2019) 72:1145–57. doi: 10.3233/JAD-190263
6. Ostojic SM, Korovljev D, Stajer V. Dietary creatine and cognitive function in U.S. adults aged 60 years and over. *Aging Clin Exp Res.* (2021) 33:3269–74. doi: 10.1007/s40520-021-01857-4
7. Dong X, Li S, Chen J, Li Y, Wu Y, Zhang D. Association of dietary ω-3 and ω-6 fatty acids intake with cognitive performance in older adults: national health and nutrition examination survey (NHANES) 2011-2014. *Nutr J.* (2020) 19:25. doi: 10.1186/s12937-020-00547-7
8. Denisova NA, Booth SL. Vitamin K and sphingolipid metabolism: evidence to date. *Nutr Rev.* (2005) 63:111–21. doi: 10.1111/j.1753-4887.2005.tb00129.x
9. Ferland G. Vitamin K, an emerging nutrient in brain function. *Biofactors.* (2012) 38:151–7. doi: 10.1002/biof.1004
10. Ferland G. Vitamin K and the nervous system: an overview of its actions. *Adv Nutr.* (2012) 3:204–12. doi: 10.3945/an.111.001784
11. Ferland G. Vitamin K and brain function. *Semin Thromb Hemost.* (2013) 39:849–55.
12. Presse N, Shatenstein B, Kergoat MJ, Ferland G. Low vitamin K intakes in community-dwelling elders at an early stage of Alzheimer's disease. *J Am Diet Assoc.* (2008) 108:2095–9. doi: 10.1016/j.jada.2008.09.013
13. Chouet J, Ferland G, Féart C, Rolland Y, Presse N, Boucher K, et al. Dietary vitamin K intake is associated with cognition and behaviour among geriatric patients: the CLIP study. *Nutrients.* (2015) 7:6739–50. doi: 10.3390/nu7085306
14. Kiely A, Ferland G, Ouliass B, O'Toole PW, Purtill H, O'Connor EM. Vitamin K status and inflammation are associated with cognition in older Irish adults. *Nutr Neurosci.* (2020) 23:591–9. doi: 10.1080/1028415X.2018.1536411
15. van den Heuvel EG, van Schoor NM, Vermeer C, Zwijsen RM, den Heijer M, Comijs HC. Vitamin K status is not associated with cognitive decline in middle aged adults. *J Nutr Health Aging.* (2015) 19:908–12. doi: 10.1007/s12603-015-0579-8
16. Centers for Disease Control and Prevention [CDC]. *About the National Health and Nutrition Examination Survey*. (2017). Available online at: https://www.cdc.gov/nchs/nhanes/about_nhanes.htm (accessed February 20, 2022).
17. Abufaraj M, Xu T, Cao C, Waldhoer T, Seitz C, D'Andrea D, et al. Prevalence and trends in kidney stone among adults in the USA: analyses of national health and nutrition examination survey 2007-2018 data. *Eur Urol Focus.* (2021) 7:1468–75. doi: 10.1016/j.euf.2020.08.011
18. Centers for Disease Control and Prevention [CDC]. *Dietary Interview - Total Nutrient Intakes, First Day, 2013-2014*. (2016). Available online at: https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/DR1TOT_H.htm (accessed February 20, 2022).
19. Centers for Disease Control and Prevention [CDC]. *NHANES 2013-2014 Procedure Manuals*. (2014) Atlanta, GA: Centers for Disease Control and Prevention.
20. Siener R. Nutrition and kidney stone disease. *Nutrients.* (2021) 13:1917.
21. United States Department of Agriculture [USDA]. *USDA Food and Nutrient Database for Dietary Studies*. (2021). Available online at: https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/food-surveys-research-group/docs/fndds/
22. Dong X, Jiang H, Li S, Zhang D. Low serum testosterone concentrations are associated with poor cognitive performance in older men but not women. *Front Aging Neurosci.* (2021) 13:712237. doi: 10.3389/fnagi.2021.712237
23. Centers for Disease Control and Prevention [CDC]. *Cognitive Functioning*. (2017). Available online at: https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/CFQ_H.htm (accessed February 20, 2022).
24. Sotaniemi M, Pulliainen V, Hokkanen L, Pirttilä T, Hallikainen I, Soininen H, et al. CERAD-neuropsychological battery in screening mild Alzheimer's disease. *Acta Neurol Scand.* (2012) 125:16–23. doi: 10.1111/j.1600-0404.2010.01459.x
25. Sebaldt R, Dalziel W, Massoud F, Tanguay A, Ward R, Thabane L, et al. Detection of cognitive impairment and dementia using the animal fluency test: the DECIDE study. *Can J Neurol Sci.* (2009) 36:599–604. doi: 10.1017/s0317167100008106
26. Rosano C, Perera S, Inzitari M, Newman AB, Longstreth WT, Studenski S. Digit symbol substitution test and future clinical and subclinical disorders of cognition, mobility and mood in older adults. *Age Ageing.* (2016) 45:688–95. doi: 10.1093/ageing/afw116
27. Dong X, Li S, Sun J, Li Y, Zhang D. Association of coffee, decaffeinated coffee and caffeine intake from coffee with cognitive performance in older adults: national health and nutrition examination survey (NHANES) 2011-2014. *Nutrients.* (2020) 12:840. doi: 10.3390/nu12030840
28. Wang X, Li X, Xing Y, Wang W, Li S, Zhang D, et al. Threshold effects of total copper intake on cognitive function in US older adults and the moderating effect of fat and saturated fatty acid intake. *J Acad Nutr Diet.* (2021) 121:2429–42. doi: 10.1016/j.jand.2021.06.002
29. Centers for Disease Control and Prevention [CDC]. *NHANES Tutorials Module 3 Weighting*. (2014). Available online at: https://wwwn.cdc.gov/nchs/nhanes/tutorials/module3.aspx (accessed February 20, 2022).
30. United States Department of Health and Human Services [USDHHS]. *Dietary Guidelines for Americans*. (2020). Available online at: https://www.dietaryguidelines.gov/ (accessed February 20, 2022).
31. Soutif-Veillon A, Ferland G, Rolland Y, Presse N, Boucher K, Féart C, et al. Increased dietary vitamin K intake is associated with less severe subjective

memory complaint among older adults. *Maturitas.* (2016) 93:131–6. doi: 10.1016/j.maturitas.2016.02.004
32. Popa DS, Bigman G, Rusu ME. The role of vitamin K in humans: implication in aging and age-associated diseases. *Antioxidants.* (2021) 10:566. doi: 10.3390/antiox10040566
33. Alessenko AV, Albi E. Exploring sphingolipid implications in neurodegeneration. *Front Neurol.* (2020) 11:437. doi: 10.3389/fneur.2020.00437

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Wang, Zhao, Luo, Zhang and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 19 July 2022
doi: 10.3389/fnut.2022.874118

Diet Quality and Cognitive Performance in Children Born Very Low Birth Weight

Julie Sato[1,2,3]*, *Meghan McGee*[4,5], *Nicole Bando*[4,5], *Nicole Law*[5], *Sharon Unger*[4,6,7,8] and *Deborah L. O'Connor*[4,5,7]

[1] Department of Diagnostic Imaging, The Hospital for Sick Children, Toronto, ON, Canada, [2] Department of Psychology, University of Toronto, Toronto, ON, Canada, [3] Neuroscience and Mental Health Program, The Hospital for Sick Children Research Institute, Toronto, ON, Canada, [4] Department of Nutritional Sciences, University of Toronto, Toronto, ON, Canada, [5] Department of Translational Medicine, The Hospital for Sick Children Research Institute, Toronto, ON, Canada, [6] Department of Paediatrics, University of Toronto, Toronto, ON, Canada, [7] Department of Paediatrics, Sinai Health, Toronto, ON, Canada, [8] Division of Neonatology, The Hospital for Sick Children, Toronto, ON, Canada

Children born very low birth weight (VLBW, <1,500 g) are at high risk for cognitive and academic difficulties later in life. Although early nutrition (e.g., breastfeeding) is positively correlated with IQ in children born VLBW, the association between dietary intake in childhood and cognitive performance is unknown. Thus, our study is the first to investigate the relationship between diet quality, as measured by the Healthy Eating Index-2010 (HEI-2010) and cognitive performance in a Canadian cohort of 5-year-old children born VLBW (n = 158; 47% female). Diet quality was measured using two 24-h diet recalls obtained from parents and cognitive performance was assessed using the Wechsler Preschool and Primary Scale of Intelligence-IV (WPPSI-IV). To account for additional sociodemographic factors that could influence neurodevelopment, linear regression analyses were adjusted for sex, household income above/below the poverty line, maternal education, birth weight and breastfeeding duration. Mean ± SD HEI-2010 score was 58.2 ± 12.4, with most children (67%) having diets in "need of improvement" (scores 51–80). HEI-2010 scores were not significantly associated with IQ or any other WPPSI-IV composite score. Significant predictors of IQ in our model were birth weight, sex, and maternal education. Our findings emphasize the important role of maternal education and other sociodemographic factors on neurodevelopment in children born VLBW. Further, despite not finding any significant association between HEI-2010 scores and IQ, our results highlight the need to improve diet quality in young children born VLBW. Further research is needed to confirm the impact of diet quality on cognitive performance in this vulnerable population.

Keywords: preterm, very low birth weight, diet quality, cognition, children

OPEN ACCESS

Edited by:
Maria G. Grammatikopoulou, International Hellenic University, Greece

Reviewed by:
Rosaura Leis, University of Santiago de Compostela, Spain
Alan Emond, University of Bristol, United Kingdom

***Correspondence:**
Julie Sato
julie.sato@sickkids.ca

Specialty section:
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

Received: *11 February 2022*
Accepted: *16 June 2022*
Published: *19 July 2022*

Citation:
Sato J, McGee M, Bando N, Law N, Unger S and O'Connor DL (2022) Diet Quality and Cognitive Performance in Children Born Very Low Birth Weight. Front. Nutr. 9:874118. doi: 10.3389/fnut.2022.874118

INTRODUCTION

Very low birth weight (VLBW, <1,500 g) infants are born early in the third trimester of pregnancy, a critical period of brain development, putting them at higher risk of cognitive impairments and academic underachievement later in life (1–3). Convergent findings support overall lower academic performance and IQ scores in preterm [<37 weeks gestational age (GA)] compared to full-term

children, with one meta-analysis reporting almost a 12-point difference in IQ among school-aged children born preterm, independent of socioeconomic status [SES; (4)]. Several factors, as a consequence of preterm birth, increase the risk of sub-optimal neurodevelopment, including inadequate nutrient intake and morbidity during initial hospitalization [e.g., sepsis, necrotizing enterocolitis, chronic lung disease; (5–7)]. After hospital discharge, SES factors play a central role in the neurodevelopment of preterm infants, but other factors such as childhood nutrition have not yet been explored (8, 9).

While the role of post-discharge diet on neurodevelopment in infants born VLBW is unknown, evidence from studies of full-term infants suggests that a moderate association exists between diet and academic outcomes (8). In full-term children, different components of the diet (e.g., fruits and vegetables, breakfast consumption) are associated with cognition (8, 10, 11). In one large Canadian cohort (n = 5,200) of grade 5 students, higher diet quality scores, assessed by the Diet Quality Index-International and Healthy Eating Index (HEI), were associated with a reduced likelihood of failing school literacy assessments (12). More recently, a cross-sectional analysis in a Spanish cohort of children and adolescents (n = 1,371, 8–18 years) found better adherence to a Mediterranean diet, as measured by the KIDMED diet quality index, was associated with higher academic performance (13). A Mediterranean diet is typically rich in vegetables, fruit, nuts, fish and olive oil. Importantly, these findings remained significant after adjusting for various confounders including maternal education, sex, age, birth weight and GA. Infants born VLBW, however, were not included in these studies.

In preterm and VLBW children, the only studies investigating the relation between diet and cognition have focused on nutrient intake during early infancy. In one study, greater breastmilk intake during initial hospitalization in preterm infants (<37 weeks GA) was associated with higher verbal IQ in adolescence [n = 50, 13–19 years; (14)]. In boys, breastmilk intake was also linked with higher IQ scores, as well as total brain and white matter volume. Importantly, in the seminal meta-analytic paper by Anderson et al. (1), the authors found that low birth weight infants who were breastfed derived a greater cognitive benefit than normal birth weight infants, even after adjusting for covariates such as SES or maternal education. More recent systematic reviews and a cluster-randomized control trial also support the positive association between breastfeeding and neurodevelopment that persists after adjustment for maternal IQ in both full-term and preterm children (15, 16).

Given the high risk of academic underachievement and cognitive difficulties in children born VLBW, elucidating modifiable factors that impact cognitive outcomes in this population is an essential step for informing supports and strategies for families and educators (17, 18). Further, preschool age represents a critical time period to investigate the association between diet and cognitive development, as dietary preferences are established early in childhood (19), and cognitive ability at this age is associated with later academic achievement in full-term children (20, 21). Thus, our study estimated diet quality in children born VLBW using the HEI-2010 (22, 23) to evaluate associations with cognitive performance. In line with the full-term literature, we hypothesized that higher HEI-2010 scores (reflecting closer conformance with dietary guidelines) would correspond with higher IQ scores.

METHODS

Participants

All surviving children and their families who consented to the original randomized trial were approached for the present follow-up study when the children were 5 years of age. The feeding intervention, study protocol and outcomes have been published (24–27). Briefly, VLBW infants were enrolled between October 2010 and December 2012, from four tertiary neonatal intensive care units in Ontario, Canada. Infants were eligible if their birth weight was <1,500 g, and if families consented within 4 days of birth. Exclusion criteria included chromosomal or congenital anomalies that could affect neurodevelopment. At the 5-year follow-up, written informed consent was obtained from parents and verbal assent from children.

Exposures: Diet Quality, Sociodemographic and Lifestyle Characteristics

A 24-h dietary recall was completed with parents during the study visit and parents were contacted 1 week later to complete a second recall by phone or email as described in detail previously (28). Briefly, the 24-h dietary recall asks parents to detail everything their child ate and drank in the previous day using the Automated Self-Administered 24-h (ASA24) Dietary Assessment tool, Canadian version (2016). The HEI-2010, a validated measure of diet quality in both children and adults (22, 23), was used to assess diet quality. Importantly, this tool considers diet quality distinctly from the quantity of food consumed (i.e., food groups are considered per 1000 kcal consumed).

The HEI-2010, created by the U.S. Department of Agriculture, consists of 12 components: nine adequacy components (that should be consumed in adequate amounts for optimal health) and three moderation components (that should be consumed in limited amounts for optimal health; see **Supplementary Tables 1, 2** for the HEI-2010 Components and Standards for Scoring). Overall diet quality is reflected in the total index score, which is the sum of the 12 dietary components. HEI-2010 scores range from 0 to 100, with higher scores reflecting greater adherence to dietary requirements and better diet quality. Diets with HEI-2010 scores >80 are categorized as being "healthy", with scores between 51 and 80 reflecting diets that "need improvement", and scores <51 considered "poor" diets (22). HEI-2010 scores were calculated from the two non-consecutive 24-h dietary recalls using the multivariate Markov Chain Monte Carlo approach, which adjusts for weekday vs. weekend, season and recall sequence. A validated scoring algorithm was used to calculate individual HEI-2010 scores.

Outcome Measures: Cognitive Performance

The primary outcome measure for this study was cognitive performance assessed using Full-Scale IQ on the Wechsler Preschool and Primary Scale of Intelligence-IV [WPPSI-IV; (29)]; the version based on Canadian norms. The WPPSI-IV is a standardized measure of intelligence for children between 2 years and 6 months through to 7 years and 7 months of age. Secondary outcome measures included the following composite scores of the WPPSI-IV: verbal comprehension index (VCI), vocabulary acquisition index (VAI), visual spatial index (VSI), fluid reasoning index (FRI), processing speed index (PSI), and working memory index (WMI). All WPPSI-IV composite scores are calculated as standard scores with a mean of 100 and a standard deviation of 15, with "low average" scores defined as scores <90 (29). In line with other experts in the field, children who were unable to complete the WPPSI-IV assessment due to severe disability or who performed below the threshold of the test, were assigned a score of 49 (24, 30–32).

Demographic and Clinical Variables

Demographic and clinical factors including sex, birth weight, household income above/below the poverty line, maternal education, maternal ethnicity, and duration of breastfeeding were collected as described in detail previously (24). Briefly, sex and birth weight were collected from medical records and maternal education and ethnicity, as well as household income were reported from standardized parent questionnaires at birth. Maternal education was dichotomized as mothers having a university degree or not and household income above/below the poverty line was based on 2006 Statistics Canada family size-adjusted cut-off values (33). Duration of breastfeeding was calculated as the last recorded date that any breastmilk was provided, up to 18 months corrected age. During initial hospitalization, daily volumes of both parenteral and enteral nutrition were prospectively recorded in all infants. Enteral feed type during the intervention was characterized as receiving >50% of enteral feeds as either donor milk, preterm formula or mother's breastmilk.

Statistical Analyses

Normality of data were confirmed visually using histogram distributions and analytically using the Shapiro-Wilk test. Normally distributed variables were described as mean (standard deviation or 95% confidence interval [CI]) or median (interquartile range) when not normally distributed. Independent samples *t*-tests were used to compare differences between sexes for continuous outcome variables, and categorical variables were compared using chi-square tests.

Linear regression models were computed for primary and secondary outcomes. The association between HEI-2010 scores and WPPSI-IV composite scores was analyzed using linear regression models adjusted for sex, birth weight, breastfeeding duration, income above/below the poverty line, and maternal education (university degree or not). Interactions between exposure variables and sex were assessed in linear regression models to test whether the association between diet quality and IQ varied by sex. If the interaction was not statistically significant, it was removed from the model and the analyses were re-run. Multicollinearity was assessed using variance inflation factors with a cut-off value >10 considered to represent multicollinearity. Statistical analyses were performed in R 3.5.1 (34) using the rms statistical package. All hypothesis tests were two-sided and considered statistically significant if *p*-values were <0.05.

TABLE 1 | Birth and parental baseline characteristics of children and families who participated in follow-up at 5 years.

Characteristic	*n*	Mean (SD) or *n* (%)
Sex		
Female	158	74 (47)
Male		84 (53)
Birth weight, grams	158	1,013 (264)
Birth gestational age, weeks	158	28 (3)
Mother's ethnicity		
European	156	68 (44)
Asian		25 (16)
Middle Eastern or South Asian		29 (19)
Mixed or other		34 (22)
Mother's education		
None/high school diploma	156	31 (20)
College/vocational diploma		41 (26)
Baccalaureate		49 (31)
Post baccalaureate		35 (22)
Family living below poverty line		
No	149	120 (81)
Yes		29 (19)
Enteral feed type during initial hospitalization		
>50% donor milk	158	22 (14)
>50% preterm formula		20 (13)
>50% mother's breastmilk		116 (73)
Breastfeeding duration, days	158	223 (181)

Sensitivity Analyses

To ensure that imputation of scores did not impact study findings, sensitivity analyses were performed by excluding children who were assigned a score of 49. In addition, in-hospital enteral feed type (receiving >50% mother's breastmilk, donor milk, or preterm formula) was included as a covariate in multivariable models to test for the effect of supplemental milk provided in-hospital. We also tested the interaction between enteral feed type and maternal education (university degree or not) in linear regression models. If the interaction was not statistically significant, it was removed from the model.

RESULTS

Participant Characteristics

One hundred and fifty-eight of 316 (50% follow-up rate) eligible children born VLBW participated in the 5-year follow-up [mean (SD) age: 5.7 (0.2) years]. Birth and parental baseline

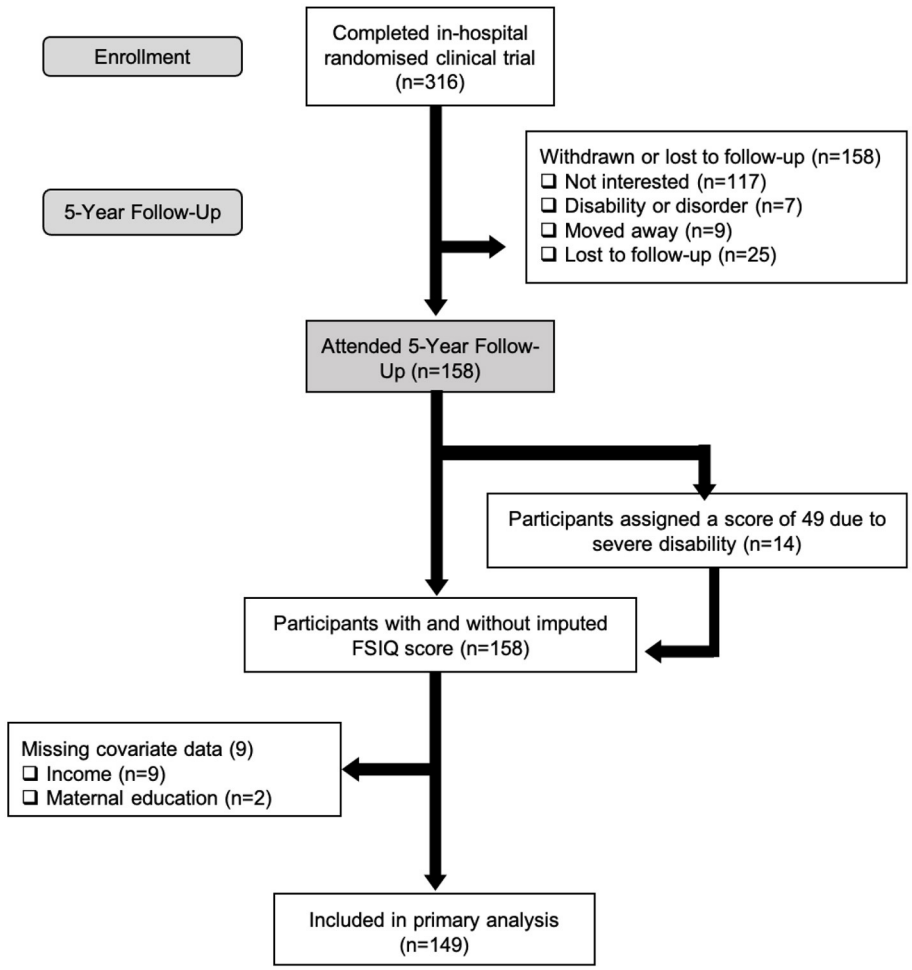


FIGURE 1 | Flow of VLBW participants through the randomized clinical trial from initial enrollment to inclusion in primary analysis.

characteristics are summarized in **Table 1**, including sex, birth weight, birth GA, mother's ethnicity, mother's education, family income, enteral feed type, and breastfeeding duration. Mean (SD) birth weight and GA were 1013 (264) g and 28 (3) weeks, respectively. Fifty percent of mothers in our sample had an education level of university or above and 20% had incomes below the poverty line. As reported previously, except for maternal education, no statistically significant differences in baseline characteristics were found between children who participated in the 5-year follow-up and those who did not (28). Mothers of children who participated in the follow-up study were more educated than mothers of children who did not ($p = 0.02$).

Cognitive Assessments

Complete WPPSI-IV data were obtained for 91.1% (144 of 158) of children born VLBW. Fourteen children who attended the follow-up visits were unable to complete the WPPSI-IV (see **Figure 1** for participant flowchart). In these cases, an IQ score of 49 (the lower limit of IQ) was assigned. The mean (SD) of IQ was 94.3 (20.4), which is within the average range of standard IQ classification (90–109). The mean (SD) for all WPPSI-IV composite scores are summarized in **Table 2**. Males had significantly lower scores for IQ, FRI, WMI, and PSI. In addition, more males had an IQ score below 90, which according to the WPPSI-IV manual (29) is indicative of low average or borderline level cognitive ability ($p = 0.006$).

Diet Quality

HEI-2010 diet quality scores ranged from 19.2 to 87.1, with a mean (SD) of 58.2 (12.4). As previously reported (28), most children had diet scores that fell within the "poor" (43/158 children) or "needs improvement" (106/158 children) diet quality categories. Only 6% of our cohort had HEI-2010 scores that were above 80, indicative of a good quality diet meeting recommended age-specific guidelines. There were no sex differences in HEI-2010 total scores.

TABLE 2 | WPPSI-IV composite scores for 5-year-old children born VLBW.

WPPSI-IV composite scores	All participants, mean (SD), range ($n = 158$)	Female, mean (SD), range ($n = 74$)	Male, mean (SD), range ($n = 84$)	*p*-value
Full-scale IQ	94.3 (20.4), 49–130	98.9 (18.8), 49–128	90.4 (21.1), 49–130	0.007*
VCI	96.1 (20.9), 49–138	98.9 (19.0), 49–138	93.5 (22.3), 49–133	0.056
VSI	94.1 (17.7), 49–138	95.5 (17.5), 49–124	92.8 (18.0), 49–132	0.298
FRI	93.1 (18.6), 49–133	97.8 (16.8), 49–131	89.0 (19.3), 49–133	0.002*
WMI	97.1 (19.4), 49–137	100.6 (17.7), 49–131	94.0 (20.4), 49–137	0.014*
PSI	91.2 (20.3), 49–126	95.7 (21.2), 49–126	87.2 (18.6), 49–123	$4.98 \times 10^{\wedge -4*}$
VAI	96.6 (19.2), 49–132	98.5 (18.1), 49–132	94.9 (20.0), 49–132	0.197
Low average scores <90, No./Total (%) Full scale IQ	55/158 (34.8)	17/74 (23.0)	38/84 (45.2)	0.006*

VCI, verbal comprehension index; VAI, vocabulary acquisition index; VSI, visual spatial index; FRI, fluid reasoning index; PSI, processing speed index; WMI, working memory index.
**Statistically significant between sexes.*

Association Between Exposure and Outcome Variables

Of the 158 children who were tested, 149 children were included in the primary analyses due to missing covariate data (**Figure 1**). HEI-2010 scores were not significantly associated with IQ scores (**Table 3**). However, statistically significant variables in our model were birth weight, sex, and maternal education. Every additional gram of birth weight was associated with a gain of 0.02 points in IQ (95% CI, 0.01–0.03). On average, males had IQ scores that were 7.7 points below females (95% CI, 1.5–13.8). The strongest predictor of IQ in our model was maternal education. Children whose mothers had a university degree were found to have an IQ of 9.1 points greater, or a 0.6 standard deviation difference, than children whose mothers were college-educated or had no post-secondary education (95% CI, 1.8–16.2). The interaction between HEI-2010 scores and sex were assessed in the adjusted linear regression model to test whether the association between diet quality and IQ varied by sex. This interaction term, however, was not significant ($p = 0.83$) and therefore removed from the model. Further, the associations between the different components of the HEI-2010 (i.e., total fruits, total vegetables, whole grains, etc.) with IQ were also investigated in adjusted linear regression models but revealed no significant associations (**Supplementary Table 3**).

Secondary outcome variables (other WPPSI-IV composite scores) were regressed on HEI-2010 scores and revealed non-significant associations ($p > 0.05$). These findings remained unchanged in a sensitivity analysis that excluded children who were unable to complete the assessments and who would have otherwise been assigned a score of 49 (**Supplementary Table 4**). In sensitivity analyses, in-hospital enteral feed type (e.g., >50% mother's breastmilk, donor milk, or preterm formula) was included in the General linear model (GLM) as a covariate but was not statistically significant.

DISCUSSION

Our findings demonstrate that diet quality at 5 years of age was not correlated with IQ or any other WPPSI-IV composite measures in children born VLBW. There are no comparable data in the preterm or VLBW populations, but our findings are inconsistent with the few studies who report a positive association between diet quality and cognition in full-term children, even after adjusting for a range of confounding factors (12, 35, 36). Unlike our findings, Khan et al. (36) in a cross-sectional study from the United States assessing diet quality using the HEI-2005 version, found positive associations with executive function in full-term children ($n = 65$, 7–9 years). Similarly, Haapala et al. (35) in a large Finnish cohort of full-term children ($n = 428$, aged 6–8 years), found that poor diet quality assessed using the Baltic Sea Diet Score, was associated in a dose-dependent manner with lower cognitive scores on the Raven's Colored Progressive Matrices; this relation was strongest for the boys. Similar to the HEI-2010, the Baltic Sea diet score assesses diet quality but is specific to Nordic dietary recommendations. Both full-term studies, however, did not directly measure IQ and were performed in older cohorts of children, which may explain why we did not find significant associations between diet quality and cognitive performance in our study (35, 36). Another reason for this discrepancy is the index of diet quality used in these studies, which makes comparison difficult. Further, with the exception of the previously mentioned studies (35, 36), few other studies have used validated measures of diet quality, such as the HEI-2010 in children. Thus, future studies should focus on investigating diet quality using validated tools to better understand its impact on cognition in young children.

In this cohort, the average HEI-2010 score was 58.2 out of 100, with the majority of children (94%) having diets in the "needs improvement" or "poor quality" range. Although diet quality scores were low in our sample of children born VLBW, they are similar to those reported in Khan et al.'s cohort of

TABLE 3 | Associations between diet quality, sociodemographic variables and IQ in children born VLBW ($n = 149$).

	Full-scale IQ	
Factor	**β (S.E.)**	***p*-value**
HEI-2010 score, per point	−0.026 (0.129)	0.839
Birth weight, per g	0.017 (0.006)	0.006*
Sex, reference = female	−7.670 (3.105)	0.015*
Breastfeeding duration, per day	0.004 (0.009)	0.642
Income below the poverty line, reference = no	−2.595 (4.277)	0.545
Maternal education, reference = no university	9.059 (3.635)	0.014*

Results presented are beta coefficients (standard error) from linear regression models for HEI-2010 scores and IQ adjusted for sex, birth weight, income, maternal education, and breastfeeding duration. HEI-2010, Healthy Eating Index 2010, VLBW, very low birth weight.

**Statistically significant variables in regression model.*

7–9-year-old full-term children (mean HEI-2005 score: 66.4) and Florence et al.'s cohort of 10–11-year-old full-term children (mean DQI-I score: 62.4), both of which found significant associations between diet and cognitive performance. However, the lack of variability in our diet scores may be one reason why we did not find significant associations between diet quality and IQ. It is also possible that the association between diet and cognition, may become more apparent with increasing age as IQ scores begin to stabilize over childhood (12, 36). Our results may also indicate that other factors are more critical in supporting neurodevelopment in children born VLBW that were not assessed in this study, such as early growth (9, 37–39). More specifically, it is important to consider the confounding role of SES factors related to neurodevelopment and diet quality in children such as maternal education.

In this cohort, maternal education was the strongest predictor of IQ, with children whose mothers had a university degree, compared to no university degree, having a 9-point gain in IQ. SES indicators such as maternal education have been reported to be predisposing risk factors of preterm birth and have also been linked with adverse cognitive and academic outcomes in both preterm and full-term populations (40, 41). Our findings are similar to those of Voss et al. (41), who reported that 10–13-year-old children born preterm ($n = 148$, mean: birth weight = 798 g, GA = 27.1 weeks) from mothers with low educational background (high school or less) were almost 22 times more likely to have an IQ score $\leq$85 compared to those from more highly-educated mothers (college or higher). The reasons for this association may be due to many factors, including parenting style and quality of cognitive stimulation (41). Given the current and previous findings that maternal education is predictive of cognitive performance in preterm children (42, 43), early interventions focused on supporting families with low maternal education may help attenuate cognitive impairment related to preterm birth.

Despite previous findings that children born preterm are consistently below full-term children in neurocognitive outcomes, encouragingly mean IQ scores were within the average range in our cohort. Males, however, had significantly lower IQ, FRI, WMI and PSI scores compared to females and consistently underperformed in all other cognitive domains. This is in line with previous developmental studies, showing females have a slight IQ advantage in early to mid-childhood due to earlier maturational processes (44, 45). Consistent with this, Lean et al. (46) found males born very preterm were at higher risk of poor intellectual outcomes compared to very preterm-born females at 5 years of age. Thus, future studies and interventions should consider the heightened "triple-risk" of being born male, preterm and to socioeconomically disadvantaged mothers, to mitigate later cognitive risk.

Our study has many strengths, including using the HEI-2010, a measure of diet quality that has been validated in young children (22). In addition, dietary intake was assessed using two 24-h recalls that were adjusted for usual intake using the NCI method. To our knowledge, this is the first study to assess the association between diet quality and cognitive performance in young children born VLBW. Further, our study adjusted for sociodemographic variables such as maternal education and household income, which many other studies fail to take into account. However, despite the many strengths of our study, there are some limitations to consider. Only 50% of the survivors from the original trial participated in this 5-year follow-up. Great efforts were made to contact all 316 families who participated in the initial trial, however, due to various reasons indicated in **Figure 1**, many families were unable or not interested in participating in this follow-up study. While sample retention is a common challenge faced in prospective cohort studies, we acknowledge that our sample may not be representative of all children born VLBW. However, our sample represented diversity in educational attainment and household income, as well as similar incidence of major morbidity to national data of VLBW infants (47). Another limitation is the lack of a matched control group in our study. Although this study was performed cross-sectionally (i.e., association between diet quality and cognitive performance at 5 years), children were part of a prospective cohort study, from birth, in which they were recruited from birth. Follow-up studies such as ours are costly and time-intensive, making it difficult to include a control group. We did, however, find that average diet quality scores in full-term children were similar to our results (12, 36). In addition, we also acknowledge the possibility of other unmeasured confounding variables, such as the amount and quality of preschool education, that may influence the relations between diet and cognition. We did, however, collect and control for various other sociodemographic factors that could influence neurodevelopment such as sex, household income above/below the poverty line, maternal education, birth weight and breastfeeding duration. Further research is also necessary to assess the impact of specific micronutrients (e.g., iron) and its association with neurodevelopment, which was not assessed in the current study.

Diet quality, assessed using the HEI-2010, was not associated with IQ in 5-year-old children born VLBW. Although this is inconsistent with previous studies reporting a positive association between diet quality and cognition in older cohorts of full-term children, our study is the first to assess this association in 5-year-old children born VLBW. While it is unclear which factors contribute to these discrepant findings, the age range of our cohort, the measure of diet quality used, or the lack of variability in the diet quality scores (most within the "needs improvement" range), all may contribute to these differences. In addition, previous studies did not directly assess the association between diet quality and IQ, which may explain differences with our study findings. Maternal education, however, was independently associated with cognitive performance, and was the strongest predictor of IQ in our model. Maternal education is an important predictor of neurodevelopment in children born VLBW and should be considered in future interventions to target those at risk for cognitive difficulties. Finally, most children in our study had diets that were "in need of improvement", which has important implications for the health and developmental outcomes in this vulnerable population. Future studies are needed to confirm these findings and assess the impact of diet quality on cognitive performance in young children born VLBW.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because deidentified individual participant data will not be made available in order to protect the privacy and confidentiality of our participants. We do not have consent from participating families to share their anonymized data, nor do we have permission from the research ethics boards of our participating hospitals. Requests to access the datasets should be directed to deborah.oconnor@utoronto.ca.

ETHICS STATEMENT

The Hospital for Sick Children's Research Ethics Board (REB# 1000053053) approved the study protocol. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JS, MM, SU, and DO'C contributed to conception and design of the study. JS, MM, and NB were involved in data collection. JS and MM performed the statistical analysis. JS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This study was funded by the Canadian Institutes of Health Research (FHG 129919) and the SickKids Research Institute Restracomp Scholarship to JS.

ACKNOWLEDGMENTS

The authors thank the study families for their participation and ongoing support of this work. The authors also thank Aisling Conway for assisting with study visits, and Michael Jory for setting up and managing our study database (The Hospital for Sick Children).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.874118/full#supplementary-material

REFERENCES

1. Anderson PJ. Neuropsychological outcomes of children born very preterm. *Semin Fetal Neonatal Med.* (2014) 19:90–6. doi: 10.1016/j.siny.2013.11.012
2. Bhutta AT, Cleves MA, Casey PH, Cradock MM, Anand KJ. Cognitive and behavioral outcomes of school-aged children who were born preterm: a meta-analysis. *JAMA.* (2002) 288:728–37. doi: 10.1001/jama.288.6.728
3. Joseph RM, O'Shea TM, Allred EN, Heeren T, Hirtz D, Jara H, et al. Neurocognitive and academic outcomes at age 10 years of extremely preterm newborns. *Pediatrics.* (2016) 137:e20154343. doi: 10.1542/peds.2015-4343
4. Kerr-Wilson CO, Mackay DF, Smith GC, Pell JP. Meta-analysis of the association between preterm delivery and intelligence. *J Public Health.* (2012) 34:209–16. doi: 10.1093/pubmed/fdr024
5. Cormack BE, Harding JE, Miller SP, Bloomfield FH. The influence of early nutrition on brain growth and neurodevelopment in extremely preterm babies: a narrative review. *Nutrients.* (2019) 11:2029. doi: 10.3390/nu11092029
6. Rees CM, Pierro A, Eaton S. Neurodevelopmental outcomes of neonates with medically and surgically treated necrotizing enterocolitis. *Arch Dis Child Fetal Neonatal Ed.* (2007) 92:F193–8. doi: 10.1136/adc.2006.099929
7. Schlapbach LJ, Aebischer M, Adams M, Natalucci G, Bonhoeffer J, Latzin P, et al. Impact of sepsis on neurodevelopmental outcome in a Swiss National Cohort of extremely premature infants. *Pediatrics.* (2011) 128:e348–57. doi: 10.1542/peds.2010-3338
8. Burrows T, Goldman S, Pursey K, Lim R. Is there an association between dietary intake and academic achievement: a systematic review. *J Hum Nutr Diet.* (2017) 30:117–40. doi: 10.1111/jhn.12407
9. Wong HS, Edwards P. Nature or nurture: a systematic review of the effect of socio-economic status on the developmental and cognitive outcomes of children born preterm. *Matern Child Health J.* (2013) 17:1689–700. doi: 10.1007/s10995-012-1183-8
10. Hoyland A, Dye L, Lawton CL. A systematic review of the effect of breakfast on the cognitive performance of children and adolescents. *Nutr Res Rev.* (2009) 22:220–43. doi: 10.1017/S0954422409990175
11. Nyaradi A, Li J, Hickling S, Foster J, Oddy WH. The role of nutrition in children's neurocognitive development, from pregnancy through childhood. *Front Hum Neurosci.* (2013) 7:97. doi: 10.3389/fnhum.2013.00097
12. Florence MD, Asbridge M, Veugelers PJ. Diet quality and academic performance. *J Sch Health.* (2008) 78:209–41. doi: 10.1111/j.1746-1561.2008.00288.x
13. Esteban-Cornejo I, Izquierdo-Gomez R, Gómez-Martínez S, Padilla-Moledo C, Castro-Piñero J, Marcos A, et al. Adherence to the Mediterranean diet and academic performance in youth: the UPandDOWN study. *Eur J Nutr.* (2016) 55:1133–40. doi: 10.1007/s00394-015-0927-9
14. Isaacs EB, Fischl BR, Quinn BT, Chong WK, Gadian DG, Lucas A. Impact of breast milk on intelligence quotient, brain size, and white matter development. *Pediatr Res.* (2010) 67:357–62. doi: 10.1203/PDR.0b013e3181d026da

15. Horta BL, Loret de Mola C, Victora CG. Breastfeeding and intelligence: a systematic review and meta-analysis. *Acta Paediatr.* (2015) 104:14–9. doi: 10.1111/apa.13139
16. Kramer MS, Aboud F, Mironova E, Vanilovich I, Platt RW, Matush L, et al. Breastfeeding and child cognitive development: new evidence from a large randomized trial. *Arch Gen Psychiatry.* (2008) 65:578–84. doi: 10.1001/archpsyc.65.5.578
17. Beck S, Wojdyla D, Say L, Betran AP, Merialdi M, Requejo JH, et al. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ.* (2010) 88:31–8. doi: 10.2471/BLT.08.062554
18. Huddy CL, Johnson A, Hope PL. Educational and behavioural problems in babies of 32-35 weeks gestation. *Arch Dis Child Fetal Neonatal Ed.* (2001) 85:F23–8. doi: 10.1136/fn.85.1.F23
19. McGuire S. Institute of Medicine (IOM) early childhood obesity prevention policies. *Adv Nutr.* (2012) 3:56–7. doi: 10.3945/an.111.001347
20. Clark CA, Woodward LJ. Neonatal cerebral abnormalities and later verbal and visuospatial working memory abilities of children born very preterm. *Dev Neuropsychol.* (2010) 35:622–42. doi: 10.1080/87565641.2010.508669
21. Tramontana MG, Hooper SR, Selzer SC. Research on the preschool prediction of later academic achievement: a review. *Dev Rev.* (1988) 8:89–146. doi: 10.1016/0273-2297(88)90001-9
22. Guenther PM, Kirkpatrick SI, Reedy J, Krebs-Smith SM, Buckman DW, Dodd KW, et al. The Healthy Eating Index-2010 is a valid and reliable measure of diet quality according to the 2010 Dietary Guidelines for Americans. *J Nutr.* (2014) 144:399–407. doi: 10.3945/jn.113.183079
23. Guenther PM, Casavale KO, Reedy J, Kirkpatrick SI, Hiza HA, Kuczynski KJ, et al. Update of the healthy eating index: HEI-2010. *J Acad Nutr Diet.* (2013) 113:569–80. doi: 10.1016/j.jand.2012.12.016
24. O'Connor DL, Gibbins S, Kiss A, Bando N, Brennan-Donnan J, Ng E, et al. Effect of supplemental donor human milk compared with preterm formula on neurodevelopment of very low-birth-weight infants at 18 months: a randomized clinical trial. *JAMA.* (2016) 316:1897–905. doi: 10.1001/jama.2016.16144
25. Unger S, Gibbins S, Zupancic J, O'Connor DL. DoMINO: donor milk for improved neurodevelopmental outcomes. *BMC Pediatr.* (2014) 14:123. doi: 10.1186/1471-2431-14-123
26. Ng D, Unger S, Asbury M, Kiss A, Bishara R, Bando N, et al. Neonatal morbidity count is associated with a reduced likelihood of achieving recommendations for protein, lipid, and energy in very low birth weight infants: a prospective cohort study. *J Parenter Enteral Nutr.* (2018) 42:623–32. doi: 10.1177/0148607117710441
27. Asbury MR, Unger S, Kiss A, Ng D, Luk Y, Bando N, et al. Optimizing the growth of very-low-birth-weight infants requires targeting both nutritional and nonnutritional modifiable factors specific to stage of hospitalization. *Am J Clin Nutr.* (2019) 110:1384–94. doi: 10.1093/ajcn/nqz227
28. McGee M, Unger S, Hamilton J, Birken CS, Pausova Z, Kiss A, et al. Associations between diet quality and body composition in young children born with very low body weight. *J Nutr.* (2020) 150:2961–8. doi: 10.1093/jn/nxaa281
29. Wechsler D. *Wechsler Preschool and Primary Scale of Intelligence. Canadian Manual (WPPSI®–IV CDN)*. 4th ed. San Antonio, TX: Pearson (2012).
30. Belfort MB, Anderson PJ, Nowak VA, Lee KJ, Molesworth C, Thompson DK, et al. Breast milk feeding, brain development, and neurocognitive outcomes: a 7-year longitudinal study in infants born at less than 30 weeks' gestation. *J Pediatr.* (2016) 177:133–9.e1. doi: 10.1016/j.jpeds.2016.06.045
31. Stoll BJ, Hansen NI, Adams-Chapman I, Fanaroff AA, Hintz SR, Vohr B, et al. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *JAMA.* (2004) 292:2357–65. doi: 10.1001/jama.292.19.2357
32. Wadhawan R, Oh W, Perritt RL, McDonald SA, Das A, Poole WK, et al. Twin gestation and neurodevelopmental outcome in extremely low birth weight infants. *Pediatrics.* (2009) 123:e220–7. doi: 10.1542/peds.2008-1126
33. Statistics Canada. *Income Research Paper Series, low income cut-offs for 2006 and low income measures for 2005*. Report No.: 75F0002MIE.
34. R Core Team. *R: a Language and Environment for Statistical Computing*. Vienna, Austria (2020). Available online at: https://www.R-project.org/
35. Haapala EA, Eloranta AM, Venäläinen T, Schwab U, Lindi V, Lakka TA. Associations of diet quality with cognition in children - the Physical Activity and Nutrition in Children Study. *Br J Nutr.* (2015) 114:1080–7. doi: 10.1017/S0007114515001634
36. Khan NA, Raine LB, Drollette ES, Scudder MR, Kramer AF, Hillman CH. Dietary fiber is positively associated with cognitive control among prepubertal children. *J Nutr.* (2015) 145:143–9. doi: 10.3945/jn.114.198457
37. Cooke RW, Foulder-Hughes L. Growth impairment in the very preterm and cognitive and motor performance at 7 years. *Arch Dis Child.* (2003) 88:482–7. doi: 10.1136/adc.88.6.482
38. Der G, Batty GD, Deary IJ. Effect of breast feeding on intelligence in children: prospective study, sibling pairs analysis, and meta-analysis. *BMJ.* (2006) 333:945. doi: 10.1136/bmj.38978.699583.55
39. Ehrenkranz RA, Dusick AM, Vohr BR, Wright LL, Wrage LA, Poole WK. Growth in the neonatal intensive care unit influences neurodevelopmental and growth outcomes of extremely low birth weight infants. *Pediatrics.* (2006) 117:1253–61. doi: 10.1542/peds.2005-1368
40. Sirin SR. Socioeconomic status and academic achievement: a meta-analytic review of research. *Rev Educ Res.* (2005) 75:417–53. doi: 10.3102/00346543075003417
41. Voss W, Jungmann T, Wachtendorf M, Neubauer AP. Long-term cognitive outcomes of extremely low-birth-weight infants: the influence of the maternal educational background. *Acta Paediatr.* (2012) 101:569–73. doi: 10.1111/j.1651-2227.2012.02601.x
42. Patra K, Greene MM, Patel AL, Meier P. Maternal education level predicts cognitive, language, and motor outcome in preterm infants in the second year of life. *Am J Perinatol.* (2016) 33:738–44. doi: 10.1055/s-0036-1572532
43. ElHassan NO, Kaiser JR. Parenteral nutrition in the neonatal intensive care unit. *Neoreviews.* (2011) 12:e130–40. doi: 10.1542/neo.12-3-e130
44. Lynn R, Kanazawa S. A longitudinal study of sex differences in intelligence at ages 7, 11 and 16 years. *Pers Individ Dif.* (2011) 51:321–4. doi: 10.1016/j.paid.2011.02.028
45. Irwing P, Lynn R. Sex differences in means and variability on the progressive matrices in university students: a meta-analysis. *Br J Psychol.* (2005) 96(Pt 4):505–24. doi: 10.1348/000712605X53542
46. Lean RE, Paul RA, Smyser CD, Rogers CE. Maternal intelligence quotient (IQ) predicts IQ and language in very preterm children at age 5 years. *J Child Psychol Psychiatry.* (2018) 59:150–9. doi: 10.1111/jcpp.12810
47. The Canadian Neonatal Network. *CNN annual reports 2010Ű2015*. Available online at: http://www.canadianneonatalnetwork.org/portal/ (accessed May 01, 2019).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Sato, McGee, Bando, Law, Unger and O'Connor. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fnut.2022.935001

A Combination of Green Tea, Rhodiola, Magnesium, and B Vitamins Increases Electroencephalogram Theta Activity During Attentional Task Performance Under Conditions of Induced Social Stress

Neil Bernard Boyle, Louise Dye, Clare Louise Lawton and Jac Billington*

School of Psychology, University of Leeds, Leeds, United Kingdom

OPEN ACCESS

Edited by:
Andrew Scholey, Swinburne University of Technology, Australia

Reviewed by:
Andrey Stoyanov Marchev, Bulgarian Academy of Sciences, Bulgaria
Estela Jiménez López, University of Castilla–La Mancha, Spain

****Correspondence:***
Neil Bernard Boyle
n.b.boyle@leeds.ac.uk

Specialty section:
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

Received: *03 May 2022*
Accepted: *21 June 2022*
Published: *22 July 2022*

Citation:
Boyle NB, Dye L, Lawton CL and Billington J (2022) A Combination of Green Tea, Rhodiola, Magnesium, and B Vitamins Increases Electroencephalogram Theta Activity During Attentional Task Performance Under Conditions of Induced Social Stress. Front. Nutr. 9:935001. doi: 10.3389/fnut.2022.935001

Background: A combination of green tea, rhodiola and magnesium with B vitamins has previously been reported to significantly increase EEG resting state theta, attenuate subjective stress, anxiety and mood disturbance, and heighten subjective and autonomic arousal under acute psychosocial laboratory stress. Here we examine the capacity of green tea and rhodiola extract administered in combination or in isolation with magnesium and B vitamins to moderate spectral brain activity during attentional task performance under stress.

Materials and Methods: One-hundred moderately stressed adults received oral supplementation of (i) Mg + B vitamins + green tea + rhodiola; (ii) Mg + B vitamins + rhodiola; (iii) Mg + B vitamins + green tea; or (iv) placebo, in a double-blind, randomised, placebo-controlled, parallel-group design (Clinicaltrials.gov: NCT03262376; 25/0817). Participants completed an attention switching and emotionally threatening attentional bias task after stress induction (Trier Social Stress Test). Spectral alpha and theta brain activity and event related potentials (ERPs) were recorded during cognitive task performance by electroencephalogram (EEG; BioSemi ActiveTwo 64 channel).

Results: The combined treatment of Mg + B vitamins + green tea + rhodiola significantly increased frontal midline theta vs. placebo and rhodiola in isolation during the attention switching task, specifically in anticipation of a change in task performance parameter. The combined treatment also significantly increased contralateral theta activation in relation to viewing emotionally threatening images in the left (vs. placebo and rhodiola in isolation) and right parietal (vs. placebo) regions. Further, this treatment demonstrated significantly heightened ipsilateral left parietal theta activation in relation to viewing emotionally threatening images. The combined treatment attenuated a decrease in alpha power during the attentional bias task evident in comparator treatments, but this did not reach significance. No significant effects of treatments on behavioural performance or ERP were found.

Conclusion: The combination of Mg + B vitamins + green tea + rhodiola increased spectral theta brain activity during the execution of two attentional tasks suggestive of a potential to increase attentional capacity under conditions of stress. Further examination of these ingredients in relation to attentional performance under stress is warranted to ascertain if functional benefits suggested by theta activation can be shown behaviourally.

Keywords: stress, green tea, rhodiola, magnesium, EEG

INTRODUCTION

Oscillatory brain activity measured by electroencephalogram (EEG) is an emerging marker for assessing the functional benefits of nutrient intake. Significant modulation of neural EEG activity has been demonstrated after acute intake of both green tea (1–3) and rhodiola (4). We have recently reported that combining green tea and rhodiola extract and administering with magnesium (Mg) and a B vitamin complex (B_6, B_9, and B_{12}) significantly increased resting state frontal midline theta activity under stress conditions compared to placebo, and rhodiola extract combined with Mg and B vitamins (5); *post hoc* exploratory analysis also demonstrated the combined treatments superiority to green tea in isolation combined with Mg and B vitamins. Increased frontal midline theta is associated with a relaxed, alert state and anxiolytic action (6) suggesting the combination of these ingredients has the potential to enhance coping capacity and offer protection from the impairing effects of stress exposure. Further, the fully combined treatment was significantly more efficacious in the alleviation of the negative effects of stress exposure on subjective state. Here we report further data from this study on the combined effects of green tea, rhodiola extract, Mg and B vitamins on spectral frequency and event related potentials (ERPs) as measured by EEG during the completion of two attentional tasks under conditions of acute stress.

Attentional tasks sensitive to stress exposure and that engage neural processes previously shown to be moderated by green tea or rhodiola intake were selected for examination. Attention switching tasks typically require respondents to repeatedly perform a task on some trials then switch to another task when cued to do so, thus requiring the effortful suppression of a dominant or distracting response (1, 7). Attention switching performance is sensitive to impairment by stress (8). Further, acute intake of L-theanine – a constituent found almost exclusively in green tea – has been demonstrated to modulate alpha band activity associated with the deployment of attentional resources and suppression of distracting information (1, 9). Attentional bias tasks measure the degree to which attention is selectively focussed on a certain type of stimuli over another. Typically, threatening or rewarding stimuli are compared to neutral stimuli to determine the level of vigilance toward or avoidance of specific stimuli categories. Attentional bias performance is moderated by stress and anxiety (10). Rhodiola intake has also been shown to activate theta band activity whilst viewing emotional/threatening stimuli which has been interpreted as enhanced cognitive processing capacity (4). These demonstrated neural effects are indicative of potential functional benefits of green tea and rhodiola intake during the completion of attentional operations. Moreover, the previously demonstrated combined effects of these ingredients in the activation of EEG activity associated with a relaxed, attentive state and reduction of the subjective impact of stress exposure suggests a potential to offer direct protection from the impairing effects of stress by increasing stress coping capacity.

This randomised, placebo controlled trial examined the effects of a combination of green tea, rhodiola extract, Mg and B vitamins on attentional performance and associated spectral frequency EEG alpha and theta responses, and attention related ERPs, after acute stress provocation. The combined treatment was compared to placebo, and rhodiola or green tea combined with Mg and B vitamins in isolation to ascertain the potential capacity of the ingredients to affect behavioural attentional performance, and associated spectral brain activity, under stress. The combined treatment was expected to offer greater behavioural cognitive functional benefit which would be reflected in the associated EEG activity compared to placebo and treatments administering green tea and rhodiola in isolation.

MATERIALS AND METHODS

Study Design

The study conformed to a double blind, randomised, placebo controlled, parallel group design comprising four treatment arms: (i) ***COMBINED:*** Mg + vitamins B_6, B_9, B_{12} + green tea + rhodiola extract; (ii) ***GREEN TEA*****:** Mg + vitamins B_6, B_9, B_{12} + green tea; (iii) ***RHODIOLA:*** Mg + vitamins B_6, B_9, B_{12} + rhodiola extract; (iv) ***PLACEBO***. The design and primary and secondary outcomes of the study were pre-registered at Clinicaltrials.gov (NCT03262376; August 25th 2017). The study was approved by the University of Leeds, School of Psychology Research Ethics Committee (17-0235; granted October 30th 2017) and conducted according to the principles of Good Clinical Practice and the Declaration of Helsinki (11).

Primary Outcomes

The effect of treatment on EEG spectral frequency oscillatory brain activity during the rested state [previously published (5)] and during completion of attentional tasks under conditions of stress exposure.

Secondary Outcomes

The effect of treatment on subjective state, salivary cortisol, cardiovascular parameters [previously published (5)], cognitive performance and attentional event related potentials (ERPs).

Participants

One hundred healthy adults (men = 36; women = 64) were recruited from the University of Leeds and local community. Eligible participants were identified from responses to an online eligibility screening questionnaire [N = 535; the full CONSORT diagram is previously published (5)]. Inclusion and exclusion criteria were as follows:

Inclusion Criteria

- Reporting moderate subjective stress levels [score of ≥13–≤25 on *stress* subscale of the Depression Anxiety and Stress Scale (DASS) (12)].
- ≥18–≤50 years of age (premenopausal if female indicated by self-reported lack of menopause symptoms or associated absence of period for 12 months+).
- BMI ≥18 and ≤30 kg/m^2.
- Healthy [free from significant physical or psychiatric disorders (self-report)].

Exclusion Criteria

- Intake of prescribed medication except contraceptives.
- Hypertension (self-report or resting blood pressure >160/95 mmHg).
- Intake of regular nutritional supplementation.
- Smoking >5/day.
- Pregnant or lactating.
- Previous exposure to a laboratory stress protocol.
- Night-working/shift work.
- Recreational drug use.
- Previous brain surgery or severe brain injury requiring hospital treatment.
- Any form of neurological condition (e.g., epilepsy).

The eligibility criteria were confirmed at a face-to-face screening and written informed consent attained from all participants. An honorarium of £60 was paid for completion of the study protocol.

Treatments

Magnesium and B vitamins were administered in a single pressed tablet form [Mg (150 mg elemental) + vitamins B_6 (0.7 mg), B_9 (0.1 mg), B_{12} (0.00125 mg)]. Teadiola® (Sanofi) – a commercially available combined green tea/rhodiola extract formulation – was employed for the green tea and rhodiola treatments. To permit administration of the two ingredients, both combined and in isolation, the manufacturer was requested to split the formulation into separate treatments: dry extracts of green tea leaf [*Camellia sinensis* L. 125 mg containing 40% L-theanine (50 mg)], and rhodiola root (*Rhodiola rosea* L. 222 mg, or 1,887 mg equivalent plant) standardised in rosavins and salidrosides. To ensure blinding these separate treatments were administered in opaque capsule form. Identical placebo equivalent forms (cellulose crystalline) were produced for all treatments. All treatments were manufactured by an affiliate of Sanofi.

Randomisation

Respondents were allocated a 6 digit/string ID code[1] at the face-to-face screening visit. Eligible respondents were subsequently randomly allocated to treatment by an independent statistician using an adaptive randomisation scheme to ensure a balanced representation of age and gender across the treatment arms (13).

Blinding

Participants were allocated to treatment arms by the independent statistician using 4 treatment codes generated by an independent technician at Sanofi. The treatments were prepared at the Leeds site by a laboratory manager not directly involved in testing. All treatments were supplied in identical forms and identifiable to the experimenters only by a packing number.

Final data sets were locked upon completion of data collection and initial EEG processing and duplicate copies logged. The Leeds team and statistician remained blinded until data locking and completion of primary statistical analyses. Treatments codes were broken to complete *a priori post hoc* comparisons as per the fixed hierarchical statistical analysis approach employed (detailed in section "Statistical Analyses").

Measures

Cognitive Tasks

Two attentional cognitive tasks were employed to permit EEG measurement of neural processing and cognitive performance under stress, and to assess the effects of treatment on these outcomes. Both cognitive tasked were presented using PsychToolBox plugin for MATLAB on a desktop computer and an iiyama Prolite B2483HS 24″ display (1920 × 1080 full HD 1080p, 2.1 megapixel, 60 Hz refresh rate).

Attention Switching Task

The attention switching task was originally develop by Wylie et al. (7) and combines an attention switching paradigm with a Go/No-Go task. Letter-number pairs were presented on a horizontal plane in the centre of the screen for 2 s (120 ms inter-stimulus delay). Each character was 1° to the left or right of the central fixation point (randomly determined). The letters were taken from a set of 4 vowels (A, E, I, and U) and four consonants (G, K, M, and R). The numbers were taken from a set of 4 even (2, 4, 6, and 8) and 4 odd (3, 5, 7, and 9) numbers. Letter-number pairs were presented in one of two alternating colours every three trials. Respondents were required to make a Go/No-Go choice based upon the colour of the letter-number pair with the change in colour cueing the switch in task-set. For example, when the letter-number pairs were red, respondents were required to respond when the letter was a consonant (Go), but not when the letter was a vowel (No-Go). Alternatively, when the letter-number pairs switched to white, respondents were required to respond when the number was odd (Go), but not when the number was even (No-Go). *Switch* trials were the first letter-number pairs presented after the task-switch (i.e., the Go/No-Go colour switch). *Nested* and *pre-switch* were the subsequent repeat trials within the same task-set. A total of 216 trials were presented

[1] www.random.org

(72 three trial task sets). Performance was measured by reaction time (RT) and accuracy across each trial type. See **Figure 1** for stimulus configuration.

Attentional Bias Task

A simple dot probe task comprising neutral (n = 24) and emotionally threatening (n = 24) images taken from the International Affective Picture System (IAPS) (14) was employed. A central fixation cross was shown for 500 ms followed by two images – one neutral and one threatening – presented simultaneously on a horizontal plane in the centre of the screen 1.5° to the left or right of the central fixation point. The images measured 150 mm width by 80 mm height and were shown for 500 ms. After a 500 ms cue-target delay, a target – the letter E or F – appeared on the left or right in the centre of the location previously occupied by one of the displayed images. Participants were asked to press E or F on the keyboard dependent upon which letter target was displayed. The presentation of the target letter coincided with the position of the emotionally threatening image (congruent trial), or the position of the neutral image (incongruent trial) with a 50% probability. The pairing of neutral and emotionally threatening images was held constant for all participants. Each stimulus image pair was shown three times across the task. A total of 144 trials were presented comprising 72 congruent and 72 incongruent trials. Target type (E or F), location of emotionally threatening/neutral image and target (left or right of fixation) were all counterbalanced and trials were presented in random order. Participants were instructed to look at the fixation cross and discriminate the target as quickly and accurately as possible. A congruency score was calculated by subtracting mean RT for congruent trials from incongruent trials. A congruency score below zero is indicative of attentional avoidance and scores above zero of attentional vigilance. See **Figure 2** for stimulus configuration.

Electroencephalogram Methodology

Electroencephalogram was recorded continuously with a BioSemi ActiveTwo EEG system (BioSemi, Amsterdam) from 64 electrodes at a 1,024 Hz sampling rate. Four electrooculograms (EOG) electrodes were used to record eye movements and were placed lateral and dorsal to each eye. Two EOGs were placed on the mastoids. EEG recordings comprised four separate blocks: two resting state measures (580 s), attention switching task (550 s), and attentional bias task (530 s). All tasks were completed in sequential order in one session. The resting state data is reported elsewhere (5).

Prior to analysis, EEG data were pre-processed in MATLAB using both EEGLAB (15) and Fieldtrip (16). Data were initially down sampled to 512 Hz. All participants' data were checked by eye and exceptionally bad channels removed. Continuous data was then re-referenced to the average of all 64 channels (with exception of removed bad channels) and data destined for spectral analysis were high-pass finite impulse response (FIR) filtered using a 1 Hz cut-off. Independent component analysis (ICA) (17) was used to identify eye-movement components in the data. Prior to spectral analysis, data was high-pass finite impulse response (FIR) filtered using a 0.1 Hz cut-off, and ICA components derived from the 1 Hz filtering set were used to remove eye-movement artefacts from 0.1 Hz filtered set. Data were segmented into epochs and baseline corrected using the following methodology:

Attention Switching Task

Switch, nested and pre-switch trials were considered individual epochs. Epochs were extracted −0.1 to 1.7 s to trial onset (appearance of stimulus). Baseline period: −100 to −20 ms (epoch shown in **Figure 1**).

Attentional Bias Task

Each trial (comprised of crosshair, picture presentation, and response letter presentation) was considered an individual epoch. Epoch length −0.1 to 3.3 s relative to crosshair onset. Baseline period: −100 to −20 ms (epoch shown in **Figure 2**).

After defining epochs a MATLAB implementation of the Fully Automated Statistical Thresholding for EEG Artefact Rejection [FASTER (18)] was used to remove artefacts in the data. This included (1) thresholded interpolation of bad channels via identification of global and local artefacts in channel data and (2) removal of bad trials on the basis of remaining eye and muscle movement noise and voltage spikes. Spectral power was estimated across each epoch in the range of 2–40 Hz in 0.1 Hz steps using a Hann taper with a variable time window (four cycles at each frequency). Frequencies of specific interest – alpha (8–12 Hz) and theta (4–7 Hz) – were extracted for statistical analysis. Alpha and theta EEG frequency data were extracted for each cognitive task using the following methodology:

Attention Switching Task

Alpha and theta values were extracted from each trial epoch – switch, nested and pre-switch trials – and averaged for each participant.

Attentional Bias Task

Alpha and theta values were extracted from each epoch and further binned into 250 ms time bands to permit comparisons of alpha/theta activity over the course of the trial.

Event-Related Potential Methodology

High-density event-related potentials (ERPs) were extracted from frontal and parietal sites during completion of the attention switching task. The componentry of the ERPs was extracted using the following electrode configuration: Left Frontal: "AF3," "F3," "F1," Right Frontal: "AF4," "F2," "F4"; Left Parietal: "PO3," "PO7," "O1," Right Parietal: "PO4," "PO8," "O2." To permit ERP componentry identification, data from these site-specific electrodes were collapsed across treatment arms and averaged into a single average waveform for the left and right hemisphere sites for each trial type (switch, nested, and pre-switch). The waveforms were visually inspected and their componentry broadly defined in accordance with the components originally identified for this specific attention switching task by Wylie et al. (7) The ERP components extracted for statistical analyses are shown in **Table 1**.

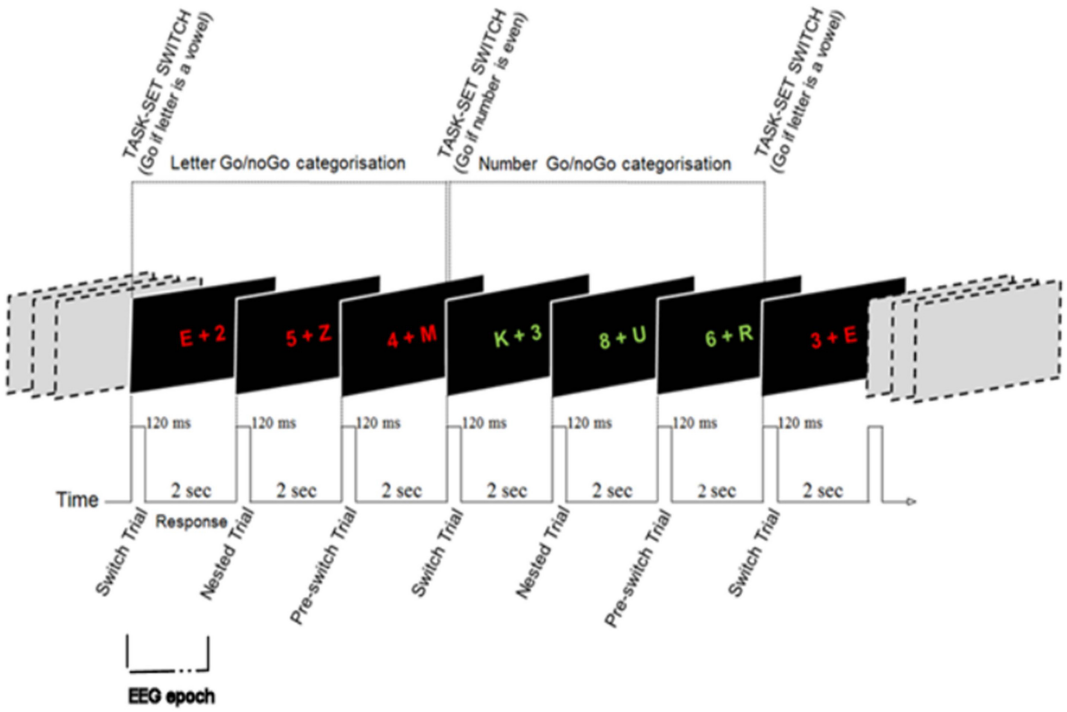


FIGURE 1 | Attention switching task stimulus configuration [adapted with permission from Wylie et al. (7)] showing seven consecutive trials/EEG epochs.

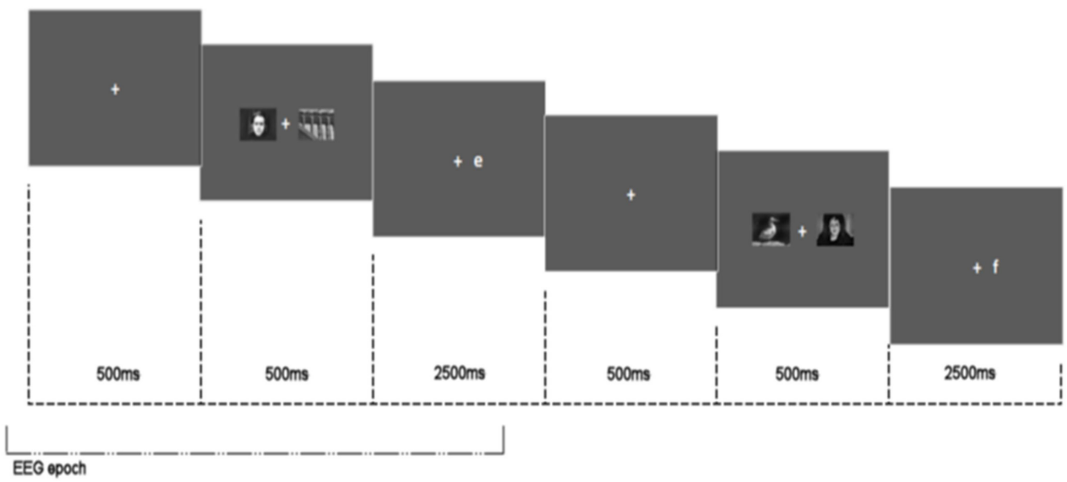


FIGURE 2 | Attentional bias task stimulus configuration showing two consecutive trials and EEG epoch.

TABLE 1 | Attention switching task event related potential component summary [with onset and offset timings (ms)] by region.

Region	Component	P1	N1	P2	LP1	LP2			
Frontal	Onset (ms)	0.1284	0.2340	0.2827	0.8	1.4			
	Offset (ms)	0.1684	0.2740	0.3227	1.3	1.650			
	Component	**P1**	**N1**	**P2**	**N275**	**P400**	**P550**	**LP1**	**LP2**
Parietal	Onset (ms)	0.058	0.116	0.218	0.274	0.370	0.544	0.8	1.4
	Offset (ms)	0.098	0.156	0.258	0.314	0.570	0.564	1.3	1.65

Stress Protocol

The Trier Social Stress Test (TSST) was employed to experimentally induce acute stress. The TSST protocol has been defined elsewhere (19). Briefly, participants completed two extemporaneous 5 min socially evaluated tasks – presenting themselves as a job candidate and completing a maths subtraction task – in front of an unresponsive panel of two female confederates.

Procedure

The study was undertaken at the Human Appetite Research Unit (HARU), School of Psychology, University of Leeds, United Kingdom. All participants completed the study protocol between 11 and 3 pm, fasted 1 h prior to commencement of the study protocol, and avoided alcohol and vigorous exercise 24 h prior to the visit. The test day procedural timeline is shown in **Figure 3**. Upon arrival participants relaxed in the testing cubicle. A number of physiological and subjective measures were collected throughout the visit. These measures are reported elsewhere (5). After 15 min the allocated treatment was administered with water and consumption witnessed and checked by the experimenter. Participants relaxed in the cubicle for 30 min before being taken to a separate room and introduced to the TSST task. Participants returned to the testing cubicle for a 5 min anticipation period. Participants then completed the TSST (lasting ∼ 10 min). Following stress induction participants were taken immediately to the EEG laboratory for fitting of the BioSemi and a clear signal established, participants completed 10 min of resting state measures. The attentional switching and attentional bias task were then undertaken in sequential order.

Statistical Analyses

All statistical analyses were performed using SAS (Statistical Analysis System, Version 9.2; SAS Institute, Inc., Cary, NC, United States) and Fieldtrip (16). Cognitive performance outcome residual plots were inspected for deviations from normality and residual outlying data points removed (±2.58 SD). One participant was removed from the study and replaced due to a fault in the EEG system preventing measurement. The primary analyses were performed in the randomised population, defined as all randomised subjects who received at least one dose of the treatment and for whom a reliable EEG signal could be obtained.

A sample size power calculation was conducted based upon previous evidence of the modulation of resting state and attentional performance-related EEG alpha wave activity by L-theanine (1–3, 9). Reported effect sizes ranged from 0.803 to 1.141 (Cohen's *d*). Assuming an effect size of 0.9, $\alpha = 0.05$, and a power of 80%, it was estimated that a sample of 25 participants per arm (100 total) would be required to find an effect of this magnitude.

For all EEG analysis (spectral frequency and ERP), individual trials for each condition were averaged for each participant prior to statistical analysis in Fieldtrip or mean extraction for ROI analysis. A whole brain approach was initially adopted comparing oscillatory activity between the COMBINED vs. PLACEBO treatments as a primary analysis step. For this, independent sample *T*-tests (Monte Carlo approach 500 random permutations for all electrodes) were employed in Fieldtrip (16) to compare oscillatory alpha and theta power magnitude (μV) across the brain. Secondary regions of interest (ROIs) analyses were carried out on ROIs in which a significant difference was observed in this primary analysis step ($p < 0.05$). ROIs selected for further analyses were informed by the whole brain result but with ROIs defined *a priori* on the basis of previous literature. Regions were defined in this way in order to prevent circularity and bias in data analysis (20) and analysed using a six-sequence mixed effect ANCOVA in SAS as per the procedure outlined below.

A mixed-effects ANCOVA was employed with treatment and gender entered as fixed model effects and participant ID entered as a random effect. Age, gender, BMI, trait anxiety (STAI-Y2), and DASS stress score were entered as covariates in all models and removed or retained based on the Akaike Information Criterion (AIC). To control the family-wise type 1 error rate, a fixed sequence testing procedure (six-steps) was used to assess the effect of each treatment arm within each primary outcome. The six-step sequence adhered to the following order: (1) COMBINED vs. PLACEBO; (2) COMBINED vs. RHODIOLA; (3) GREEN TEA vs. PLACEBO; (4) RHODIOLA vs. PLACEBO; (5) COMBINED vs. GREEN TEA; and (6) RHODIOLA vs. GREEN TEA. The significance at a level of 0.05 was tested only if the results of the previous step reached significance. Secondary endpoints related to the secondary objectives were also tested using the same fixed sequence procedure. Exploratory (*post hoc*) analyses of the comparisons not reached in the fixed sequence due to non-significance of previous steps are briefly summarised in the discussion for exploratory purposes only and to aid future investigation of the ingredients.

RESULTS

A descriptive summary of the final sample is shown in **Table 2**. One way ANOVAs revealed no significant differences between the study conditions across the participant characteristics of Age [$F(3,96) = 2.318$, $p = 0.08$], BMI [$F(3,96) = 0.359$, $p = 0.78$], STAI-Y2 [(trait anxiety); $F(3,96) = 2.185$, $p = 0.10$], and DASS Stress score [$F(3,96) = 0.906$, $p = 0.44$].

Electroencephalogram Outcomes

Attention Switching Task

Alpha

Whole brain analysis of oscillatory alpha band activity revealed no significant differences across the electrode sites between the COMBINED and PLACEBO treatments during execution of the attention switching task. Heightened alpha at FCz and surrounding electrodes was evident in the COMBINED vs. PLACEBO treatment but this comparison did not reach significance ($p > 0.05$). Accordingly, no ROI analyses were undertaken.

Theta

Whole brain analysis of oscillatory theta band activity revealed significantly heightened theta activity during the execution of the

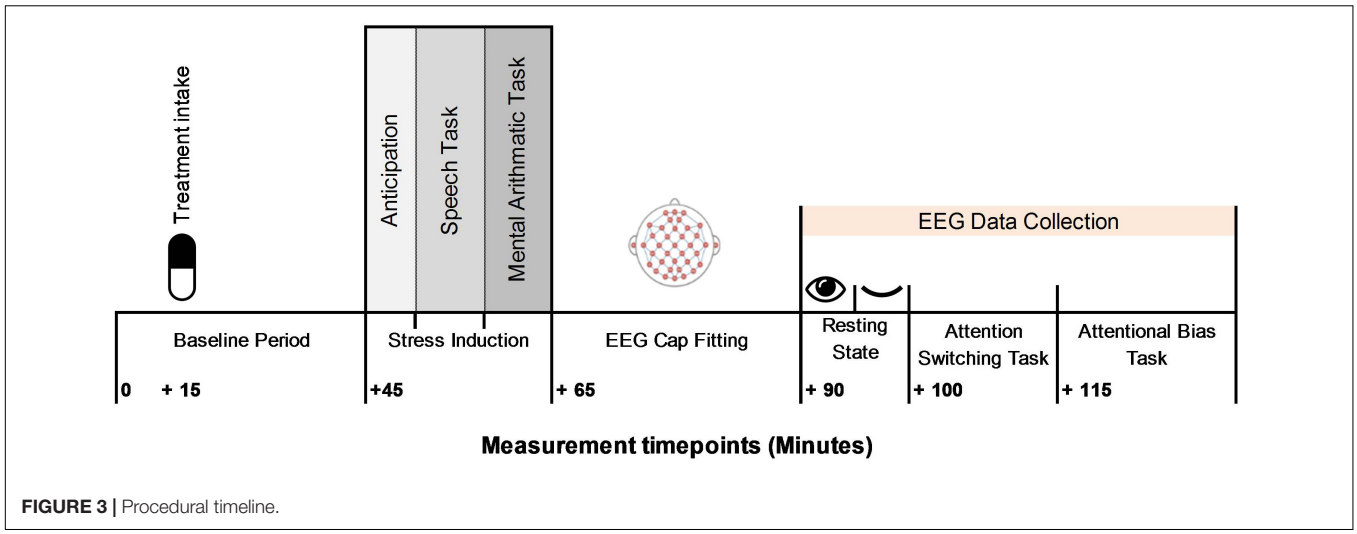


FIGURE 3 | Procedural timeline.

TABLE 2 | Summary characteristics of sample receiving each treatment.

Treatment	*N*	Sex	Age	BMI	STAI-Y2	DASS stress score
			Mean (SEM)			
Green tea	25	16F 9M	26.44 (1.57)	22.9 (0.56)	38.72 (1.46)	16.6 (0.68)
Rhodiola	25	17F 8M	26.2 (1.13)	23.25 (0.63	40.48 (1.24)	15.68 (0.63)
Combined	25	14F 11M	22.2 (0.74)	22.93 (0.52)	42.48 (1.43)	17.12 (0.74)
Placebo	25	17F 8M	25.56 (1.55)	23.69 (0.56)	43.12 (1.37)	15.96 (0.66)

attention switching task ($p < 0.05$). This effect predominantly consisted of an overall heightened frontal midline theta in the COMBINED vs. PLACEBO treatment during performance of the attention switching task (**Figure 4A**). To permit further analyses, frontal midline theta values were extracted from channels "Cz," "Fz," "FCz," "FC1," "FC2," and combined and averaged separately for switch, nested and pre-switch trials in each treatment. The COMBINED treatment heightened frontal midline theta compared to the PLACEBO during execution of the three attention switching trials (**Figure 4B**). This increased theta in the COMBINED treatment reached significance during the pre-switch trials, $t(95) = 2.46$; $p = 0.02$. The COMBINED treatment also significantly heightened frontal midline theta during pre-switch trials compared to RHODIOLA treatment, $t(95) = 2.93$; $p = 0.004$. No significant difference between the GREEN TEA treatment and PLACEBO was found for pre-switch trials. Accordingly, no further comparisons are reported.

Attentional Bias Task

Alpha

Whole brain analyses of oscillatory alpha band activity over the time course of the attentional bias task revealed significant differences in alpha power in response to stimulus presentation compared to baseline in the COMBINED treatment vs. PLACEBO. There was a strong contralateral central-parietal alpha increase when emotionally threatening images were presented in either the left and right visual fields in the COMBINED treatment (**Figures 5A,B**). The COMBINED treatment also demonstrated heightened ipsilateral alpha activity in the central-parietal cortex for left presented emotionally threatening images (**Figure 5A**), but not right. For both left and right presented emotionally threatening images, there was significantly greater ipsilateral alpha activation in the frontal cortex in the COMBINED treatment compared to PLACEBO.

To permit further exploration of the data, contralateral frontal and centro-parietal ROIs corresponding with the locus of alpha activity were extracted using the following configurations, representative of both frontal and parietal brain regions: Right Frontal: channels "F6," "F8," "FT8," "FC6"; Left Frontal: channels "F5," "F7," "FT7," "FC5"; Right Parietal: channels "CP2," "CP4," "CP6," "P2," "P4," "P6"; Left Parietal: channels "CP1," "CP3," "CP5," "P1," "P3," "P5" (ROIs are shown mapped on the scalp in **Figure 6**). ROI analyses were undertaken on spectral activity during the temporal response peak between 0.5 and 0.75 s immediately after the presentation of the images.

The COMBINED treatment increased contralateral right centro-parietal alpha power when viewing emotionally threatening images presented in the left visual field but this did not reach significance ($p = 0.07$; **Figure 6**). Since no primary comparisons were significant no further comparisons are reported.

Theta

Whole brain analyses of oscillatory theta band activity over the time course of the attentional bias task revealed significant differences in theta power in response to stimulus

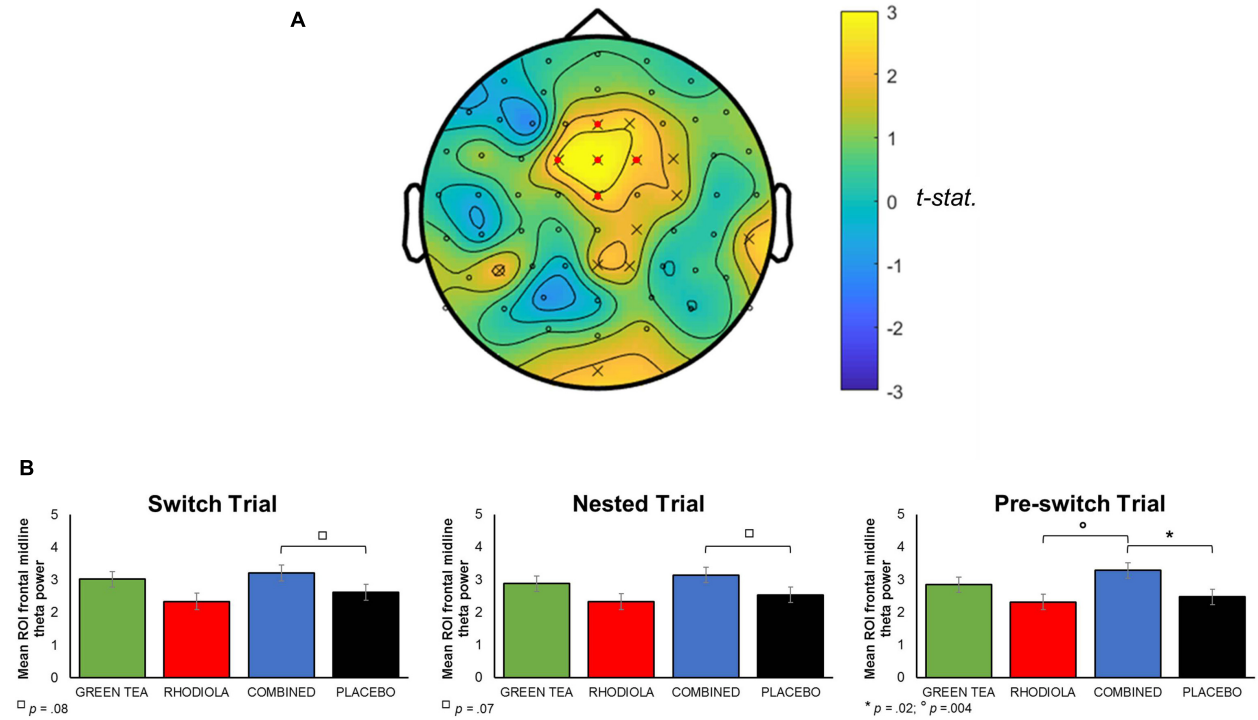


FIGURE 4 | (A) Map of t-statistic comparison of oscillatory theta band activity during attention switching task in COMBINED vs. PLACEBO treatments (positive values indicate COMBINED > PLACEBO in comparison). *A priori* defined ROI midline frontal electrodes shown in map by •. **(B)** Mean averaged ROI (SEM) frontal midline theta for each attention switching trial by treatment.

presentation compared to baseline in the COMBINED treatment vs. PLACEBO. Significantly heightened theta activation was shown in ipsilateral and contralateral central-parietal regions in the COMBINED treatment during presentation of emotionally threatening images in the left and right visual fields (**Figures 7A,B**). The effect was more pronounced in contralateral regions. This central-parietal effect in the COMBINED compared to PLACEBO treatment declined over the course of the image presentation, becoming more focussed in right frontal regions, particularly when emotionally threatening images were presented in the right visual field (ipsilateral activation; **Figure 7B**).

To permit further analyses, frontal midline and left and right parietal ROIs corresponding with the locus of theta activity were extracted using the following configurations: Frontal midline: channels "Cz," "Fz," "FCz," "FC1," "FC2"; Left Parietal: channels "C1," "C3," "C5," "CP1," "CP3," "CP5"; Right Parietal: channels "C2," "C4," "C6," "CP2," "CP4," "CP6" regions (ROIs are shown mapped on the scalp in **Figure 8**). These analyses were undertaken on spectral activity during the temporal response peak between 0.5 and 0.75 s immediately after the presentation of the images.

No significant differences in theta activation were shown between COMBINED and PLACEBO treatments in the frontal midline region ($p > 0.05$; **Figures 8A,B**). Accordingly, no further comparisons are reported for this region.

Significantly greater contralateral theta activation was shown in the left parietal region in the COMBINED treatment when emotionally threatening images were shown in the right visual field compared to PLACEBO, $t(95) = 2.94$, $p = 0.02$, and RHODIOLA, $t(95) = 2.91$, $p = 0.02$ (**Figure 8F**), treatments. The COMBINED treatment also demonstrated significantly greater contralateral theta activation in the right parietal region when emotionally threatening images were shown in the left visual field compared to PLACEBO, $t(96) = 2.77$, $p = 0.03$ (**Figure 8C**). Additionally, the COMBINED treatment demonstrated significantly greater ipsilateral theta activation in the left parietal region when emotionally threatening images were shown in the left visual field compared to PLACEBO, $t(96) = 2.64$, $p = 0.05$, and RHODIOLA, $t(96) = 2.74$, $p = 0.04$, treatments (**Figure 8E**).

Cognitive Performance

Attention Switching Task

The expected pattern of lower accuracy and slower RT on switch compared to repeated trials (nested and pre-switch) was demonstrated across the sample as a whole. However, no significant differences in accuracy or RT performance between trials were found between the COMBINED and PLACEBO treatments (**Figures 9A,B**). Accordingly, no further comparisons are reported.

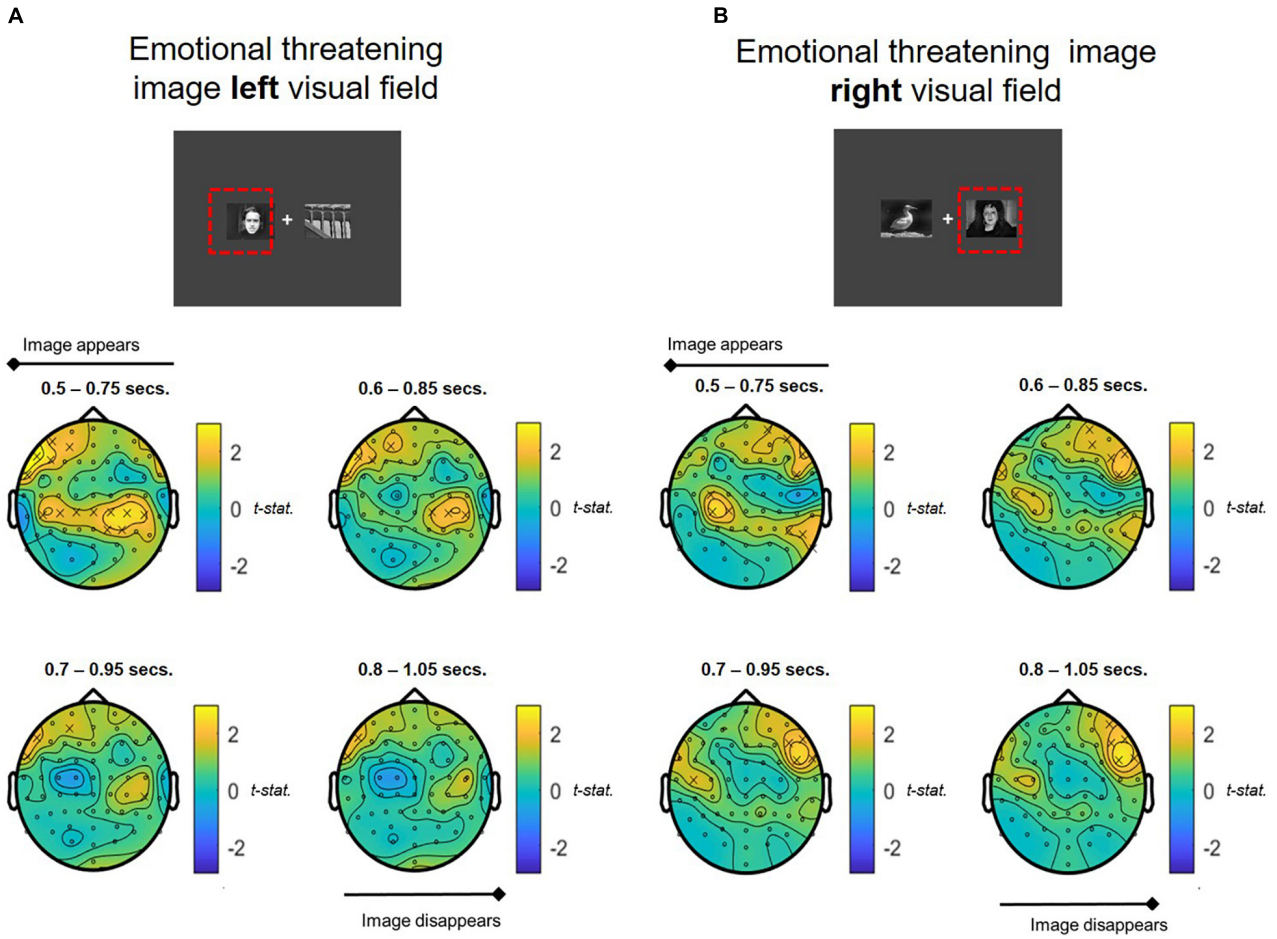


FIGURE 5 | T-statistic map of alpha band power differences between COMBINED and PLACEBO treatments during presentation of emotionally threatening images shown in the **(A)** LEFT and **(B)** RIGHT visual fields. Values > 0 indicate COMBINED treatment alpha values are higher than PLACEBO treatment. Significant electrodes ($p < 0.05$) marked with an *x*. Each figure depicts a series of *T*-tests along the time course of the presentation of the images (0.5–1.05 s, in 0.1 s steps, 0.25 s sample widths).

Attentional Bias Task

No significant difference between congruent RT, incongruent RT or congruency score was shown between the COMBINED and PLACEBO treatments. Accordingly, no further comparisons are reported. Inspection of the congruency score data (**Figure 9C**) suggests the COMBINED and PLACEBO treatments show a pattern of attentional vigilance (score > 0), RHODIOLA treatment attentional avoidance (score < 0), and GREEN TEA treatment no bias (score = ∼0). However, all congruency scores fall within a range about zero of 25 ms. The validity of congruency scores below 25 ms has been questioned and a congruency score between −25 and +25 ms suggested as an appropriately conservative criterion to indicate a lack of attentional bias (21).

Event Related Potentials

No significant differences between the COMBINED and PLACEBO treatments were shown across any of the extracted right or left frontal or parietal ERP components ($p > 0.05$). Accordingly, no further analyses are reported. Inspection of the overall ERP waveform collapsed across all treatments showed that the typical attentional ERP components expected to be shown during the completion of an attention task were demonstrated (e.g., P1, N1, and P2). However, the well-characterised ERP differentiation (7) between switch, nested and pre-switch trials typically observed in this attention switching paradigm was not demonstrated. Differentiation in ERPs for each trial type during this task typically become evident in frontal regions from ∼450 to 470 ms and parietal regions from ∼ 230 ms and show further sustained differentiation in the slow late potentials (LP1 and LP2) (7); this was not replicated here.

DISCUSSION

This study examined the capacity of green tea and rhodiola extract when administered in combination or in isolation with magnesium and B vitamins to moderate spectral brain activity during attentional task performance after acute stress provocation. As previously reported for frontal midline theta

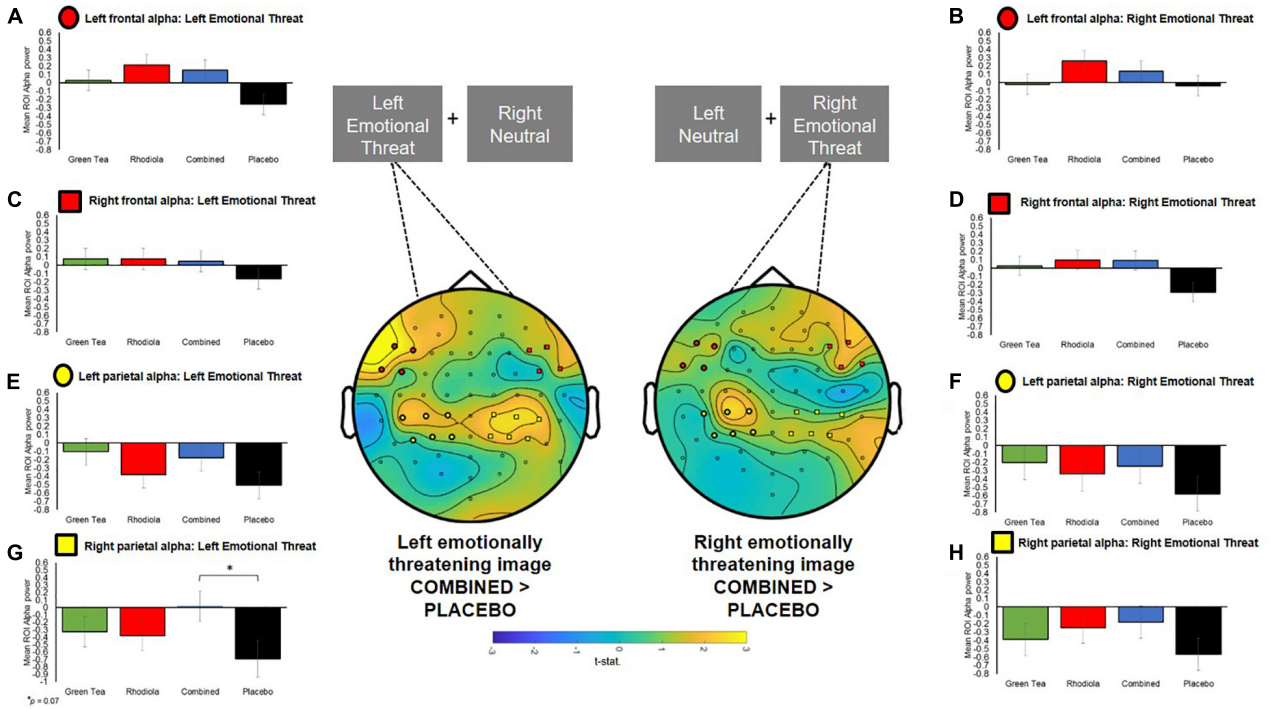


FIGURE 6 | Central figure: T-statistics maps of alpha difference between COMBINED and PLACEBO treatments 0.5–0.75 s after presentation of the images, distinguished by left and right emotionally threatening image presentation. Positive values in maps indicate COMBINED > PLACEBO. ROI electrodes symbols shown on maps and in respective summary bar charts. **(A–D)** Ipsilateral and contralateral frontal *a priori* defined ROI alpha band power when emotionally threatening image shown on left and right. **(E–H)** Ipsilateral and contralateral centro-parietal *a priori* defined ROI alpha band power when emotionally threatening image shown on left and right.

activity and subjective responses to acute stress provocation (5), the combination of these ingredients was shown to induce significantly greater effects, particularly in relation to theta activity. The study findings are discussed in relation to each attentional task employed below.

Attention Switching

Overall, spectral EEG analysis during the attention switching task showed a trend for frontal midline theta to increase in the COMBINED treatment compared to the PLACEBO treatment, with the RHODIOLA and GREEN TEA treatments showing intermediate effects. This trend of heightened theta in the COMBINED treatment reached significance during pre-switch trials vs. PLACEBO and RHODIOLA treatments. Thus, demonstrating a trial specific heightened frontal theta activation in the COMBINED treatment during execution of the attention switching pre-switch trials. Contrary to this, alpha band spectral activity showed no treatment difference during the attention switching task.

Frontal theta activity has been implicated in the operation of numerous domains of cognitive function (6). Heightened frontal theta activity is most commonly associated with executive function and working memory performance, but also correlates to a diverse range of cognitive processes including: error processing (22), attention (23), and episodic memory (24). Tasks typically shown to increase frontal midline theta are those characterised by non-specific, focussed or sustained attention or concentration, or resistance to intrusion (6). This association with a broad range of higher-order cognitive functions has led to the proposal that frontal theta is the putative "language" of a prefrontal network, in which action monitoring and top-down control mechanisms interact to optimise performance (25, 26). Heightened frontal midline theta may be particularly relevant to the realisation of a need to exert greater cognitive control, for example, when required to implement adaptive control in situations incorporating conflicting stimulus-response requirements (27). This may account for the observed increased heightened frontal midline theta in the COMBINED treatment reaching significance in the pre-switch trials, due to the anticipated need to exert greater cognitive control for the impending switch in No-Go response on the following switch trial. This heightened frontal midline theta in the COMBINED treatment is indicative of enhanced processing capacity during the execution of the attention switching task. Since performance on attention switching tasks is vulnerable to the impairing effects of stress (8), this effect may be due to the previously demonstrated capacity of the COMBINED treatment to induce a relaxed focussed state and reduce subjective rating of stress and anxiety (5).

Evidence of potential theta activity-related enhanced attentional capacity in the COMBINED treatment was not reflected in behavioural performance on the attention switching

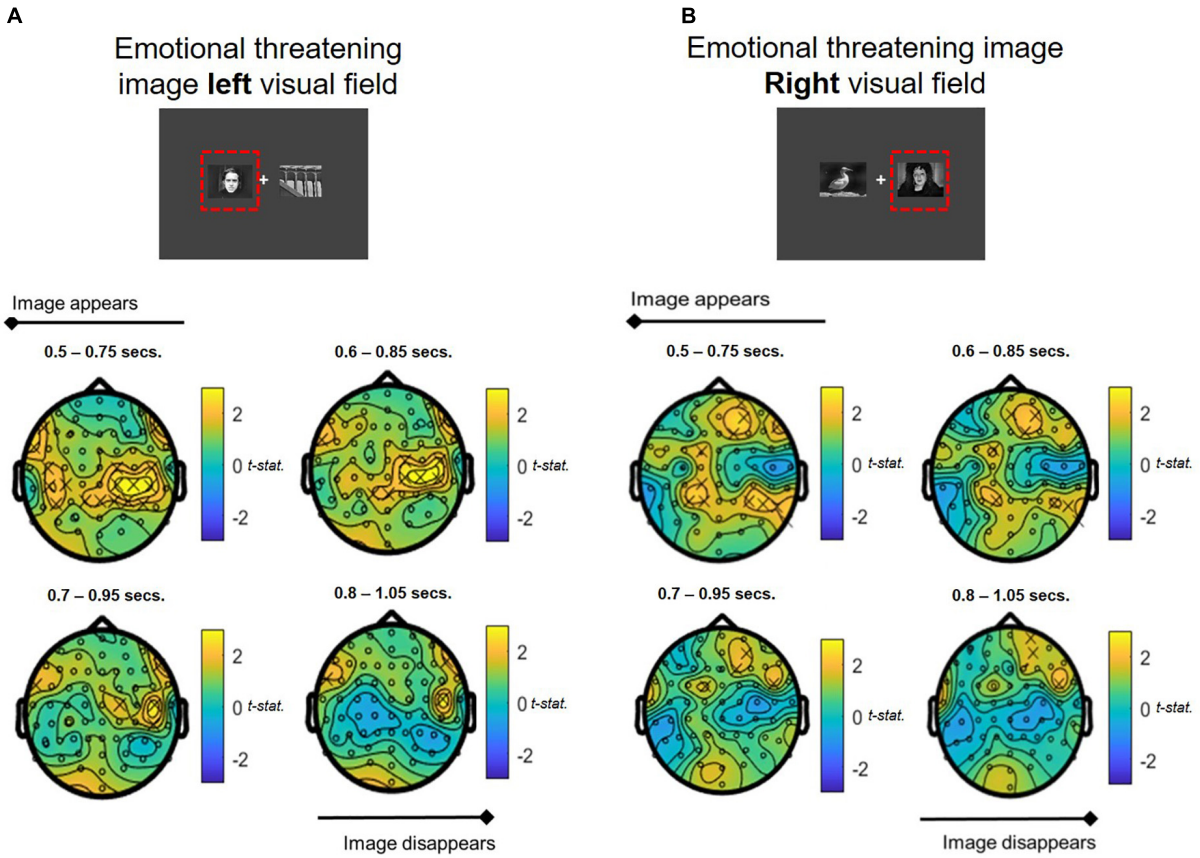


FIGURE 7 | Theta band power differences between COMBINED and PLACEBO treatments during presentation of emotionally threatening and neutral images when stress images shown in **(A)** LEFT and **(B)** RIGHT visual fields. Values > 0 indicate COMBINED treatment theta values are higher than PLACEBO treatment. Significant electrodes ($p < 0.05$) marked with an *x*. Each figure depicts a series of *T*-tests along the time course of the presentation of the images (0.5–1.05 s, in 0.1 s steps, 0.25 s sample widths).

task. The COMBINED treatment demonstrated a tendency toward higher accuracy and slower RTs – a cognitive performance pattern previously associated with heightened frontal midline theta (28) – but no significant performance differences between treatments were found. The attention switching task may not have been sensitive enough to demonstrate any potential enhancing effects of increased attentional capacity suggested by increased theta activation. However, this task employs cognitive processes closely associated with frontal theta activity – namely: focussed attention, executive control, resistance to intrusion – and is therefore a prime candidate task to confirm any positive behavioural effects of treatment. Attention switching tasks have also been previously shown to be sensitive to the enhancing effects of micronutrient interventions [e.g., green tea (29)]. However, the employment of alternative tasks may better demonstrate the potential behavioural enhancements of the COMBINED treatment.

The COMBINED treatment did not significantly moderate alpha activity during the attention switching task despite evidence of constituent ingredients previously being shown to increase alpha activity during attentional performance [L-theanine (1–3)]. Alpha activity has traditionally been associated general alertness and arousal states (30). A role for alpha has further been proposed in operation of selective attentional mechanisms (31); specifically, during attentional processing that requires the inhibition of non-essential/distracting stimuli (1). However, existing evidence of alpha activity involvement in selective attentional processing, and the capacity of green tea constituents to moderate this activity, primarily relates to the anticipatory suppression of distracting visual stimulation during the switching of attention between sensory modalities, particularly between visual and aural stimuli (1, 32). These findings were specific to the inferior and posterior parietal cortex, suggesting a principal engagement with visual processing regions (32). Therefore, the attention switching task employed here may not have modulated alpha activity due to less engagement of visuo-spatial attentional processes. Further, the capacity of green tea constituents to moderate alpha activity may be specific to the attentional switching tasks that primarily engage visuo-spatial processing.

Attentional Bias

Overall, whole brain spectral EEG analysis during the attentional bias task showed a trend for theta to be higher for the

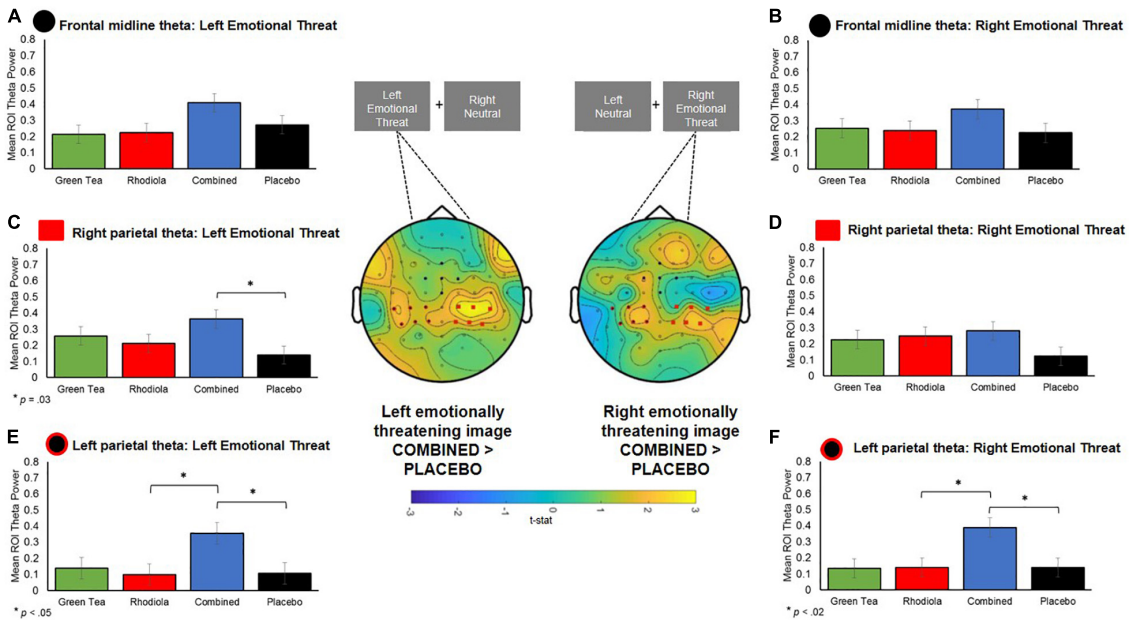


FIGURE 8 | Central figure: T-statistic maps of spectral theta activity difference between COMBINED and PLACEBO treatments 0.5–0.75 s after presentation of the images, distinguished by left and right emotionally threatening image presentation. Positive values in maps indicate COMBINED > PLACEBO. ROI electrodes symbols shown on maps and in respective summary bar chart. **(A,B)** Theta band activity in *a priori* defined ROI midline frontal region for left and right presented images. **(C,D)** RIGHT parietal *a priori* defined ROI theta activity when emotionally threatening image shown on left and right. **(E,F)** LEFT parietal *a priori* defined ROI theta activity when emotionally threatening image shown on left and right.

COMBINED compared to PLACEBO treatment. This effect was predominantly seen in the contralateral (and sometimes ipsilateral) central parietal area. ROI analysis confirmed that this was a strong effect, with left and right images eliciting significant contralateral activation and ipsilateral in the right parietal region a short time after the emotionally threatening images were presented. The COMBINED treatment elicited significantly greater contralateral left parietal theta activation vs. PLACEBO and the RHODIOLA treatments and contralateral right parietal theta activation vs. PLACEBO treatment. *Post hoc* analyses showed the COMBINED treatments also significantly heightened left parietal contralateral theta activation vs. the GREEN TEA treatment ($p = 0.02$); suggesting the combination of the ingredients may be crucial in the observed effects. Significantly higher ipsilateral left parietal activation was also shown in the COMBINED treatment vs. PLACEBO and RHODIOLA treatments.

The attentional processing of affective stimuli is prioritised by the visual attention system to permit prioritised perceptual processing of threat-relevant stimuli – whilst paying less attention to non-threat relevant stimuli (33). This permits the rapid preparation of adaptive behavioural responses, such as avoidance of threat. Increased theta activation is associated with the viewing of affective stimuli such as emotional or threatening images, and facial expressions (34). Early theta activity in response to viewing threatening stimuli is commonly shown in the occipital and parietal-occipital lobes with later responses focussed in the frontal region (35–37); a regional and temporal specificity replicated here. These findings suggest the COMBINED treatment enhanced attentional processing function during an emotionally threatening attentional task. The greater contralateral activation – relative to the presentation of emotionally threatening images in the left and right visual field – suggest this effect was most pronounced for emotionally threatening stimuli. Such attentional vigilance to threatening stimuli is known to be heightened by stress states, with stress induction shown to narrow attentional processes toward threat salient stimuli (38, 39). However, we have previously reported the COMBINED treatment significantly attenuated subjective stress immediately prior to the attentional bias task (5), so a heightened stress response does not account for the difference in theta activation observed in the COMBINED treatment. The COMBINED treatment also demonstrated significantly increased ipsilateral left parietal theta when emotionally threatening images were presented in the left visual field. This is suggestive of increased attentional processing of non-emotionally threatening stimuli which may indicate a general increase in attentional processing function in the COMBINED treatment irrespective of the affective content of stimuli.

Evidence of increased attentional processing capacity in the COMBINED treatment during the attention bias task was not supported by the behavioural data which demonstrated no significant or meaningful (21) difference between treatments in responsiveness to stimuli. It is important to note the observed increased theta activity relates to the early processing of the attentional bias task images. The heightened theta peaked immediately upon viewing the images (0.5–0.75 ms) and subsided as the trial progressed. Therefore, this activity related to early

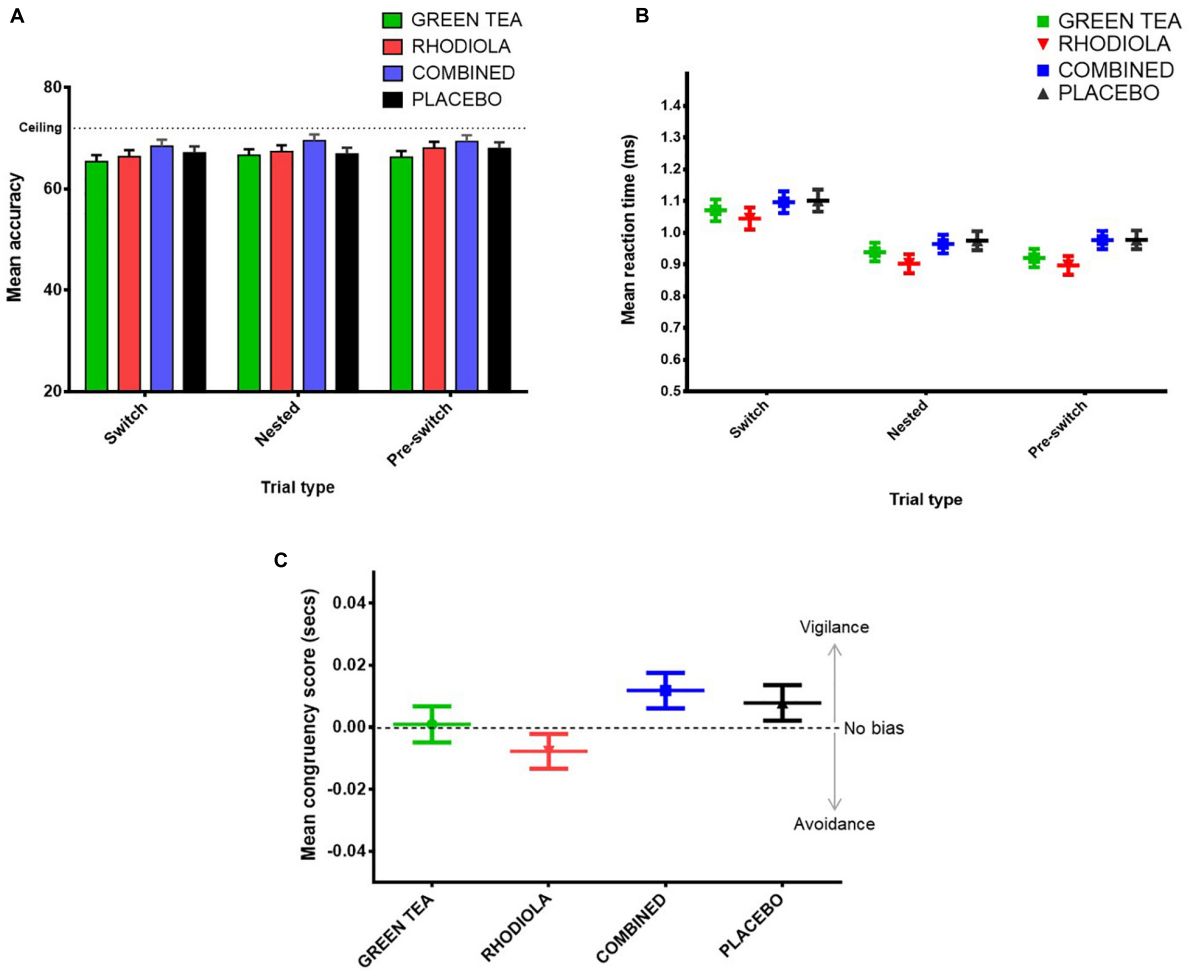


FIGURE 9 | Attention switching task **(A)** mean accuracy and **(B)** RT (ms) by trial type and treatment, and **(C)** attentional bias task attentional congruency score (incongruent trials RT – congruent trials RT) by treatment.

attentional processing of emotionally threatening images may not have been sufficient to impact upon behavioural responses which were temporally removed from initial attentional processing-related theta activation. The stimulus latency employed on the attentional bias task (500 ms) was specifically selected as shorter stimulus exposure times (≤150 ms) have been shown to be less sensitive to demonstration of a relationship between threat processing and theta activity (40). However, a reduction in the delay between stimulus presentation and behavioural response (≥150–≤500 ms) may increase the likelihood of demonstrating any behavioural effects of the heightened attentional capacity suggested by the theta response in the COMBINED treatment.

Spectral EEG analysis during the attentional bias task also demonstrated significantly higher alpha band activity in left and right parietal regions (and ipsilateral in the left parietal cortex) in the COMBINED treatment compared to the PLACEBO treatment. This significance did not follow through into the *a priori* ROI analysis; however, it is evident from **Figure 6** that the whole brain results are driven by a larger contra and ipsilateral decrease in alpha band activity in the PLACEBO group, compared to all other treatments and particularly the COMBINED group treatment. Decreased alpha power has traditionally been associated with increased task engagement, for example heightened perceptual judgement or attentiveness (41, 42). The degree of suppression of alpha is considered to index the level of task demand or relevance (30, 43–45). Whilst the significance of alpha modulation by the COMBINED treatment did not retain significance in the ROI analysis, the mean alpha power values indicate that this difference was predominantly the result of a decrease in alpha power in the PLACEBO treatment being attenuated in the COMBINED treatment, and to a lesser extent RHODIOLA and GREEN TEA treatments. This is suggestive of a greater level of attentional engagement with the stress imagery in the PLACEBO treatment. However, the proposed relationship between alpha inhibition and attentional engagement is largely reflective of the operation of non-affective attentional processes; the relationship between alpha activity and affective attentional processes is inconsistent and at times contradictory (46), including evidence of no modulation of alpha power (47, 48), increased alpha power

(49, 50) and decreased alpha power (51, 52) during the processing of affective stimuli. Therefore, the lack of significant ROI treatment differences and the inconsistent pattern of relationship between affective attentional processing and alpha activity limits further interpretation of the findings.

Attention Switching Event-Related Potential

No significant effects of treatment on attention related ERPs during the attention switching task were found. The lack of significant treatment differences for specific attention-related ERPs during this task is perhaps not surprising considering the lack of behavioural performance differentiation between treatments. The enhanced spectral EEG activity observed in the COMBINED treatment may therefore represent a general increase in attentional functional capacity rather than relating to specific indices of attentional processing on the attention switching task that would be expected to be shown in the ERP data, and further reflected in behavioural performance outputs. The lack of any treatment effects may also be due to the absence of the well-characterised differentiation between switch, nested and pre-switch trials typically observed in this attention switching paradigm. This lack of differentiation between trial types may be as a result of this attention switching task being performed under conditions of stress; a context in which, to the authors' knowledge, the characteristic ERP response for this task has yet to be confirmed.

Limitations and Future Directions

We acknowledge the proposed enhanced attentional capacity suggested by the modulation of theta activity during the employed attentional tasks was not supported by the behavioural or ERP data. This disassociation between EGG spectral activity proposed to index enhanced neural functional capacity and observed behavioural performance is not uncommon in the literature [e.g., (1, 9)]. However, behavioural confirmation of the observed EEG effects of the COMBINED treatment would strengthen the evidence of the potential cognitive enhancing effects of this combination of ingredients. The employed tests may not have been sensitive enough to capture any behavioural improvements associated with the enhanced cognitive capacity indicated by the EEG data. However, both employed tasks have been widely proven to be sensitive to the effects of stress and extensively applied and well characterised in EEG experimentation. Future attempts to confirm the potential for this combination of ingredients to enhance attentional performance should consider the use of a multi-modal visuo-spatial attentional switching task, and vary the delay between exposure to emotionally threatening images and behavioural response for the attentional bias task.

Timing of exposure to the attentional tasks may have been a potential factor in the lack of observed effects on behavioural performance. Impaired cognitive performance under stress can often occur only during the period when cortisol and sympathetic arousal are concurrently activated (53, 54). We have previously reported that the cortisol response was still elevated during completion of the attentional tasks, but the sympathetic response had returned to baseline levels (5). The timing of cognitive testing was constrained by the methodological requirements of the EEG, however, cognitive effects may be more likely demonstrated if cognitive testing is undertaken during peak sympathetic and cortisol activation. Increasing the sensitivity of the attentional tasks to the modulating effects of acute stress may increase the likelihood that the enhanced theta activation observed has more room to confer protective effects on detriments in performance.

Future studies of the observed effects should consider recording dietary intake prior to participation to assess any impact of basal dietary status on measured outcomes; for example, via the completion of a daily food intake diary. However, the accuracy of self-reported dietary intake recall is often questioned, especially when considering the small margins of scale in levels of available vitamin, minerals and botanicals relevant here. Biochemical assessment of baseline levels would be required to give a more meaningful insight into baseline levels. However, this was not feasible in a trial of this size.

Conclusion

We further demonstrate the combination of green tea and rhodiola extract, magnesium and a B vitamin complex (B_6, B_9, and B_{12}) offers potential functional benefits under conditions of stress. This combination of ingredients has previously been demonstrated to induce a relaxed, attentive state (indexed by increased resting theta activity) and attenuate subjective stress and anxiety under conditions of acute stress (5). Here we further demonstrate the capacity of these ingredients to modulate, predominantly, theta activity during the execution of two distinct attentional tasks. These findings suggest increased attentional capacity following the intake of the COMBINED treatment. Further examination of these ingredients in relation to attentional performance is warranted to demonstrate the potential of this heightened attentional capacity to protect or enhance behavioural performance under condition of stress.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available. Due to its proprietary nature the study data cannot be made openly available. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the School of Psychology Research Ethics Committee, University of Leeds. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was an investigator led trial sponsored by the University of Leeds research team. This study received funding from Sanofi. The funder was not involved in the study design, collection, analysis, interpretation of data, writing of this article, or decision to submit it for publication.

ACKNOWLEDGMENTS

We acknowledge the contribution of Fiona Croden (HARU lab manager) and stress panel confederates to conducting the trial, and all the participants that generously gave their time.

REFERENCES

1. Gomez-Ramirez M, Higgins BA, Rycroft JA, Owen GN, Mahoney J, Shpaner M, et al. The deployment of intersensory selective attention: a high-density electrical mapping study of the effects of theanine. *Clin Neuropharmacol.* (2007) 30:25–38. doi: 10.1097/01.wnf.0000240940.13876.17
2. Juneja LR, Chu DC, Okubo T, Nagato Y, Yokogoshi H. L-theanine - a unique amino acid of green tea and its relaxation effect in humans. *Trends Food Sci Technol.* (1999) 10:199–204. doi: 10.1016/s0924-2244(99)00044-8
3. Nobre AC, Rao A, Owen GN. L-theanine, a natural constituent in tea, and its effect on mental state. *Asia Pacific J Clin Nutrit.* (2008) 17:167–8.
4. Dimpfel W. Neurophysiological effects of rhodiola rosea extract containing capsules (a double-blind, randomised, placebo-controlled study). *Int J Nutrit Food Sci.* (2014) 3:157–65. doi: 10.11648/j.ijnfs.20140303.14
5. Boyle NB, Billington J, Lawton C, Quadt F, Dye L. A combination of green tea, rhodiola, magnesium and B vitamins modulates brain activity and protects against the effects of induced social stress in healthy volunteers. *Nutrit Neurosci.* (2021):1–15. [Online ahead of print]. doi: 10.1080/1028415X.2021.1909204
6. Mitchell DJ, McNaughton N, Flanagan D, Kirk IJ. Frontal-midline theta from the perspective of hippocampal "theta". *Prog Neurobiol.* (2008) 86:156–85. doi: 10.1016/j.pneurobio.2008.09.005
7. Wylie GR, Javitt DC, Foxe JJ. Task switching: a high-density electrical mapping study. *NeuroImage.* (2003) 20:2322–42. doi: 10.1016/j.neuroimage.2003.08.010
8. Plessow F, Kiesel A, Kirschbaum C. The stressed prefrontal cortex and goal-directed behaviour: acute psychosocial stress impairs the flexible implementation of task goals. *Exp Brain Res.* (2012) 216:397–408. doi: 10.1007/s00221-011-2943-1
9. Gomez-Ramirez M, Kelly SP, Montesi JL, Foxe JJ. The effects of L-theanine on alpha-band oscillatory brain activity during a visuo-spatial attention task. *Brain Topogr.* (2009) 22:44–51. doi: 10.1007/s10548-008-0068-z
10. Bar-Haim Y, Lamy D, Pergamin L, Bakermans-Kranenburg MJ, van Ijzendoorn MH. Threat-related attentional bias in anxious and nonanxious individuals: a meta-analytic study. *Psychol Bull.* (2007) 133:1–24. doi: 10.1037/0033-2909.133.1.1
11. World Medical Association. World medical association declaration of helsinki: ethical principles for medical research involving human subjects. *JAMA.* (2013) 310:2191–4. doi: 10.1001/jama.2013.281053
12. Lovibond SH, Lovibond PF. *Manual for the Depression and Anxiety Stress Scales*. 2nd ed. Balwyn, VIC: Psychology Foundation (1995).doi: 10.1037/t01004-000
13. Colavincenzo J. *SAS$Global Forum*. Las Vegas, NV: Pharmaceutical SAS User Group (2021).
14. Lang PJ, Bradley MM, Cuthbert BN. *International Affective Picture SYSTEM (IAPS): Affective Ratings of Pictures and Instruction Manual.* (Vol. Technical Report A-8). Gainesville, FL: University of Florida (2008).
15. Delorme A, Makeig S. EEGLAB: an open source toolbox for analysis of single-trial EEG dynamics including independent component analysis. *J Neurosci Methods.* (2004) 134:9–21. doi: 10.1016/j.jneumeth.2003.10.009
16. Oostenveld R, Fries P, Maris E, Schoffelen JM. FieldTrip: open source software for advanced analysis of MEG, EEG, and invasive electrophysiological data. *Comput Intell Neurosci.* (2011) 2011:156869. doi: 10.1155/2011/156869
17. Delorme A, Sejnowski T, Makeig S. Enhanced detection of artifacts in EEG data using higher-order statistics and independent component analysis. *NeuroImage.* (2007) 34:1443–9. doi: 10.1016/j.neuroimage.2006.11.004
18. Nolan H, Whelan R, Reilly RB. FASTER: fully automated statistical thresholding for EEG artifact rejection. *J Neurosci Methods.* (2010) 192:152–62. doi: 10.1016/j.jneumeth.2010.07.015
19. Kirschbaum C, Pirke KM, Hellhammer DH. The trier social stress test - A tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology.* (1993) 28:76–81. doi: 10.1159/000119004
20. Kriegeskorte N, Simmons WK, Bellgowan PSF, Baker CI. Circular analysis in systems neuroscience: the dangers of double dipping. *Nat Neurosci.* (2009) 12:535–40. doi: 10.1038/nn.2303
21. Zvielli A, Bernstein A, Koster EHW. Dynamics of attentional bias to threat in anxious adults: bias towards and/or away? *PLoS One.* (2014) 9:e104025–104025. doi: 10.1371/journal.pone.0104025
22. Luu P, Tucker DM, Makeig S. Frontal midline theta and the error-related negativity: neurophysiological mechanisms of action regulation. *Clin Neurophysiol.* (2004) 115:1821–35. doi: 10.1016/j.clinph.2004.03.031
23. Makeig S, Westerfield M, Jung TP, Enghoff S, Townsend J, Courchesne E, et al. Dynamic brain sources of visual evoked responses. *Science.* (2002) 295:690–4. doi: 10.1126/science.1066168
24. Nyhus E, Curran T. Functional role of gamma and theta oscillations in episodic memory. *Neurosci Biobehav Rev.* (2010) 34:1023–35. doi: 10.1016/j.neubiorev.2009.12.014
25. Cohen MX. Error-related medial frontal theta activity predicts cingulate-related structural connectivity. *NeuroImage.* (2011) 55:1373–83. doi: 10.1016/j.neuroimage.2010.12.072
26. van Driel J, Ridderinkhof KR, Cohen MX. Not all errors are alike: theta and alpha EEG dynamics relate to differences in error-processing dynamics. *J Neurosci.* (2012) 32:16795–806. doi: 10.1523/JNEUROSCI.0802-12.2012
27. Cavanagh JF, Frank MJ. Frontal theta as a mechanism for cognitive control. *Trends Cogn Sci.* (2014) 18:414–21. doi: 10.1016/j.tics.2014.04.012
28. Gevins A, Smith ME, McEvoy L, Yu D. High-resolution EEG mapping of cortical activation related to working memory: effects of task difficulty, type of processing, and practice. *Cerebral Cortex.* (1997) 7:374–85. doi: 10.1093/cercor/7.4.374
29. Camfield DA, Stough C, Farrimond J, Scholey AB. Acute effects of tea constituents L-theanine, caffeine, and epigallocatechin gallate on cognitive function and mood: a systematic review and meta-analysis. *Nutrit Rev.* (2014) 72:507–22. doi: 10.1111/nure.12120
30. Klimesch W, Doppelmayr M, Russegger H, Pachinger T, Schwaiger J. Induced alpha band power changes in the human EEG and attention. *Neurosci Lett.* (1998) 244:73–6. doi: 10.1016/s0304-3940(98)00122-0
31. Foxe JJ, Simpson GV, Ahlfors SP. Parieto-occipital similar to 10 Hz activity reflects anticipatory state of visual attention mechanisms. *Neuroreport.* (1998) 9:3929–33. doi: 10.1097/00001756-199812010-00030

32. Fu KM, Foxe JJ, Murray MM, Higgins BA, Javitt DC, Schroeder CE. Attention-dependent suppression of distracter visual input can be cross-modally cued as indexed by anticipatory parieto-occipital alpha-band oscillations. *Brain Res Cognit Brain Res.* (2001) 12:145–52. doi: 10.1016/s0926-6410(01)00034-9
33. Brosch T, Pourtois G, Sander D. The perception and categorisation of emotional stimuli: a review. *Cognit Emot.* (2010) 24:377–400. doi: 10.1080/02699930902975754
34. Gűntekin B, Başar E. A review of brain oscillations in perception of faces and emotional pictures. *Neuropsychologia.* (2014) 58:33–51. doi: 10.1016/j.neuropsychologia.2014.03.014
35. Aftanas LI, Golocheikine SA. Human anterior and frontal midline theta and lower alpha reflect emotionally positive state and internalized attention: high-resolution EEG investigation of meditation. *Neurosci Lett.* (2001) 310:57–60. doi: 10.1016/s0304-3940(01)02094-8
36. Aftanas LI, Pavlov SV. Trait anxiety impact on posterior activation asymmetries at rest and during evoked negative emotions: EEG investigation. *Int J Psychophysiol.* (2005) 55:85–94. doi: 10.1016/j.ijpsycho.2004.06.004
37. Aftanas LI, Pavlov SV, Reva NV, Varlamov AA. Trait anxiety impact on the EEG theta band power changes during appraisal of threatening and pleasant visual stimuli. *Int J Psychophysiol.* (2003) 50:205–12. doi: 10.1016/s0167-8760(03)00156-9
38. Rued HA, Hilmert CJ, Strahm AM, Thomas LE. The influence of stress on attentional bias to threat: an angry face and a noisy crowd. *Psychonomic Bull Rev.* (2019) 26:943–50. doi: 10.3758/s13423-018-1538-2
39. Arnsten AFT. Cognition, emotion and autonomic responses: the integrative role of the prefrontal cortex and limbic structures. In: Uylings HBM, Van Eden CG, Bruin J.P.C. De, Feenstra MGP, Pennartz CMA editors. *Progress in Brain Research*. (Vol. 126), Amsterdam: Elsevier Science (2000). p. 183–92.
40. Putman P. Resting state EEG delta-beta coherence in relation to anxiety, behavioral inhibition, and selective attentional processing of threatening stimuli. *Int J Psychophysiol.* (2011) 80:63–8. doi: 10.1016/j.ijpsycho.2011.01.011
41. Bazanova OM, Vernon D. Interpreting EEG alpha activity. *Neurosci Biobehav Rev.* (2014) 44:94–110. doi: 10.1016/j.neubiorev.2013.05.007
42. Niedermeyer E, Lopes da Silva F. *Electroencephalography: Basic Principles, Clinical Applications, and Related Fields*. 5th ed. Philadelphia, PA: Williams & Wilkins (2004).
43. Klimesch W. EEG alpha and theta oscillations reflect cognitive and memory performance: a review and analysis. *Brain Res Rev.* (1999) 29:169–95. doi: 10.1016/s0165-0173(98)00056-3
44. Boiten F, Sergeant J, Geuze R. Event-related desynchronization: the effects of energetic and computational demands. *Electroencephalogr Clin Neurophysiol.* (1992) 82:302–9. doi: 10.1016/0013-4694(92)90110-4
45. Klimesch W, Pfurtscheller G, Mohl W, Schimke H. Event-related desynchronization, ERD-mapping and hemispheric differences for words and numbers. *Int J Psychophysiol.* (1990) 8:297–308. doi: 10.1016/0167-8760(90)90020-E
46. Uusberg A, Uibo H, Kreegipuu K, Allik J. EEG alpha and cortical inhibition in affective attention. *Int J Psychophysiol.* (2013) 89:26–36.
47. Balconi M, Lucchiari C. EEG correlates (event-related desynchronization) of emotional face elaboration: a temporal analysis. *Neurosci Lett.* (2006) 392:118–23. doi: 10.1016/j.neulet.2005.09.004
48. Müller MM, Keil A, Gruber T, Elbert T. Processing of affective pictures modulates right-hemispheric gamma band EEG activity. *Clin Neurophysiol.* (1999) 110:1913–20. doi: 10.1016/S1388-2457(99)00151-0
49. Aftanas LI, Reva NV, Varlamov AA, Pavlov SV, Makhnev VP. Analysis of evoked EEG synchronization and desynchronization in conditions of emotional activation in humans: temporal and topographic characteristics. *Neurosci Behav Physiol.* (2004) 34:859–67. doi: 10.1023/B:NEAB.0000038139.39812.eb
50. Aftanas LI, Varlamov AA, Pavlov SV, Makhnev VP, Reva NV. Time-dependent cortical asymmetries induced by emotional arousal: EEG analysis of event-related synchronization and desynchronization in individually defined frequency bands. *Int J Psychophysiol.* (2002) 44:67–82. doi: 10.1016/s0167-8760(01)00194-5
51. De Cesarei A, Codispoti M. Affective modulation of the LPP and α-ERD during picture viewing. *Psychophysiology.* (2011) 48:1397–404.doi: 10.1111/j.1469-8986.2011.01204.x
52. Everhart DE, Demaree HA. Low alpha power (7.5–9.5 Hz) changes during positive and negative affective learning. *Cognit Affect Behav Neurosci.* (2003) 3:39–45. doi: 10.3758/CABN.3.1.39
53. Elzinga BM, Roelofs K. Cortisol-induced impairments of working memory require acute sympathetic activation. *Behav Neurosci.* (2005) 119:98–103. doi: 10.1037/0735-7044.119.1.98
54. Tollenaar MS, Elzinga BM, Spinhoven P, Everaerd WAM. The effects of cortisol increase on long-term memory retrieval during and after acute psychosocial stress. *Acta Psychol.* (2008) 127:542–52. doi: 10.1016/j.actpsy.2007.10.007

Conflict of Interest: LD has received consultancy and honoraria for work in the area of stress from Sanofi.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Boyle, Dye, Lawton and Billington. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 10 August 2022
DOI 10.3389/fnut.2022.951415

Check for updates

OPEN ACCESS

EDITED BY
Andrew Scholey,
Swinburne University of Technology, Australia

REVIEWED BY
Anchalee Rawangkan,
University of Phayao, Thailand
Keiko Unno,
University of Shizuoka, Japan
Ren-You Gan,
Institute of Urban Agriculture (CAAS), China

*CORRESPONDENCE
Zhonghua Liu
zhonghua-liu@hunau.edu.cn
Shuxian Cai
caishuxian@hunau.edu.cn

SPECIALTY SECTION
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

RECEIVED 24 May 2022
ACCEPTED 27 July 2022
PUBLISHED 10 August 2022

CITATION
Xie X, Wan J, Zheng X, Pan W, Yuan J, Hu B, Feng M, Liu Z and Cai S (2022) Synergistic effects of epigallocatechin gallate and l-theanine in nerve repair and regeneration by anti-amyloid damage, promoting metabolism, and nourishing nerve cells. *Front. Nutr.* 9:951415. doi: 10.3389/fnut.2022.951415

COPYRIGHT
© 2022 Xie, Wan, Zheng, Pan, Yuan, Hu, Feng, Liu and Cai. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Synergistic effects of epigallocatechin gallate and l-theanine in nerve repair and regeneration by anti-amyloid damage, promoting metabolism, and nourishing nerve cells

Xinya Xie[1,2,3], Juan Wan[1,2,3], Xin Zheng[1,2,3], Wenjing Pan[1,2,3], Jiayi Yuan[1,2,3], Baozhu Hu[1,2,3], Meiyan Feng[1,2,3], Zhonghua Liu[1,2,3]* and Shuxian Cai[1,2,3]*

[1]National Research Center of Engineering Technology for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha, China, [2]Key Laboratory of Ministry of Education for Tea Science, Hunan Agricultural University, Changsha, China, [3]Co-Innovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha, China

Green tea has significant protective activity on nerve cells, but the mechanism of action is unclear. Epigallocatechin gallate (EGCG) and *N*-ethyl-L-glutamine (L-theanine) are the representative functional components of green tea (*Camellia sinensis*). In this study, an AD model of $A\beta_{25-35}$-induced differentiated neural cell line PC12 cells was established to study the synergistic effect of EGCG and L-theanine in protecting neural cells. The results showed that under $A\beta_{25-35}$ stress conditions, mitochondria and axons degenerated, and the expression of cyclins was up-regulated, showing the gene and protein characteristics of cellular hyperfunction. EGCG + L-theanine inhibited inflammation and aggregate formation pathways, significantly increased the percentage of G0/G1 in the cell cycle, downregulated the expression of proteins such as p-mTOR, Cyclin D1, and Cyclin B1, upregulated the expression of GAP43, Klotho, p-AMPK, and other proteins, promoted mitochondrial activity and energy metabolism, and had repair and regeneration effects on differentiated nerve cells. The synergistic mechanism study showed that under the premise that EGCG inhibits amyloid stress and inflammation and promotes metabolism, L-theanine could play a nourish nerve effect. EGCG + L-theanine keeps differentiated nerve cells in a quiescent state, which is beneficial to the repair and regeneration of nerve cells. In addition, EGCG + L-theanine maintains the high-fidelity structure of cellular proteins. This study revealed for the first time that the synergistic effect

of EGCG with L-theanine may be an effective way to promote nerve cell repair and regeneration and slow down the progression of AD. Our findings provide a new scientific basis for the relationship between tea drinking and brain protection.

KEYWORDS

EGCG, L-theanine, synergistic effects, β-amyloid stress, neuroinflammation, cell cycle regulation

Introduction

Proteotoxic stress, mitochondrial dysfunction, and genomic instability lead to cellular hyperactivity and are closely associated with age-related degenerative diseases such as atherosclerosis, type 2 diabetes, osteoporosis, and Alzheimer's disease (1–3). Under various stressful conditions, cells promote mTOR or other growth factors sensitive growth-stimulating pathways, causing cells to exhibit hyperactivity.

Following damage to the genome and epigenome of cells, many senescence-related secretions are mainly transcribed through NF-κB, secreting interleukins, growth factors, proteases, cytokines, matrix-degrading enzymes, and metalloproteinases into the extracellular space. Furthermore, it mediates insulin resistance, blocks cellular signaling feedback, accelerates senescence in neighboring cells, and mediates the development of senescent phenotypes. Pathological amyloid Aβ is the most neurotoxic agrin with a high aggregation rate. Many drugs can effectively inhibit the formation of Aβ, but clinical studies have no effect. Earlier studies identified Aβ amyloid plaques in the extracellular matrix, and recent studies have also found low levels of amyloid plaques in the intracellular Golgi apparatus, endoplasmic reticulum, and mitochondria (4, 5). Nerve cell axons are rich in mitochondria and prone to degeneration of nerve cells and axons under β-amyloid stress (6).

Alzheimer's disease is a neurodegenerative disease characterized by dysregulation of the neuronal cell cycle, leading to cell death (7). Cell cycle markers are present in degenerating AD neurons in G1, S, G2, or M phases, indicating that terminally differentiated neurons re-enter the cell cycle. When the cell cycle is reactivated, the function and integrity of terminally differentiated neurons are disrupted. Furthermore, inappropriate activation of cyclins alters the function of DNA-binding proteins, thereby affecting the overall structure and further DNA damage by exacerbating oxidative damage by various endonucleases (8). Therefore, AD and other related neurodegenerative diseases can be effectively treated by inhibiting amyloid stress, regulating cell cycle, keeping cells in a quiescence state, improving energy metabolism, and promoting axonal growth (9, 10).

Currently, there is much literature on the neuroprotective effects of green tea (*Camellia sinensis*) and its core functional components Epigallocatechin gallate (EGCG) and *N*-ethyl-L-glutamine (L-theanine) (11–14). The molecular structures of EGCG and L-theanine are shown in **Figure 1**. EGCG is a flavonoid that belongs to the gallate-esterified flavan-3-ols. EGCG accounts for about 9–13% of the dry tea weight of tea leaves, and 50–80% of the total catechins. EC, EGC, and ECG each account for about 5–15% of the total catechins (15). EGCG significantly protects biological macromolecular structures such as proteins and phospholipids by inhibiting the formation of β-sheet, a toxic seed structure of aggregates (16, 17). EGCG is a mitochondria-targeted drug that modulates mitochondrial metabolism, including mitochondrial biogenesis, and mitochondrial-mediated cell cycle and apoptosis (18, 19). In addition, EGCG can also promote axonal growth in PC12 cells (20). L-theanine, a non-protein water-soluble amino acid, is characteristically found in tea plants (21). L-theanine content accounts for about 1–2% of the dry weight of tea leaves, and the content of L-theanine in high-theanine tea tree varieties is about 4% or more (22). There are 26 amino acids identified in tea, of which 6 are non-protein amino acids. L-theanine

Abbreviations: $A\beta_{25-35}$, β-amyloid fragment 25-35; EGCG, (-)-epigallocatechinegallate; L-theanine, *N*-ethyl-L-glutamine; FTIR, Fourier transform infrared spectroscopy; ThT, thioflavin T; TEM, transmission electron microscope; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; ATP, adenosine triphosphate; ROS, reactive oxygen species; GAP43, growth associated protein-43; TNF-α, tumor necrosis factor-α; BACE1, beta-site amyloid cleaving enzyme-1; Aβ42, amyloid 1-42; IL-6, interleukin-6; NF-kB, nuclear factor kappa-B; mTOR, mammalian target of rapamycin; P-mTOR, phospho-mammalian target of rapamycin; Sirt1, Sirtuin1; UPs, ubiquitin conjugated proteins; 4-HNE, 4-hydroxynonenal; RAGE, receptor for advanced glycation endproducts; p62, sequestosome 1; SASP, the senescence-associated secretory phenotype; MMP, mitochondrial membrane potential; LDH, lactate dehydrogenase; JC-1, it is an ideal fluorescent probe widely used to detect mitochondrial membrane potential; DAPI, It is a fluorescent dye that can bind strongly to DNA and is often used in fluorescence microscopy; TPM, trans per kilobase of exon model per million mapped reads; BDNF, brain-derived neurotrophic factor; 50 μM $A\beta_{25-35}$, $A\beta_{25-35}$; 50 μM $A\beta_{25-35}$/50 μM EGCG, $A\beta_{25-35}$/EGCG; 50 μM $A\beta_{25-35}$/50 μM L-theanine, $A\beta_{25-35}$/L-theanine; 50 μM $A\beta_{25-35}$/50 μM EGCG + 50 μM L-theanine, $A\beta_{25-35}$/EGCG + L-theanine.

accounts for about 50–60% of the total amino acids in tea (22). L-theanine has cerebral protective activities such as anti-anxiety, anti-depression, memory promotion, prevention of vascular dementia, and nutritional nerve (23–26). L-theanine can inhibit glutamate receptors, increase intracellular glutamine and glutathione concentrations, attenuate oxidative damage, and have neuroprotective effects (24). In the SAPM8 brain aging model, L-theanine significantly inhibits the concentration of Aβ42 and down-regulates the Aβ42 formation pathways such as PS1, BACE1, and p16 (27).

Epigallocatechin gallate is mainly stored in the vacuoles of fresh tea leaves and has protective effects on tea trees such as anti-ultraviolet rays and anti-insect pests. L-theanine is synthesized in the roots of tea trees, and has a high content in the actively growing tea buds and other tissues, providing carbon and nitrogen sources for the growth of tea trees (28, 29). The repairing effect of EGCG and the growth-promoting effect of L-theanine are beneficial to the growth of tea tree shoots. This study speculates that EGCG and L-theanine have a synergistic effect on neuroprotection. PC12, a cell line derived from rat pheochromocytoma, has been widely used as an *in vitro* model to study neuronal apoptosis and differentiation (30). We established an AD cell model through PC12 cells to study the synergistic protective effect of EGCG and L-theanine on differentiated neural lineage cells.

Materials and methods

Materials

Both EGCG (≥99%) and L-theanine (≥99%) were purchased from Sigma-Aldrich (St Louis, MO, United States). In addition to the secondary antibody (Abcam, CA, United Kingdom), the following primary antibodies were used for western blot analysis: anti-GAPDH, anti-Histone H3, anti-CyclinD1, anti-CyclinB1, anti-IL6, anti-AMPK, anti-mTOR, anti-Sirt1, anti-NF-κB, anti-Nrf2, and anti-KEAP1 (Cell Signaling Technology, Boston, United States), anti-Klotho (Novusbio, Colorad, United States), both RAGE and β-Amyloid (Santa Cruz Biotechnology, San Cruise, United States), anti-p62 (Epitomics, Burlingame, United States), anti-4-HNE (Millipore, Boston, United States), Anti-Multi Ubiquitin mAb (MBL, Tokyo, Japan). Western chemiluminescent horseradish peroxidase substrate (Thermo Fisher Scientific, Waltham, MA, United States), protease inhibitor mixture (Beyotime, Shanghai, China), BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA, United States), and radioimmunoprecipitation assay (RIPA) lysis buffer (KeyGEN BioTECH, Nanjing, China) were also used in the experiments.

Incubation and preparation of different $A\beta_{25-35}$ protein samples

Epigallocatechin gallate (1 mM), L-theanine (1 mM), and EGCG (1 mM) + L-theanine (1 mM) were mixed with $A\beta_{25-35}$ (1 mM) at a concentration of 1 mM, respectively, and incubated in a 37°C incubator for 7 days (31). After incubation, they were diluted 20 times and added to cells for use. That is, the final concentrations used in cell experiments were 50 μM $A\beta_{25-35}$, 50 μM $A\beta_{25-35}$/50 μM EGCG, 50 μM $A\beta_{25-35}$/50 μM L-theanine, and 50 μM $A\beta_{25-35}$/50 μM EGCG + 50 μM L-theanine. In the description of the results that follow in the article, 50 μM $A\beta_{25-35}$, 50 μM $A\beta_{25-35}$/50 μM EGCG, 50 μM $A\beta_{25-35}$/50 μM L-theanine, and 50 μM $A\beta_{25-35}$/50 μM EGCG + 50 μM L-theanine are abbreviated as $A\beta_{25-35}$, $A\beta_{25-35}$/EGCG, $A\beta_{25-35}$/L-theanine, and $A\beta_{25-35}$/EGCG + L-theanine, respectively.

Fluorescence detection of thioflavin T

Thioflavin T fluorescent probes bind to β-sheet-rich molecules to emit fluorescence. ThT fluorescent probes are ideal for detecting amyloid aggregates in protein misfolding research (32, 33). Mixed 10 μL of aliquots of $A\beta_{25-35}$ solution (1 mM) with 90 μL of ThT (80 μg/mL) in PBS. The fluorescence was measured at λex and λem of 440 and 485 nm, respectively, using a fluorescence microplate reader (Thermo, Waltham, MA, United States).

Cell culture

Rat pheochromocytoma cell lines (PC12 cells) were from Peking Union Medical College, Cell Bank (Beijing, China) and grown in Dulbecco's modified Eagle medium (DMEM) (Cromwell, CT, United States) containing 10% fetal bovine serum (FBS) (BI, IL), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 95% air and 5% CO_2. During cell culture, cells were passaged approximately every 2 days.

$A\beta_{25-35}$ insult, drug treatment, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay

PC12 cells were seeded in a 96-well cell culture plate at a density of 10^4/well and cultured for 24 h. Then the cells were treated with different $A\beta_{25-35}$ protein samples ($A\beta_{25-35}$, $A\beta_{25-35}$/EGCG, $A\beta_{25-35}$/L-theanine, and $A\beta_{25-35}$/EGCG + L-theanine) for 24 h. Cell viability was detected by MTT

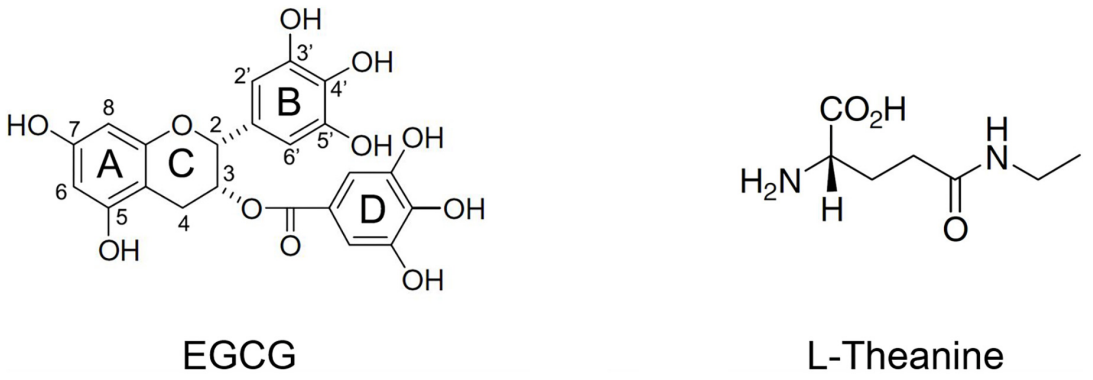


FIGURE 1
Molecular structures of the active ingredients Epigallocatechin gallate (EGCG) and *N*-ethyl-L-glutamine (L-theanine) in tea.

colorimetric assay. Briefly, the supernatants were exchanged with a medium containing 0.5 g/ml MTT for 4 h, the superments were removed, and 150 μL/well dimethyl sulfoxide (DMSO) was added. The absorbance was detected at 570 nm. The relative cell viability was expressed as the mean percentage of absorbance in treated vs. control cells. The value of the control was set at 100%.

Transmission electron microscopic examination

Cell samples from different treatment groups were collected and double-fixed in 2.5% glutaraldehyde solution containing Millonig's phosphate buffer (pH = 7.3). Examined and photographed on a Hitachi HT-7700 electron microscope after double staining with 3% uranyl acetate and lead nitrate.

Flow measurement of cell cycle and apoptosis

After collecting cells, washed twice with pre-chilled PBS. The cell cycle determination steps were as follows: added 2 mL of 70% ethanol, vortex to mix the cells, incubated at −20°C for 2 h, removed the ethanol by centrifugation, and washed twice with PBS, divided the cells into 5 mL flow cytometry tubes, added 500 μL PI/Rnase staining buffer, mixed the cells, incubated at room temperature in the dark for 15 min, stored at 4°C, and detected by flow cytometry within 1 h. The steps for apoptosis assay were as follows: added 1 × cell binding buffer, divided the cells into 5 mL flow cytometry tubes, each tube contained 1×10^5 cells, added 5 μL each of FITC Annexin V and PI dye, and protected from light until incubated for 15 min at room temperature, added 400 μL 1 × cell-binding buffer to each tube, and detected by flow cytometry (FACSCalibur system, BD Biosciences) within 1 h.

Fourier transform infrared spectroscopy spectral analysis

Cell samples from different treatment groups were collected for freeze-drying and infrared spectroscopy analysis. Cells protein samples were prepared by freeze-drying method, and vacuum freeze-dried at −42°C for 24 h. Samples were detected by Fourier transform Infrared spectroscopy (FTIR) (34, 35). The spectral range was 4000–400 cm^{-1}. It was recorded in reflectance mode with a spectral resolution of 4 cm^{-1}, accumulating 64 scans per spectrum. Amide I bands (1700–1600 cm^{-1}) were used to determine the secondary structure of proteins in cell samples from different treatment groups, and spectra of original untreated spectra were synthesized and analyzed using omnic software (Thermo Nicolet, Waltham, United States).

JC1 detection of cell mitochondrial membrane potential

Mitochondrial membrane potential was detected using a fluorescent dye mitochondrial membrane potential detection kit (JC-1) staining kit (Beyotime, Shanghai, China) (36). PC12 cells were seeded on 24-well cell slides at a density of 5×10^4/well, and the cells were cultured according to the experimental method in Sections "Cell culture" and "$A\beta_{25-35}$ insult, drug treatment, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay." Subsequent experimental operations were carried out in strict accordance with the kit instructions. After the experiment, the staining of cells in different treatment groups was observed by s fluorescence microscope (Zeiss, Axio scope. A1). In the dual-color staining of JC-1, the higher the ratio of red/green fluorescence intensity, the higher the mitochondrial membrane potential.

DAPI fluorescent staining

After cell experiment, the cells were washed three times in the dark with PBS, mounted with Antifade Mounting Medium with DAPI (Beyotime, Shanghai, China), and then photographed with a fluorescence microscope (Zeiss, Axio scope. A1).

LDH cytotoxicity assay

The lactate dehydrogenase kit (Beyotime, Shanghai, China) detected lactate dehydrogenase activity released by cells in different treatment groups. PC12 cells were seeded in a 96-well cell culture plate at a density of 1×10^4/well, and cells were cultured according to the experimental methods in Sections "Cell culture" and "$A\beta_{25-35}$ insult, drug treatment, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay." After the experiment, the cell culture medium of different treatment groups was collected and centrifuged (400 g, 5 min) to remove dead cells in the culture medium. Finally, the LDH in the culture medium was detected strictly according to the operation method of the kit.

Adenosine triphosphate detection

Usually, a drop in ATP levels indicates impaired or decreased mitochondrial function (37). PC12 cells were seeded in a 6-well culture plate at 1.5×10^5/well, and cultured according to the experimental methods in Sections "Cell culture" and "$A\beta_{25-35}$ insult, drug treatment, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay." After the experiment, the medium was discarded, washed twice with pre-cooled PBS, 200 μL of cell lysate was added to each well, centrifuged (4°C, 12000 rpm, 20 min), and the supernatant was collected. The subsequent ATP detection experiments were performed according to the Adenosine triphosphate (ATP) detection kit (Beyotime, Shanghai, China).

Western blotting assay

After cell experiments were completed, cells were washed twice with pre-chilled PBS, an appropriate lysis buffer containing 1 × cocktail (Fermentas) and RIPA buffer (Pplygen), and the cells were lysed for 30 min on ice, vortexing every 10 min. The supernatant was collected by centrifugation (4°C, 12000 rpm, 20 min). Protein concentrations were determined using the BCA kit (Thermo, Waltham, United States), and equal amounts of protein (30 μg) per sample were separated by SDS-PAGE electrophoresis. After electrophoresis, the proteins on the gel were transferred to PVDF (Millipore, Boston, United States) membranes and blocked with TBST buffer containing 5% non-fat milk at room temperature for 1 h with slow shaking. Primary antibodies were added, overnight at 4°C. After washing with TBST for 5 min × 5 times at room temperature, the PVDF membranes were incubated with appropriate secondary antibodies for 1 h. After washing with TBST for 5 min × 5 times, the PVDF membranes were reacted with an enhanced chemiluminescence substrate (Piece Rockford, United States) for 2∼5 min. The luminescence signals were then detected by a chemiluminescence gel imaging system, and finally the optical density (OD) value of each band was analyzed using Image-J software.

Enzyme-linked immunosorbent assay

The TNF-α expression levels in the cell protein samples of different treatment groups were detected strictly according to the ELISA kit (Jiangsu Feiya Biological Technology Co. Ltd.) operation method.

BODIPY fluorescent staining

PC12 cells were seeded on 24-well cell slides at a density of 5×10^4/well, and the cells were cultured according to the experimental method in Sections "Cell culture" and "$A\beta_{25-35}$ insult, drug treatment, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay." After the experiment, the cells were washed twice with PBS, and 200 Ml/well of fixative was added for 10 min. After washing twice with PBS, BODIPY staining solution (Invitrogen, New York, United States) was added and incubated for 30 min in an incubator. Finally, the staining of cells in different treatment groups was observed by s fluorescence microscope (Zeiss, Axio scope. A1).

RNA sequencing and gene expression analysis

After the cell experiment, the collected cell samples were immediately pre-frozen in liquid nitrogen for 2 min, frozen in dry ice and sent to BGI (Shenzhen, China[1]) for transcriptome sequencing (RNA-seq) detection. The expression level of each gene was calculated using the number of fragments per kilobase of transcript (FPKM) after normalizing the expression level of each gene. Differentially expressed genes (DEGs) were screened by the screening conditions of $|$ fold changes$| \geq 2$ and Q value ≤ 0.05, and correlation analysis was performed in different treatment groups. In order to more comprehensively explore

1 www.genomics.cn

the mechanism of action from the transcriptome level, with |fold changes| $\geq$ 1.2 and Q value $\leq$ 0.05 as the screening conditions, more DEGs were screened for network interaction analysis. In addition, to explore the synergistic mechanism of EGCG and L-theanine, according to the DEGs of the $A\beta_{25-35}$/EGCG + L-theanine treatment group, a heat map analysis of the expression of related genes in different treatment groups was performed. Finally, STRING[2] and Cytoscape (United States, v3.8.2) software were used to analyze the network interaction and visualize visualization of DEGs in different blocks in the heat map.

Statistical analysis

Using GraphPad Prism 8.01 software (GraphPad Software Inc., San Diego, United States), combined with Turkey's multiple comparison test, a one-way ANOVA test was used to analyze the significance of differences. The results were expressed as mean $\pm$ standard deviation. The variation was judged significant at $p < 0.05$ and highly significant at $p < 0.01$.

Results

Epigallocatechin gallate and L-theanine synergistically protect PC12 cells from Aβ $_{25-35}$-induced injury

PC12 cells were incubated with different concentrations of EGCG or L-theanine for 24 h. EGCG had no significant effect on cell viability in the 50 μM concentration range, but significantly decreased cell viability at 100 μM concentration ($p < 0.01$). L-theanine promoted cell growth in a concentration-dependent manner in the 100 μM range. The above results revealed that EGCG had no growth-promoting effect on cells, and had cytotoxicity at a high concentration of 100 μM; while L-theanine had a cell growth-promoting effect at a concentration of 100 μM (**Figures 2A,B**).

Soluble oligopeptide β-sheet (A11+) structure, the seed structure of protein misfolding and protein aggregate formation, is the toxic core structure of amyloid (38, 39). $A\beta_{25-35}$ is the neurotoxic fragment of $A\beta_{1-40}$ and $A\beta_{1-42}$ (40, 41). ThT fluorescence probe detection was performed on different $A\beta_{25-35}$ (50 μM) protein samples. The results showed that the β-sheet (All+) structure of the $A\beta_{25-35}$ group was significantly increased ($p < 0.01$); EGCG and EGCG + L-theanine had significant inhibitory activity on β-sheet (All+) structure formation ($p < 0.01$), while the inhibitory activity of L-theanine was not significant (**Figure 2C**). EGCG interacts with Aβ, apparently forming small unstructured Aβ aggregates (42). When $A\beta_{25-35}$ was at 50 μM, EGCG had the best cytoprotective activity at 50 μM concentration. L-theanine from 10 to 100 μM had a significant effect on promoting cell growth. While under $A\beta_{25-35}$ stress conditions, different concentrations of L-theanine had no cytoprotective activity, and the difference in activity was not obvious. Therefore, we determined the concentration of EGCG and L-theanine to be 50 μM each for subsequent synergy studies. Subsequent content abbreviated 50 μM $A\beta_{25-35}$, 50 μM $A\beta_{25-35}$/50 μM EGCG, 50 μM $A\beta_{25-35}$/50 μM L-theanine and 50 μM $A\beta_{25-35}$/50 μM EGCG + 50 μM L-theanine to $A\beta_{25-35}$, $A\beta_{25-35}$/EGCG, $A\beta_{25-35}$/L-theanine and $A\beta_{25-35}$/EGCG + L-theanine, respectively.

Different $A\beta_{25-35}$ protein samples were incubated with PC12 cells for 24 h. The results of MTT and brightfield photography showed that $A\beta_{25-35}$/EGCG, $A\beta_{25-35}$/EGCG + L-theanine, and $A\beta_{25-35}$/L-theanine significantly promoted cell activity, and the $A\beta_{25-35}$/EGCG + L-theanine group had the strongest cell viability, and its activity was better than that of the control group (**Figures 2D,E**).

Epigallocatechin gallate and L-theanine synergistically inhibit amyloidogenic and inflammatory pathways

The most striking features of neurodegenerative diseases are the accumulation of protein aggregates and autophagic dysfunction (43, 44). Ubiquitin-conjugated Proteins (Ups) and p62-modified proteins can be diagnostic tools for the aggregate formation and autophagy dysfunction (45, 46). Western blotting analysis showed that $A\beta_{25-35}$ group cells produced a large amount of β-amyloid, accumulated 4-HNE, Ups and p62-modified aggregates, up-regulated TNF-α content ($p < 0.01$) and RAGE protein expression, and increased NF-κB nuclear transcription level by about 75% ($p < 0.01$). The results showed that the $A\beta_{25-35}$ group had increased cell aggregates formation and up-regulated inflammatory pathways ($p < 0.01$), while EGCG and EGCG + L-theanine had significant inhibitory effects, followed by L-theanine. Nrf2/KEAP1 is an endogenous antioxidant signaling pathway in cells, and Nrf2/KEAP1 is unstable and released under stress conditions (47). The level of nuclear translocation of Nrf2 protein decreased in $A\beta_{25-35}$ group cells ($p < 0.01$), and the unbound keap1 protein increased ($p < 0.01$); EGCG, EGCG + L-theanine, and L-theanine promoted Nrf2 nuclear translocation ($p < 0.01$) and decreased the amount of unbound keap1 protein ($p < 0.01$) (**Figures 3A,B**).

β-sheet formation is a crucial early step in amyloidogenesis (48). The infrared absorption peak spectrum and fitting analysis

2 https://cn.string-db.org/

10.3389/fnut.2022.951415

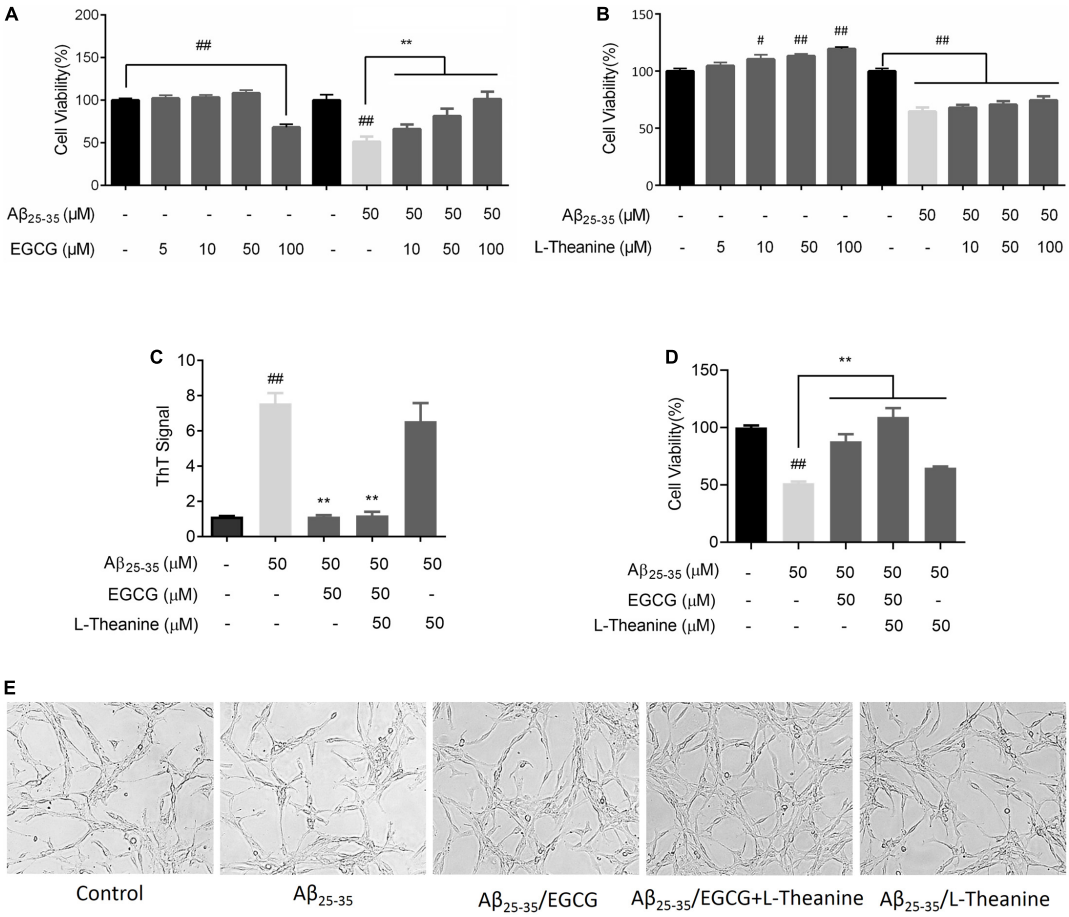


FIGURE 2
EGCG and L-theanine synergistically protect PC12 cells from Abeta$_{25-35}$-induced injury. **(A)** MTT assay of cell viability in EGCG or Aβ$_{25-35}$/EGCG treated group. **(B)** MTT detection of cells viability in L-theanine or Aβ$_{25-35}$/L-theanine treated group. **(C)** ThT assay of different Aβ$_{25-35}$ proteins samples. **(D)** MTT assay of cell viability in the synergistic group of EGCG and L-theanine. **(E)** White light photography of cell morphology. #$p < 0.05$, ##$p < 0.01$, compared with control group; **$p < 0.01$, compared with Aβ$_{25-35}$ group, $n = 6$.

results of the protein amide I band (1700–1600 cm^{-1}) showed that (Figure 3C), the infrared absorption peaks of Aβ$_{25-35}$ group shifted to low wavenumbers, and the β-sheet structure increased significantly, α-helix and disordered structures were reduced. EGCG significantly inhibited the formation of β-sheet structure and promoted the formation of α-helix structure, but still had a great influence on cellular protein structures; L-theanine inhibited the shift of the absorption peak to the lower band; the absorption peaks and secondary structures fitting peaks of the Aβ$_{25-35}$/EGCG + L-theanine group were almost identical to those of the control group.

EGCG + L-theanine targets cell cycle and promotes axonal growth

The results of DAPI nuclear staining showed that the nuclei of the Aβ$_{25-35}$ group had different morphologies, some nuclei were enlarged, and the staining was high, indicating severe DNA damage; the nuclei of Aβ$_{25-35}$/EGCG + L-theanine group were uniform in morphology and light staining, same as the control group; the Aβ$_{25-35}$/EGCG and Aβ$_{25-35}$/L-theanine groups also had some improvement (Figure 4A).

Cell cycle control is closely related to neuronal axonal growth (50). Cyclin D1 and Cyclin B1 are markers of the G1 and G2 phases of the cell cycle, respectively (51). Cyclin D1 and Cyclin B1 are in a low expression state in differentiated neural cells to maintain cell differentiation state and normal function (52). The results of Western blotting showed that the protein expressions of Cyclin D1 and Cyclin B1 in the Aβ$_{25-35}$ group were up-regulated, and the expression of Cyclin D1 increased by about 50% ($p < 0.01$); in Aβ$_{25-35}$/EGCG and Aβ$_{25-35}$/EGCG + L-theanine groups, Cyclin D1 and Cyclin B1 protein expressions were down-regulated ($p < 0.01$), and the Aβ$_{25-35}$/EGCG + L-theanine group was the most significant;

10.3389/fnut.2022.951415

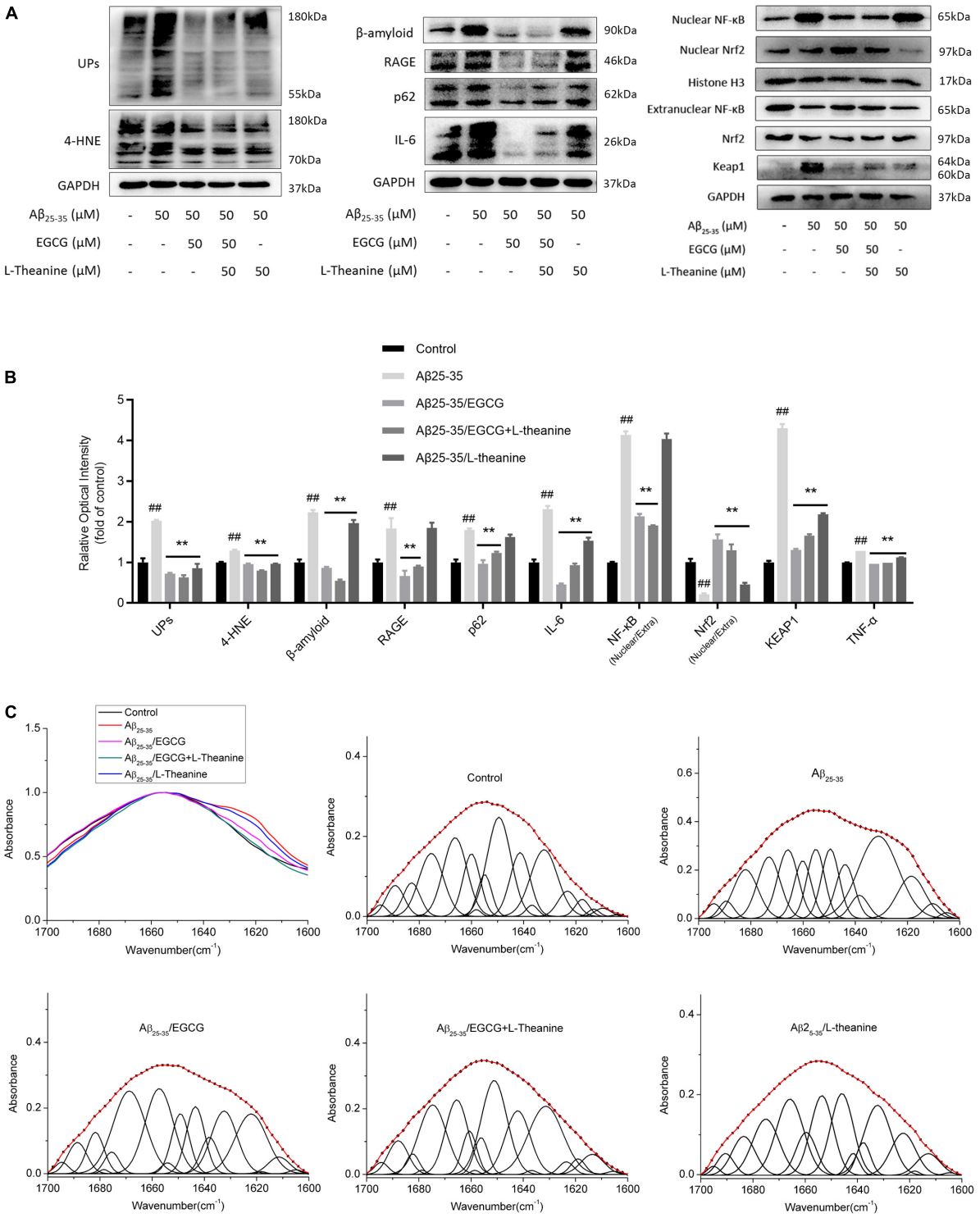


FIGURE 3

EGCG and L-theanine synergistically inhibit amyloidogenic and inflammatory pathways. **(A,B)** ELISA and Western-blotting detection and analysis of protein expression levels associated with aggregates formation and inflammation. Among them, ELISA detected TNF-α, and Western-blotting detected the remaining proteins. "Nuclear/Extra" indicates the degree of nuclear translocation of the associated proteins. **(C)** Amide I band (1700–1600 cm^{-1}) infrared spectra and Gaussian fitting analysis of cell samples in different treatment groups. In the fitted plots, amide I maxima between 1675 and 1695 cm^{-1} are generally assigned to antiparallel β-sheet/aggregated strands; and amide I maxima of 1660–1670 cm^{-1}, 1648–1660 cm^{-1}, 1640–1648 cm^{-1}, 1625–1640 cm^{-1}, and 1610–1628 cm^{-1} are assigned to 3^{10}-helix, α-helix, unordered strands, β-sheet, and aggregated strands, respectively (49). ##$p < 0.01$, compared with control group; **$p < 0.01$, compared with $A\beta_{25-35}$ group, $n = 3$.

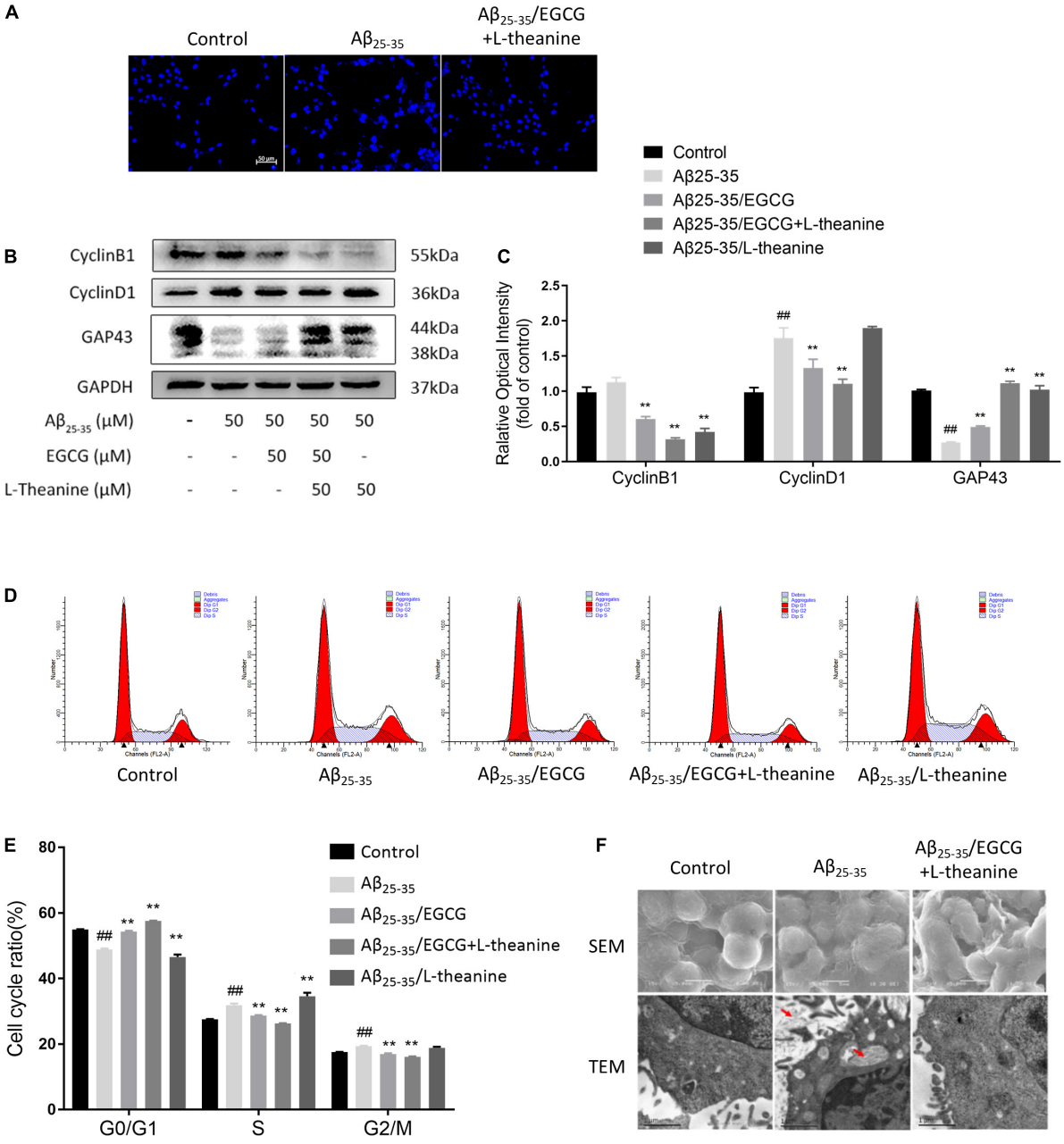


FIGURE 4
EGCG + L-theanine regulates cell cycle and promotes axonal growth. **(A)** DAPI nuclear staining observation (bar = 50 μm). **(B,C)** Western-blotting detection of Cyclin B1, Cyclin D1, and GAP43. **(D)** Flow cytometry analysis of cell cycle and apoptosis. **(E)** Statistical analysis of cell cycle. **(F)** Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis (red arrows point to the cellular fibrous aggregate fraction). ##$p < 0.01$, compared with control group; **$p < 0.01$, compared with $A\beta_{25-35}$ group, $n = 3$.

Cyclin B1 was down-regulated in the $A\beta_{25-35}$/L-theanine group ($p < 0.01$) (Figures 4B,C).

The results of cell cycle detection showed that the percentage of S phase increased by about 5% in the $A\beta_{25-35}$ group ($p < 0.01$). The $A\beta_{25-35}$/EGCG and $A\beta_{25-35}$/EGCG + L-theanine groups increased the percentage of G0/G1 phase ($p < 0.01$) and down-regulated the percentage of S phase ($p < 0.01$), which was more significant in the $A\beta_{25-35}$/EGCG + L-theanine group. The S phase percentage of $A\beta_{25-35}$/L-theanine was the highest (Figures 4D,E). The above results indicate that, under the condition of $A\beta_{25-35}$ toxic stress, EGCG + L-theanine significantly promotes the formation of the quiescent state of cells, and is beneficial to the repair of differentiated nerve cells and the growth of axons.

10.3389/fnut.2022.951415

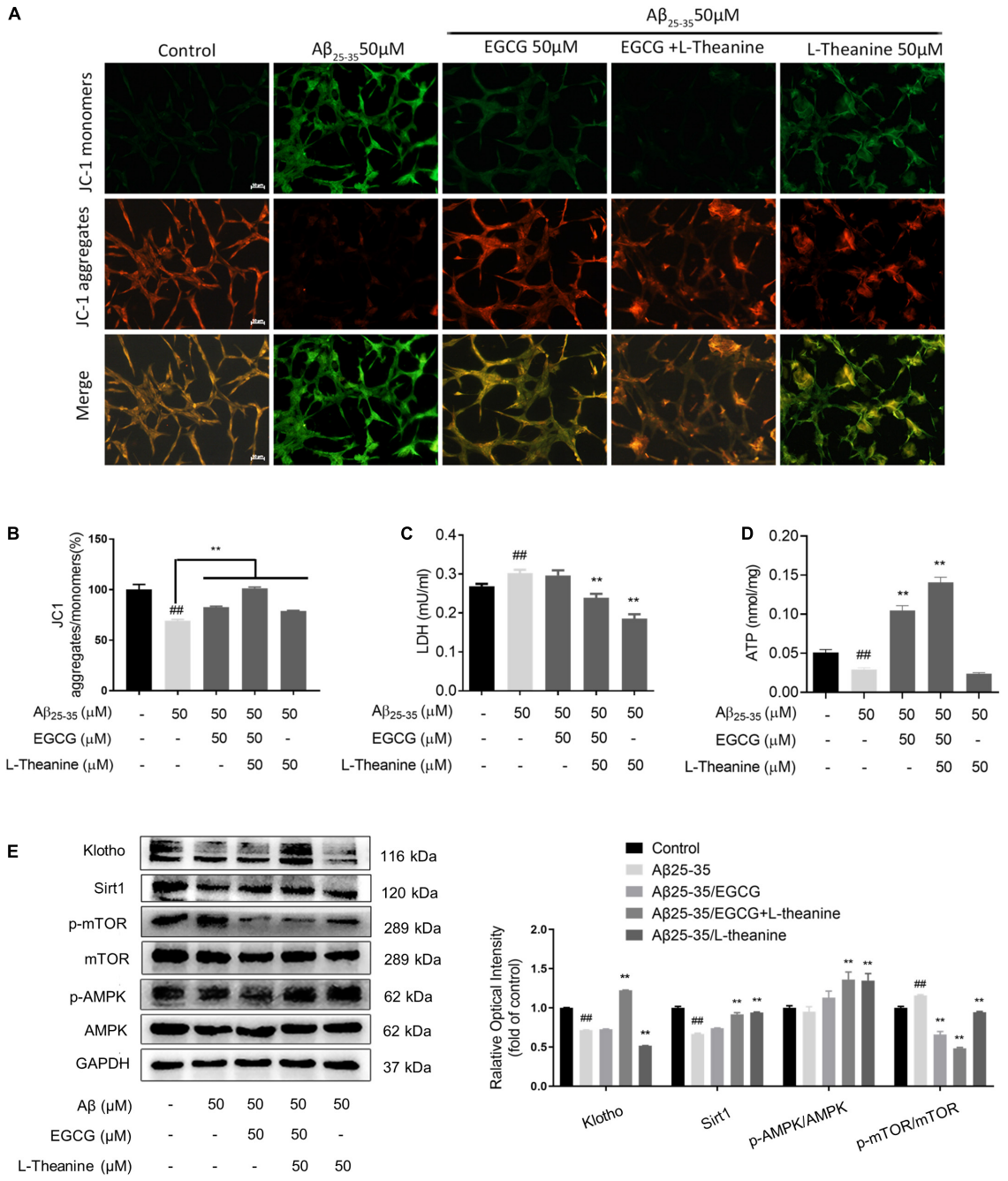


FIGURE 5

EGCG and L-theanine synergistically protect mitochondria and promote energy metabolism. **(A)** JC1 fluorescence staining of mitochondrial membrane potential. **(B)** JC1 staining statistics (bar = 50 μm). **(C,D)** Kit detection of LDH and ATP. **(E)** The expression levels of proteins related to mitochondria and energy metabolism were detected by Western blotting. ##$p < 0.01$, compared with control group; **$p < 0.01$, compared with $A\beta_{25-35}$ group, $n = 3$.

The neurite growth factor GAP43 is a nerve-specific protein involved in nerve cell growth, synapse development and nerve cell regeneration (53). The expression of GAP43 protein in the $A\beta_{25-35}$ group was decreased ($p < 0.01$); compared with the $A\beta_{25-35}$ group, the expressions of the three tea component treatment groups were all up-regulated ($p < 0.01$), among which the $A\beta_{25-35}$/EGCG + L-theanine group expression increased about 4-fold (**Figures 4B,C**).

The scanning electron microscopy (SEM) results showed that cells in the $A\beta_{25-35}$ group had flattened cell bodies and

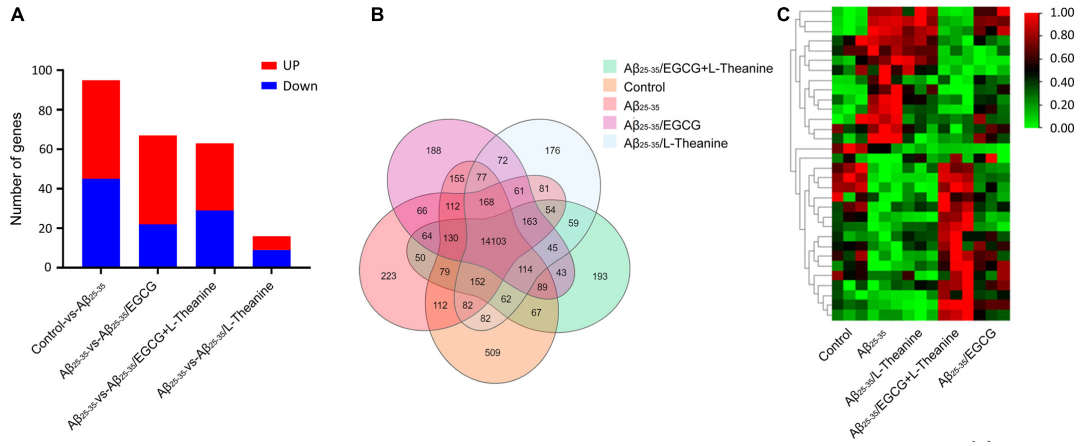


FIGURE 6
Analysis of differentially expressed genes (DEGs) in different treatment groups. **(A)** Statistical analysis of DEGs expression between groups. **(B)** Venn diagram. One of the circles represents the set of DEGs for a treatment group, areas where different circles were superimposed represent DEGs common to different treatment groups, and non-overlapped parts represent DEGs specific to the treatment group. **(C)** Heat map. The color blocks from red to blue indicate the gene expression levels from high to low.

atrophied axons; in the $A\beta_{25-35}$/EGCG + L-theanine group, the cells were plump, with rich surface structures and thick axons. Transmission electron microscopy (TEM) results showed that in the $A\beta_{25-35}$ group cells, there were fibrous aggregates in the cell membrane and intracellular organelles (the part indicated by the red arrow), mitochondrial vacuolation and nucleus heterochromatin aggregates were formed. In contrast, no fibrous aggregates were detected in the $A\beta_{25-35}$/EGCG + L-theanine-treated group cells, and the cell status was significantly improved (Figure 4F).

EGCG + L-theanine protects mitochondria and promotes energy metabolism

Decreased mitochondrial membrane potential (MMP) is a hallmark event in the early stage of apoptosis (54). The results of JC-1 staining showed that the MMP in the $A\beta_{25-35}$ group ($p < 0.01$), while the MMP in the $A\beta_{25-35}$/EGCG, $A\beta_{25-35}$/L-theanine, and $A\beta_{25-35}$/EGCG + L-theanine groups all increased ($p < 0.01$), in which EGCG and L-theanine exhibited a significant synergistic effect (Figures 5A,B). Cellular LDH content increased and ATP production decreased in $A\beta_{25-35}$ group ($p < 0.01$); cellular LDH content decreased in $A\beta_{25-35}$/EGCG + L-theanine and $A\beta_{25-35}$/L-theanine groups ($p < 0.01$) (Figure 5C); compared with $A\beta_{25-35}$ group, cellular ATP production was increased about 4-fold and 6-fold in the $A\beta_{25-35}$/EGCG and $A\beta_{25-35}$/EGCG + L-theanine groups, respectively (Figure 5D). The results indicate that EGCG and L-theanine have different protective effects on mitochondria and have synergistic effects.

The results of mitochondria and energy metabolism-related protein expression detection showed that under $A\beta_{25-35}$ stress conditions, the expression of anti-aging factor Klotho decreased ($p < 0.01$); EGCG and L-theanine alone had no effect, while EGCG + L-theanine up-regulated Klotho expression by about 65% ($p < 0.01$). During calorie restriction, Sirt1 and p-AMPK proteins are highly expressed, while p-mTOR is lowly expressed, closely related to cellular energy metabolism, maintaining the balance of cellular energy supply and demand by affecting multiple links of cellular material metabolism (55, 56). Under $A\beta_{25-35}$ stress conditions, the expression levels of Sirt1 and p-AMPK were decreased, and p-mTOR was significantly increased ($p < 0.01$); EGCG + L-theanine and L-theanine significantly up-regulated Sirt1 and p-AMPK expression levels ($p < 0.01$); in addition, the protein expression level of p-mTOR in the three tea component treatment groups was at a low level ($p < 0.01$) (Figure 5E). The results suggest that L-theanine is more effective than EGCG in activating calorie restriction-like energy metabolism pathways, which maybe related to the high bioavailability of L-theanine (57).

Transcriptome analysis of the synergistic effect of epigallocatechin gallate and L-theanine in protecting nerve cells

This study obtained 699.87 million raw reads from 15 transcriptome cDNA libraries, and the filtered clean reads accounted for more than 91.23%. Comparing valid readings with Rattus_norvegicu (GCF_000001895.5_Rnor_6.0), each sample had a total contrast of over 81.7% and a unique contrast

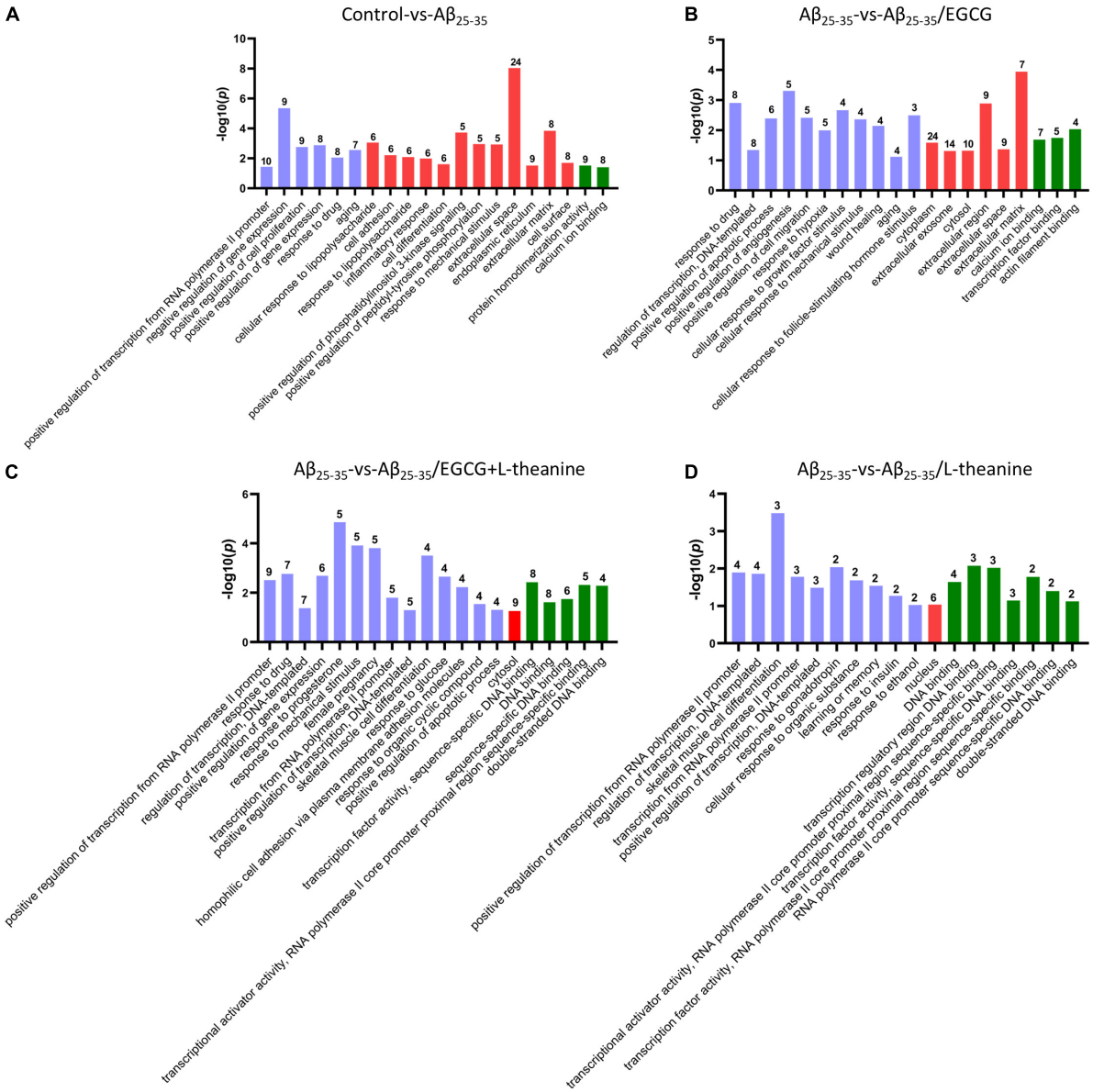


FIGURE 7
GO enrichment analysis of differentiated genes (DEGs) in PC12 cells of different treatment groups. Graphs from panels **(A–D)** represent the GO analysis of Control-vs-Aβ$_{25-35}$, Aβ$_{25-35}$-vs-Aβ$_{25-35}$/EGCG, Aβ$_{25-35}$-vs-Aβ$_{25-35}$/EGCG + L-theanine, and Aβ$_{25-35}$-vs-Aβ$_{25-35}$/L-theanine, respectively. The blue-purple, red, and green columns in the GO enrichment analysis diagrams represent the biological processes, cellular components, and molecular functions of cells in different treatment groups, respectively.

of over 76.65%. Transcriptome sequencing (RNA-seq) is high quality and suitable for subsequent bioinformatic analysis.

Differentially expressed genes enrichment analysis

Differential genes (DEGs) were screened according to the screening conditions of $|$ fold change$|$ $\geq$ 2 and Q $\leq$ 0.05. Compared with the control group, 95 DEGs were identified in the Aβ$_{25-35}$ group. Compared with the Aβ$_{25-35}$ group, 67, 63, and 16 DEGs were in Aβ$_{25-35}$/EGCG group, Aβ$_{25-35}$/EGCG + L-theanine group, and Aβ$_{25-35}$/L-theanine group, respectively (Figure 6A). The results of the Venn diagram showed that there were 14103 co-expressed genes in all treatment groups (Figure 6B). Cluster analysis of DEGs of co-expressed genes showed that the expression trends of DEGs in different treatment groups were significantly different, and Aβ$_{25-35}$/EGCG + L-theanine group showed the opposite expression trend to Aβ$_{25-35}$ (Figure 6C).

GO analysis showed that the different genes (DEGs) in Aβ$_{25-35}$ group cells were significantly enriched in transcription,

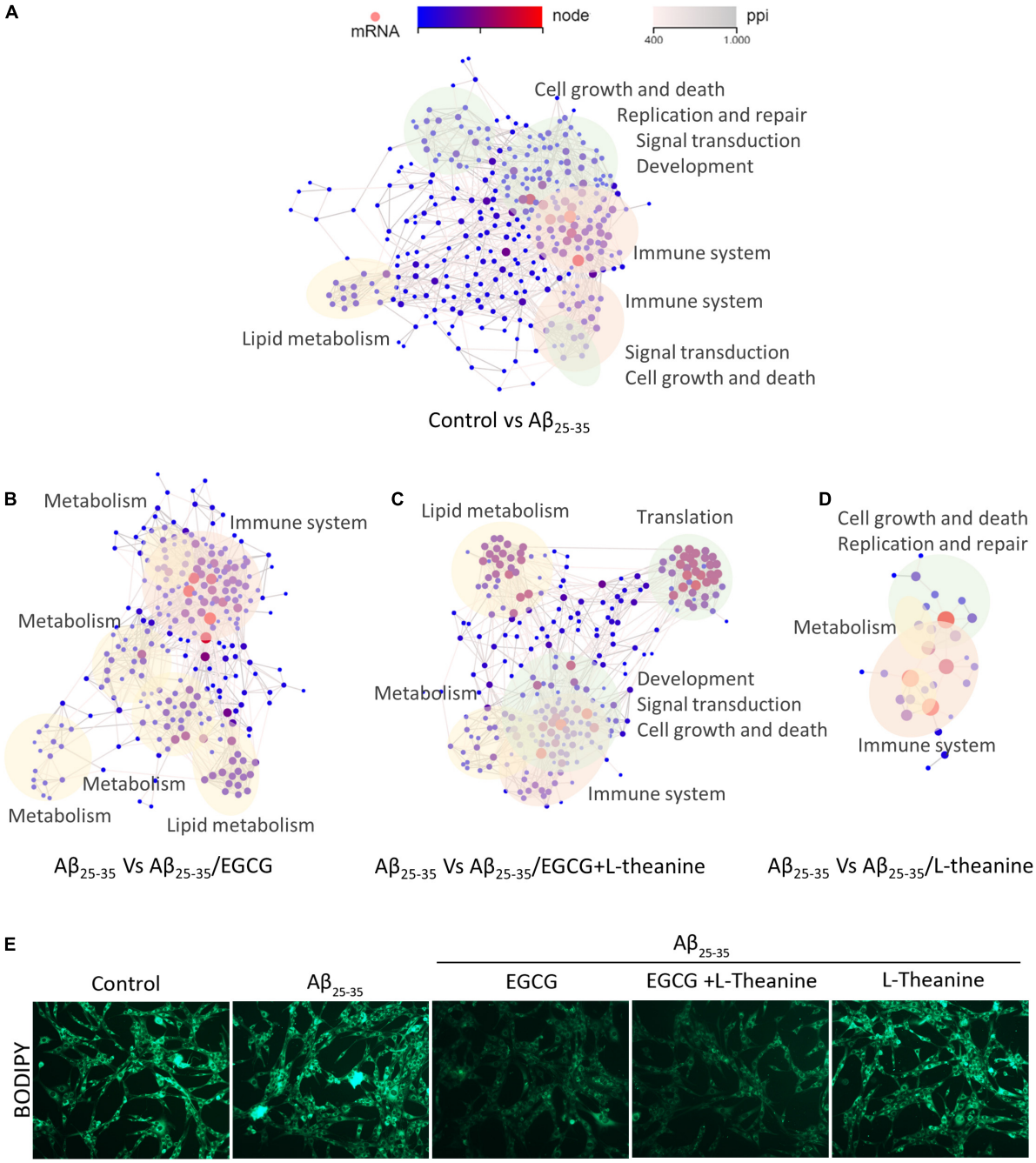


FIGURE 8
Network interaction plot (PPI) analysis of differential genes in different treatment groups. **(A–D)** Interaction analysis of differential genes in different treatment groups. The yellow blocks in the panel represent metabolisms such as lipids, carbohydrates, amino acids, and energy; green blocks represent signal transduction such as cell growth and death, cellular community and endocrine system, genetic information procession, replication, and repair, etc.; pink blocks represent signal transduction, such as inflammation and immune system. The gray lines represent functional associations of differential genes; the nodes from blue to red represent the ranges of enriched differential genes, from panels **(A–D)** are 1∼64, 1∼58, 1∼45, and 1∼9, respectively. **(E)** BODIPY staining of cell lipid droplets in different treatment groups.

cell proliferation, inflammation, aging, and extracellular matrix (Figure 7A). DEGs in the $A\beta_{25-35}$/EGCG group were mainly enriched in cell translation, extracellular matrix, wound healing, cytoplasm, and growth factor stimulation (Figure 7B). The DEGs-enriched pathways in the $A\beta_{25-35}$/L-theanine group and $A\beta_{25-35}$/EGCG + L-theanine group were similar, mainly enriched in DNA or RNA binding transcriptional activity, gonadotropins, female progesterone and cell differentiation

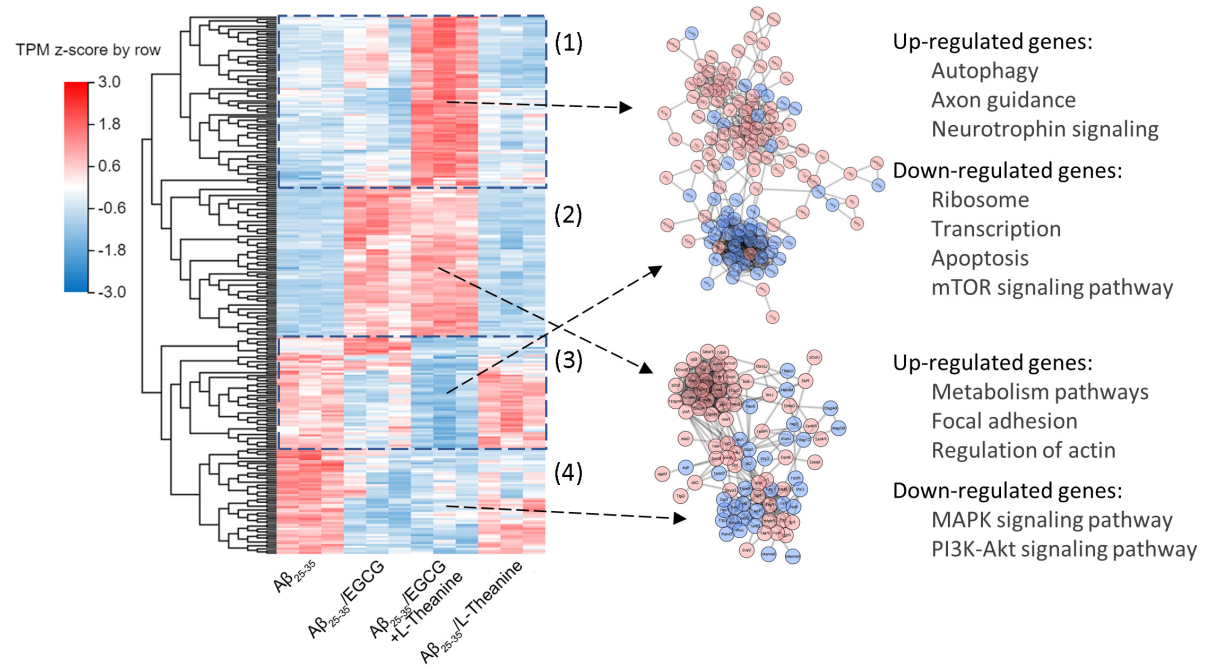


FIGURE 9
Analysis of the synergistic mechanism of EGCG and L-theanine from the transcriptome level. (1–4) The results showed that the cluster map of DEGs in the Aβ25-35-vs-Aβ25-35/EGCG + L-theanine was mainly divided into four blocks, of which (1) and (3) blocks gene expression trend was opposite to that of other treatment groups, and (2) and (4) blocks gene expression trend were very similar to that of $A\beta_{25-35}$-vs-$A\beta_{25-35}$/EGCG. The expression level of each gene was calculated using the transcripts per kilobase of exon model per million mapped reads (TPM) after normalizing the expression level of each gene.

(Figures 7C,D). Progesterone and other estrogenic pathways are neuroprotective against traumatic brain injury by promoting GAP43 expression (58). The above results indicate that $A\beta_{25-35}$ has extensive toxic effects inside and outside cells, while EGCG has extensive intracellular and extracellular repair effects, and L-theanine and EGCG + L-theanine can nourish nerve cells and promote axonal growth, and so on.

Interaction network analysis of differential genes and detection of lipid metabolism

The DEGs interaction network analysis showed that, compared with the control group, the DEGs in the $A\beta_{25-35}$ group (Control-vs-$A\beta_{25-35}$) acted on the following network: metabolism (lipids, carbohydrates, amino acids, and energy metabolism) (Figure 8A yellow blocks), genetic material related (cell growth and death, cellular community and endocrine system, genetic information procession, replication and repair, immune system, and other signal transduction) (Figure 8A green blocks) and the inflammatory immune system (Figure 8A pink blocks). The DEGs of $A\beta_{25-35}$-vs-$A\beta_{25-35}$/EGCG mainly acted on networks such as anti-inflammatory (Figure 8B pink blocks), lipid and energy metabolism pathways (Figure 8B yellow blocks). The DEGs of $A\beta_{25-35}$-vs-$A\beta_{25-35}$/L-theanine mainly act on cell cycle, immune regulation, genetic material such as DNA and RNA (Figure 8C green blocks) and immunomodulation (Figure 8C, pink blocks). The DEGs in $A\beta_{25-35}$-vs-$A\beta_{25-35}$/EGCG + L-theanine mainly interacted in cell cycle, MAPK, metabolism, signal transduction, genetic information processing, and other pathways (Figure 8D yellow, green, and pink blocks). The results indicate that L-theanine fully exerts its regulatory effects on cell cycle, immunity, DNA and RNA, and other genetic materials under EGCG inhibiting $A\beta_{25-35}$ stress and regulating metabolism.

Lipid metabolism is of particular interest due to its high concentration in CNS. Chronic accumulation of phase-separated molecular aggregates such as lipid droplets and amyloid can lead to neurotoxicity (59, 60). The result of lipid droplets BODIPY fluorescence staining showed that under $A\beta_{25-35}$ stress condition, the structure of intracellular lipid droplets became blurred and smaller, or merged into large-area lipid plaques; EGCG and EGCG + L-theanine have significantly reduced the content of intracellular lipid droplets (Figure 8E). This result was corroborated with transcriptomic EGCG promoting lipid and energy metabolism (Figure 8B).

Analysis of the synergistic mechanism of epigallocatechin gallate and L-theanine from the transcriptome level

Using the DEGs of $A\beta_{25-35}$-vs-$A\beta_{25-35}$/EGCG + L-theanine as a reference, the cluster analysis and interaction

10.3389/fnut.2022.951415

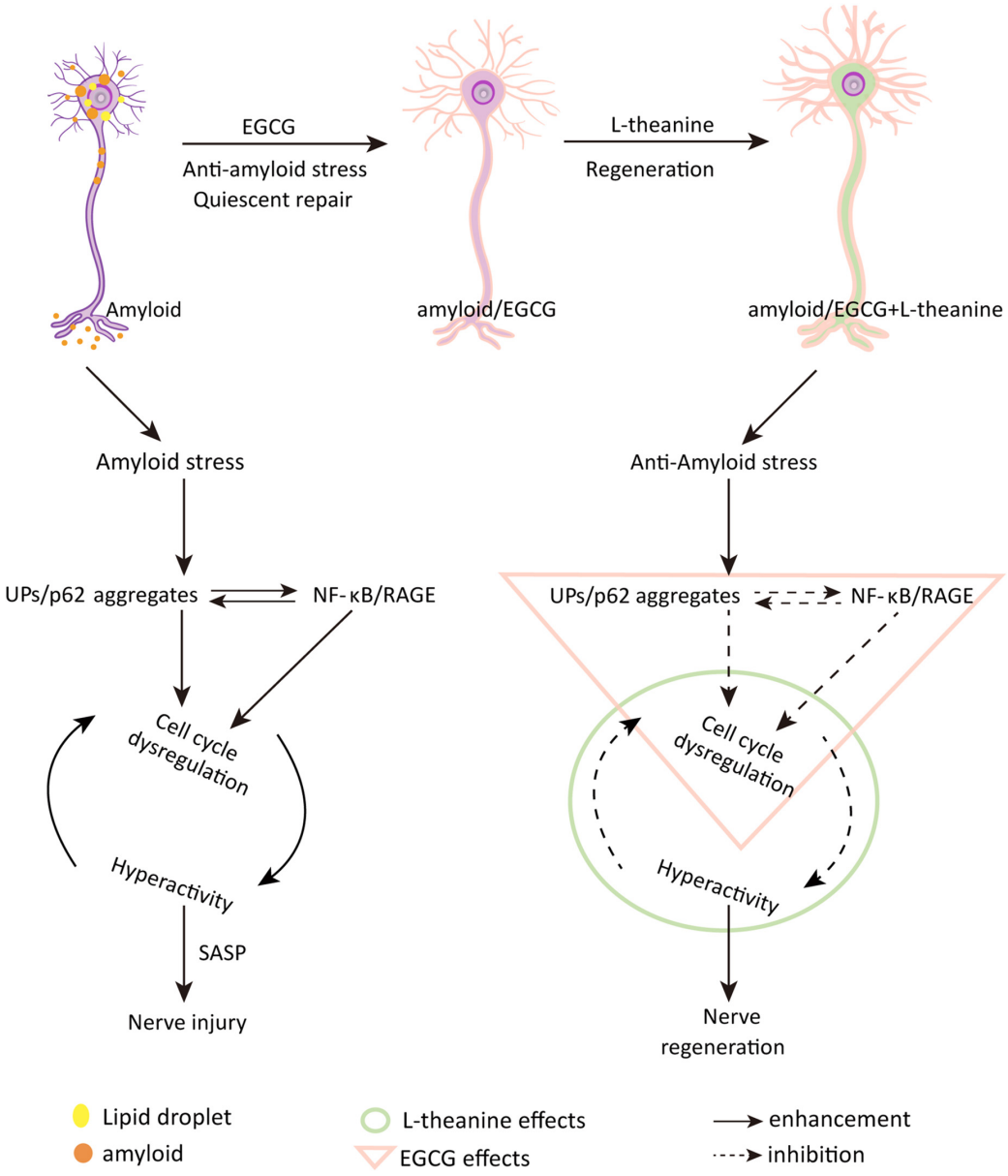


FIGURE 10
Schematic diagram of the molecular mechanism by which EGCG and L-theanine synergistically promote nerve cell repair and regeneration under amyloid stress conditions. Epigallocatechin gallate (EGCG) and *N*-ethyl-L-glutamine (L-theanine) are the representative functional components of green tea (Camellia sinensis). This study investigated the synergistic protective effect of EGCG and L-theanine on neural cells by establishing an AD model of $A\beta_{25-35}$-induced differentiated neural cell line PC12 cells. Through a series of cell and molecular biology experiments, it was found that amyloid stress mainly promotes the accumulation of UPs/p62-modified aggregates, upregulates the NF-κB/RAGE inflammatory pathway, stimulates cell-cycle dysregulation, and leads to cell hyperactivity and damage; EGCG + L-theanine can repair and regenerate amyloid-stressed differentiated neural cells. The synergistic mechanism is that under the premise that EGCG inhibits amyloid stress and inflammation and promotes metabolism, L-theanine can nourish nerves. EGCG + L-theanine increases the G0/G1 phase, down-regulates the expression of cyclins, keeps the cells in a quiescent state, and is beneficial to the repair and regeneration of the differentiated neuronal cells. In addition, EGCG + L-theanine maintains the high-fidelity structure of cellular proteins. This study revealed for the first time that the synergistic effect of EGCG with L-theanine or drinking green tea may be an effective way to promote nerve cell repair and regeneration and slow the progression of AD.

correlation of different genes between groups were carried out. The results showed that the cluster map of DEGs in the $A\beta_{25-35}$-vs-$A\beta_{25-35}$/EGCG + L-theanine was mainly divided into four blocks, of which two blocks [Figure 9 (1,3)] gene expression trend was opposite to that of other treatment groups, which was presumed to result from the

synergistic interaction between EGCG and L-theanine. The up-regulated genes were mainly related to autophagy, axon guidance and neurotrophin signaling pathways, while the down-regulated genes were mainly enriched in ribosome, transcription, apoptosis and mTOR signaling pathway. This result is consistent with the previous experimental results.

The gene expression trends of the other two blocks [**Figure 9** (2,4)] were very similar to that of $A\beta_{25-35}$-vs-$A\beta_{25-35}$/EGCG. It is speculated that these two blocks were mainly the effects of EGCG, and the up-regulatory genes were mainly enriched in metabolism pathways, focal adhesion and actin regulation, and the down-regulated genes were main enriched in MAPK and PI3K-Akt signaling pathways. Combined with the previous results (**Figure 8**), we speculated that under the premise that EGCG inhibits amyloid stress, promotes lipid metabolism and inhibits cell mitogenic activity, L-theanine could exert anti-inflammatory, promote axonal growth and nourish nerves. The synergistic effect of EGCG and L-theanine made EGCG have a milder effect on cells, thereby better maintaining the high-fidelity structure of cellular proteins (**Figure 3C**).

Discussion

This study demonstrates that EGCG and L-theanine synergistically promote repair and regeneration of differentiated neural cell line PC12 cells under amyloid stress conditions. EGCG mainly inhibits amyloid stress and inflammation and promotes lipid metabolism. In the presence of EGCG, L-theanine can fully exert its neurotrophic effect. EGCG + L-theanine targets the cell cycle, keeps nerve cells in a quiescent state under amyloid stress, promotes cell viability and axonal growth, and maintains the high-fidelity structure of cellular proteins.

Maintain the fidelity structure of nerve cell proteins

The toxicity of disease-causing protein aggregates may be due to their inherent misfolded nature and structural heterogeneity. These properties will lead to numerous aberrant interactions with various cellular components, including phospholipid bilayers, protein receptors, soluble proteins, RNA, and small metabolites, leading to cellular damage and ultimately cell death (38). Extensive literature studies have shown that EGCG binds to fold-rich aggregates, significantly inhibits β-amyloid fibrillogenesis, and is a potent remodeler of mature amyloid fibrils (16, 17). EGCG targeted and inhibited β-sheets formation of $A\beta_{25-35}$ toxic structure (**Figure 2C**), and promoted the growth of differentiated PC12 cells (**Figure 2A**). $A\beta_{25-35}$ can function as a neurotrophic factor for differentiation neurons and has important physiological roles (61, 62). $A\beta_{25-35}$ may have a certain neurotrophic effect after EGCG changes its toxic structure, which needs to be further verified.

Under $A\beta_{25-35}$ stress conditions, EGCG, L-theanine, and EGCG + L-theanine significantly downregulated neurotoxic proteins such as p62, ubiquitination and 4-HNE, and inhibited inflammatory pathways (**Figures 3A,B**). L-theanine significantly suppressed the shift of the protein infrared spectrum to lower wavenumbers, while also protecting the disordered structure of the protein (**Figure 3C**). Hub proteins, such as GAP43 and ubiquitination, are often disordered and flexible structures that perform essential signal transduction (63). In many neurodegenerative diseases, intrinsically disordered proteins form fibrillar aggregates for pathogenesis (64). EGCG + L-theanine maintained the high-fidelity structure of the neuronal protein, and the disordered structure was the same as that of the control group (**Figure 3C**). EGCG has a significant inhibitory effect on amyloid stress and significantly affects the protein amide I band spectrum, indicating that EGCG also has a more significant effect on cells. The $A\beta_{25-35}$/EGCG + L-theanine group has the best cellular protein fidelity, almost identical to the control group (**Figure 3C**). This result may be that L-theanine's weak acidity helps protect EGCG from being degraded, making EGCG bind more tightly to $A\beta_{25-35}$, and enhancing its anti-amyloid stress activity (65).

Based on the above results, we speculate that EGCG and L-theanine can promote each other, and fully exert repair and growth-promoting effects, so that EGCG + L-theanine exhibits milder and more effective cytoprotective activities.

Targets cell cycle and promotes the formation of cellular quiescence

Cell mitotic quiescence is critical for complex connected neuronal systems (66, 67). In differentiated neuronal cells, amyloid stress leads to abnormal cell cycle entry and is closely associated with neuronal inflammation and apoptosis (68–70). This study showed that the expression of cyclin marker proteins Cyclin D1 and Cyclin B1 in $A\beta_{25-35}$ group was significantly increased. In $A\beta_{25-35}$/EGCG + L-theanine group, the expression of Cyclin D1 and Cyclin B1 was significantly decreased, the percentage of cell cycle G0/G1 phase was significantly increased, the cell inflammation was down-regulated, the GAP43 protein expression was up-regulated, the p-mTOR expression was down-regulated, and the axonal growth was obvious (**Figures 3**, **4**). In quiescent cells, mTOR is deactivated,

and levels of pS6, cyclin D1, p21, and p16 are low (52). This study and literature show that EGCG + L-theanine promotes the formation of the neuronal quiescent state under amyloid stress, keeps differentiated neuronal cells in the G0 phase, and improves cell viability and axonal growth.

$A\beta_{25-35}$ significantly promoted the formation of neuronal inflammation and oxidative stress, while EGCG and EGCG + L-theanine had significant inhibitory activity (**Figures 3A,B**). The transcriptome results further demonstrated that EGCG + L-theanine enhanced the anti-inflammatory efficacy (**Figures 8**, **9**). Additionally, lipids play an important role in neurodegeneration, neuroinflammation, and psychiatric disorders. Imbalances in sphingolipid levels is associated with diseases (71). EGCG and EGCG + L-theanine significantly promote cellular lipid metabolism, energy metabolism, and caloric restriction (**Figures 5E**, **8E**). These results further demonstrate that EGCG and EGCG + L-theanine promote a quiescent state of neuronal cells under amyloid stress conditions.

Synergistic effects of epigallocatechin gallate and L-theanine in promoting nerve cell regeneration

Epigallocatechin gallate exhibited toxicity at high concentrations, while L-theanine exhibited cell growth-promoting activity. Under $A\beta_{25-35}$ stress conditions, EGCG promoted cell viability concentration-dependent, while L-theanine showed no cytoprotective activity. The bioavailability of EGCG is not high, but it has a significant anti-amyloid stress effect. L-theanine is a naturally brain-derived neurotrophic factor (BDNF) but has no inhibitory activity against amyloid stress (**Figures 2A,B**). The above results indicate that the mechanisms of action of EGCG and L-theanine have opposite effects.

Neurotrophic factors play an important role in combinatorial approaches to spinal cord repair by promoting cell survival, and maintaining axonal growth and regeneration (72). Although neurotrophins have positive effects in animal models, their short half-lives, low bioavailability, and limited permeability across the blood-brain barrier (BBB) limit their use in patients (73). The bioavailability of L-theanine is nearly 100%, and it has BDNF and neurogenesis (23, 29, 74–76). The transcriptome results showed that L-theanine mainly acts on genetic material such as DNA and RNA, and has estrogen-like and other growth-promoting effects (**Figures 7–9**). Their estrogenic pathways have significant neuroprotective effects.

Neuronal cells have abundant mitochondria, especially in axonal structures. Differentiated neural cells have higher energy requirements than normal cells, which require the transport of mitochondria to distal synapses (77). Therefore, protecting mitochondria, improving energy metabolism, and maintaining the differentiated state of neuronal axons are crucial. EGCG promoted the metabolism of lipids, carbohydrates, amino acids and energy (**Figures 8B,E**), and significantly promoted ATP production (**Figure 5D**). L-theanine inhibited LDH production and significantly promoted the phosphorylation of AMPK. EGCG + L-theanine significantly up-regulated Klotho protein expression, had the best effect on improving mitochondrial membrane potential activity and promoted mitochondrial rejuvenation (**Figure 5**).

The above results revealed that under the premise that EGCG binds to amyloid and promotes quiescent state repair of cells, L-theanine enters cells to play neuron nutrition and promote axonal growth (**Figure 10**). Drink 5–10 g of green tea daily to get adequate amounts of EGCG and L-theanine (15, 22). An epidemiological survey study showed that drinking two or more cups of green tea per day (100 mL/cup) can reduce the damage to brain memory (78). Another survey found that drinking two cups of tea daily or drinking tea continuously for more than 8–10 years can reduce the risk of Parkinson's disease by 28˜80% (79).

Conclusion

Proteotoxic stress, mitochondrial dysfunction, and genomic instability lead to cellular hyperactivity and are closely associated with age-related degenerative diseases such as atherosclerosis, type 2 diabetes, osteoporosis, and Alzheimer's disease. In the context of neurodegenerative diseases, targeting a single pathway may not be sufficient to promote long-distance axonal regeneration and functional recovery after injury. Amyloid stress-mediated neuronal degeneration results from multi-faceted effects and the synergy effect of EGCG and L-theanine exhibits multi cytoprotective activities. EGCG mainly inhibits the formation of amyloid protein rich in toxic β-sheet structure, improves lipid and energy metabolism, protects cell membrane structure, and maintains cellular protein homeostasis (**Figures 3–5**, **8**). L-theanine mainly improves the endogenous antioxidant capacity of cells and protects mitochondria and cell genetic materials (**Figures 5**, **8**). The synergistic effect of EGCG and L-theanine has multi-target protective effects on nerve cells under amyloid stress. EGCG and L-theanine promote each other and exert mild cytoprotective effects. This study revealed one of the core underlying mechanisms by which green tea delays age-related degenerative diseases such as AD.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI BioSample database, accession SAMN29216840.

Author contributions

SC performed writing the original draft. ZL and SC performed funding acquisition. XX, JW, XZ, MF, JY, WP, and BH performed methodology. ZL performed supervision. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by Guangxi Innovation Driven Development Special Fund Project (No. AA20302018-18, AA20302018-17), National Key R&D Program of China (2018YFC1604405), National Tea Industry Technology System Research Project of China (CARS-19-C01), National Natural Science Foundation Project of China (31471590 and 31100501), and Self-Science Foundation of Hunan Province, China (2019jj50237).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Dumont M, Lin MT, Beal MF. Mitochondria and antioxidant targeted therapeutic strategies for Alzheimer's disease. *J Alzheimers Dis.* (2010) 20:S633–43. doi: 10.3233/jad-2010-100507

2. Madamanchi NR, Runge MS. Mitochondrial dysfunction in atherosclerosis. *Circ Res.* (2007) 100:460–73. doi: 10.1161/01.Res.0000258450.44413.96

3. Wang FS, Wu RW, Chen YS, Ko JY, Jahr H, Lian WS. Biophysical modulation of the mitochondrial metabolism and redox in bone homeostasis and osteoporosis: how biophysics converts into bioenergetics. *Antioxidants.* (2021) 10:1394. doi: 10.3390/antiox10091394

4. Sharoar MG, Hu X, Ma XM, Zhu X, Yan R. Sequential formation of different layers of dystrophic neurites in Alzheimer's brains. *Mol Psychiatry.* (2019) 24:1369–82. doi: 10.1038/s41380-019-0396-2

5. Fiala JC. Mechanisms of amyloid plaque pathogenesis. *Acta Neuropathol.* (2007) 114:551–71. doi: 10.1007/s00401-007-0284-8

6. Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol.* (2007) 8:101–12. doi: 10.1038/nrm2101

7. Ueberham U, Hilbrich I, Ueberham E, Rohn S, Glöckner P, Dietrich K, et al. Transcriptional control of cell cycle-dependent kinase 4 by Smad proteins–implications for Alzheimer's disease. *Neurobiol Aging.* (2012) 33:2827–40. doi: 10.1016/j.neurobiolaging.2012.01.013

8. Ranganathan S, Bowser R. Alterations in G(1) to S phase cell-cycle regulators during amyotrophic lateral sclerosis. *Am J Pathol.* (2003) 162:823–35. doi: 10.1016/s0002-9440(10)63879-5

9. Kuboyama T, Yang X, Tohda C. Natural medicines and their underlying mechanisms of prevention and recovery from amyloid B-induced axonal degeneration in Alzheimer's Disease. *Int J Mol Sci.* (2020) 21:4665. doi: 10.3390/ijms21134665

10. Sparks DL, Sabbagh MN, Connor DJ, Lopez J, Launer LJ, Petanceska S, et al. Atorvastatin therapy lowers circulating cholesterol but not free radical activity in advance of identifiable clinical benefit in the treatment of mild-to-moderate AD. *Curr Alzheimer Res.* (2005) 2:343–53. doi: 10.2174/1567205054367900

11. Pervin M, Unno K, Ohishi T, Tanabe H, Miyoshi N, Nakamura Y. Beneficial effects of green tea catechins on neurodegenerative diseases. *Molecules.* (2018) 23:1297. doi: 10.3390/molecules23061297

12. Khalatbary AR, Khademi E. The green tea polyphenolic catechin epigallocatechin gallate and neuroprotection. *Nutr Neurosci.* (2020) 23:281–94. doi: 10.1080/1028415x.2018.1500124

13. Zhang J, Zhou X, Yu Q, Yang L, Sun D, Zhou Y, et al. Epigallocatechin-3-gallate (EGCG)-stabilized selenium nanoparticles coated with Tet-1 peptide to reduce amyloid-β aggregation and cytotoxicity. *ACS Appl Mater Interfaces.* (2014) 6:8475–87. doi: 10.1021/am501341u

14. Cai J, Jing D, Shi M, Liu Y, Lin T, Xie Z, et al. Epigallocatechin gallate (EGCG) attenuates infrasound-induced neuronal impairment by inhibiting microglia-mediated inflammation. *J Nutr Biochem.* (2014) 25:716–25. doi: 10.1016/j.jnutbio.2014.02.012

15. Rahmani AH, Al Shabrmi FM, Allemailem KS, Aly SM, Khan MA. Implications of green tea and its constituents in the prevention of cancer via the modulation of cell signalling pathway. *Biomed Res Int.* (2015) 2015:925640. doi: 10.1155/2015/925640

16. Ahmed R, VanSchouwen B, Jafari N, Ni X, Ortega J, Melacini G. Molecular mechanism for the (-)-epigallocatechin gallate-induced toxic to nontoxic remodeling of Aβ oligomers. *J Am Chem Soc.* (2017) 139:13720–34. doi: 10.1021/jacs.7b05012

17. Bieschke J, Russ J, Friedrich RP, Ehrnhoefer DE, Wobst H, Neugebauer K, et al. EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity. *Proc Natl Acad Sci USA.* (2010) 107:7710–5. doi: 10.1073/pnas.0910723107

18. de Oliveira MR, Nabavi SF, Daglia M, Rastrelli L, Nabavi SM. Epigallocatechin gallate and mitochondria-A story of life and death. *Pharmacol Res.* (2016) 104:70–85. doi: 10.1016/j.phrs.2015.12.027

19. Oliveira MR, Nabavi SF, Daglia M, Rastrelli L, Nabavi SM. The critical role of epigallocatechin gallate in regulating mitochondrial metabolism. *Future Med Chem.* (2018) 10:795–809. doi: 10.4155/fmc-2017-0204

20. Gundimeda U, McNeill TH, Schiffman JE, Hinton DR, Gopalakrishna R. Green tea polyphenols potentiate the action of nerve growth factor to induce

neuritogenesis: possible role of reactive oxygen species. *J Neurosci Res.* (2010) 88:3644–55. doi: 10.1002/jnr.22519

21. Li MY, Liu HY, Wu DT, Kenaan A, Geng F, Li HB, et al. Theanine: a unique functional amino acid in tea (*Camellia sinensis* L.) with multiple health benefits and food applications. *Front Nutr.* (2022) 9:853846. doi: 10.3389/fnut.2022.853846

22. Yu Z, Yang Z. Understanding different regulatory mechanisms of proteinaceous and non-proteinaceous amino acid formation in tea (*Camellia sinensis*) provides new insights into the safe and effective alteration of tea flavor and function. *Crit Rev Food Sci Nutr.* (2020) 60:844–58. doi: 10.1080/10408398.2018.1552245

23. Cho HS, Kim S, Lee SY, Park JA, Kim SJ, Chun HS. Protective effect of the green tea component, L-theanine on environmental toxins-induced neuronal cell death. *Neurotoxicology.* (2008) 29:656–62. doi: 10.1016/j.neuro.2008.03.004

24. Chen SQ, Wang ZS, Ma YX, Zhang W, Lu JL, Liang YR, et al. Neuroprotective effects and mechanisms of tea bioactive components in neurodegenerative diseases. *Molecules.* (2018) 23:512. doi: 10.3390/molecules23030512

25. Rothenberg DO, Zhang L. Mechanisms underlying the anti-depressive effects of regular tea consumption. *Nutrients.* (2019) 11:1361. doi: 10.3390/nu11061361

26. Katasonov AB. Neurobiological effects of theanine and its possible use in neurology and psychiatry. *Zh Nevrol Psikhiatr Im S S Korsakova.* (2018) 118:118–24. doi: 10.17116/jnevro2018118111118

27. Cai S, Yang H, Wen B, Zhu K, Zheng X, Huang J, et al. Inhibition by microbial metabolites of Chinese dark tea of age-related neurodegenerative disorders in senescence-accelerated mouse prone 8 (SAMP8) mice. *Food Funct.* (2018) 9:5455–62. doi: 10.1039/c8fo01512k

28. Shen JZ, Wang Y, Ding Z, Ding S, Wang H, Bi C, et al. Metabolic analyses reveal growth characteristics of young tea shoots in spring. *Sci Hortic.* (2019) 246:478–89. doi: 10.1016/j.scienta.2018.11.022

29. Yoneda Y. An L-glutamine transporter isoform for neurogenesis facilitated by L-theanine. *Neurochem Res.* (2017) 42:2686–97. doi: 10.1007/s11064-017-2317-6

30. Hidalgo FJ, Delgado RM, Zamora R. Protective effect of phenolic compounds on carbonyl-amine reactions produced by lipid-derived reactive carbonyls. *Food Chem.* (2017) 229:388–95. doi: 10.1016/j.foodchem.2017.02.097

31. Xu J, Zhou L, Weng Q, Xiao L, Li Q. Curcumin analogues attenuate Aβ25-35-induced oxidative stress in PC12 cells via Keap1/Nrf2/HO-1 signaling pathways. *Chem Biol Interact.* (2019) 305:171–9. doi: 10.1016/j.cbi.2019.01.010

32. Wolfe LS, Calabrese MF, Nath A, Blaho DV, Miranker AD, Xiong Y. Protein-induced photophysical changes to the amyloid indicator dye thioflavin T. *Proc Natl Acad Sci USA*. (2010) 107:16863–8. doi: 10.1073/pnas.1002867107

33. Biancalana M, Koide S. Molecular mechanism of thioflavin-T binding to amyloid fibrils. *Biochim Biophys Acta.* (2010) 1804:1405–12. doi: 10.1016/j.bbapap.2010.04.001

34. Goormaghtigh E, Ruysschaert JM, Raussens V. Evaluation of the information content in infrared spectra for protein secondary structure determination. *Biophys J.* (2006) 90:2946–57. doi: 10.1529/biophysj.105.072017

35. Cooper EA, Knutson K. Fourier transform infrared spectroscopy investigations of protein structure. *Pharm Biotechnol.* (1995) 7:101–43. doi: 10.1007/978-1-4899-1079-0_3

36. Chazotte B. Labeling mitochondria with JC-1. *Cold Spring Harb Protoc.* (2011) 2011:pdb.rot065490. doi: 10.1101/pdb.prot065490

37. Higgins CF, Hiles ID, Salmond GP, Gill DR, Downie JA, Evans IJ, et al. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature.* (1986) 323:448–50. doi: 10.1038/323448a0

38. Chiti F, Dobson CM. Protein misfolding, amyloid formation, and human disease: a summary of progress over the last decade. *Annu Rev Biochem.* (2017) 86:27–68. doi: 10.1146/annurev-biochem-061516-045115

39. Soto C. Alzheimer's and prion disease as disorders of protein conformation: implications for the design of novel therapeutic approaches. *J Mol Med.* (1999) 77:412–8. doi: 10.1007/s001090050371

40. Liu N, Yu Z, Xun Y, Shu P, Yue Y, Yuan S, et al. Amyloid-β25-35 upregulates endogenous neuroprotectant neuroglobin via NFκB activation in vitro. *J Alzheimers Dis.* (2018) 64:1163–74. doi: 10.3233/jad-180163

41. Zeng Z, Xu J, Zheng W. Artemisinin protects PC12 cells against β-amyloid-induced apoptosis through activation of the ERK1/2 signaling pathway. *Redox Biol.* (2017) 12:625–33. doi: 10.1016/j.redox.2017.04.003

42. Hyung SJ, DeToma AS, Brender JR, Lee S, Vivekanandan S, Kochi A, et al. Insights into antiamyloidogenic properties of the green tea extract (-)-epigallocatechin-3-gallate toward metal-associated amyloid-β species. *Proc Natl Acad Sci USA*. (2013) 110:3743–8. doi: 10.1073/pnas.1220326110

43. Kumar D, Ambasta RK, Kumar P. Ubiquitin biology in neurodegenerative disorders: from impairment to therapeutic strategies. *Ageing Res Rev.* (2020) 61:101078. doi: 10.1016/j.arr.2020.101078

44. Eldeeb MA, Ragheb MA, Soliman MH, Fahlman RP. Regulation of neurodegeneration-associated protein fragments by the N-degron pathways. *Neurotox Res.* (2022) 40:298–318. doi: 10.1007/s12640-021-00396-0

45. Schmidt MF, Gan ZY, Komander D, Dewson G. Ubiquitin signalling in neurodegeneration: mechanisms and therapeutic opportunities. *Cell Death Differ.* (2021) 28:570–90. doi: 10.1038/s41418-020-00706-7

46. Bartlett BJ, Isakson P, Lewerenz J, Sanchez H, Kotzebue RW, Cumming RC, et al. p62, Ref(2)P and ubiquitinated proteins are conserved markers of neuronal aging, aggregate formation and progressive autophagic defects. *Autophagy.* (2011) 7:572–83. doi: 10.4161/auto.7.6.14943

47. Bellezza I, Giambanco I, Minelli A, Donato R. Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochim Biophys Acta Mol Cell Res.* (2018) 1865:721–33. doi: 10.1016/j.bbamcr.2018.02.010

48. Burcham PC, Kuhan YT. Introduction of carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. *Biochem Biophys Res Commun.* (1996) 220:996–1001. doi: 10.1006/bbrc.1996.0521

49. Jackson M, Mantsch HH. The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit Rev Biochem Mol Biol.* (1995) 30:95–120. doi: 10.3109/10409239509085140

50. Marinkovic T, Marinkovic D. Obscure involvement of MYC in neurodegenerative diseases and neuronal repair. *Mol Neurobiol.* (2021) 58:4169–77. doi: 10.1007/s12035-021-02406-w

51. Lim IK. TIS21 (/BTG2/PC3) as a link between ageing and cancer: cell cycle regulator and endogenous cell death molecule. *J Cancer Res Clin Oncol.* (2006) 132:417–26. doi: 10.1007/s00432-006-0080-1

52. Blagosklonny MV. Geroconversion: irreversible step to cellular senescence. *Cell Cycle.* (2014) 13:3628–35. doi: 10.4161/15384101.2014.985507

53. Chung D, Shum A, Caraveo G. GAP-43 and BASP1 in axon regeneration: implications for the treatment of neurodegenerative diseases. *Front Cell Dev Biol.* (2020) 8:567537. doi: 10.3389/fcell.2020.567537

54. Kroemer G, Reed JC. Mitochondrial control of cell death. *Nat Med.* (2000) 6:513–9. doi: 10.1038/74994

55. Herzig S, Shaw RJ. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nat Rev Mol Cell Biol.* (2018) 19:121–35. doi: 10.1038/nrm.2017.95

56. Cantó C, Auwerx J. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Curr Opin Lipidol.* (2009) 20:98–105. doi: 10.1097/MOL.0b013e328328d0a4

57. Sharma E, Joshi R, Gulati A. l-Theanine: an astounding sui generis integrant in tea. *Food Chem.* (2018) 242:601–10. doi: 10.1016/j.foodchem.2017.09.046

58. Liu F, Liao F, Li W, Han Y, Liao D. Progesterone alters Nogo-A, GFAP and GAP-43 expression in a rat model of traumatic brain injury. *Mol Med Rep.* (2014) 9:1225–31. doi: 10.3892/mmr.2014.1967

59. Adibhatla RM, Hatcher JF. Role of lipids in brain injury and diseases. *Future Lipidol.* (2007) 2:403–22. doi: 10.2217/17460875.2.4.403

60. Saito Y, Kimura W. Roles of phase separation for cellular redox maintenance. *Front Genet.* (2021) 12:691946. doi: 10.3389/fgene.2021.691946

61. Yankner BA, Duffy LK, Kirschner DA. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science.* (1990) 250:279–82. doi: 10.1126/science.2218531

62. Pearson HA, Peers C. Physiological roles for amyloid beta peptides. *J Physiol.* (2006) 575:5–10. doi: 10.1113/jphysiol.2006.111203

63. Bondos SE, Dunker AK, Uversky VN. On the roles of intrinsically disordered proteins and regions in cell communication and signaling. *Cell Commun Signal.* (2021) 19:88. doi: 10.1186/s12964-021-00774-3

64. Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem.* (2006) 75:333–66. doi: 10.1146/annurev.biochem.75.101304.123901

65. Pirker KF, Baratto MC, Basosi R, Goodman BA. Influence of pH on the speciation of copper(II) in reactions with the green tea polyphenols, epigallocatechin gallate and gallic acid. *J Inorg Biochem.* (2012) 112:10–6. doi: 10.1016/j.jinorgbio.2011.12.010

66. Chen SD, Yang JL, Lin YC, Chao AC, Yang DI. Emerging roles of inhibitor of differentiation-1 in Alzheimer's disease: cell cycle reentry and beyond. *Cells.* (2020) 9:1746. doi: 10.3390/cells9071746

67. Liu DZ, Ander BP, Sharp FR. Cell cycle inhibition without disruption of neurogenesis is a strategy for treatment of central nervous system diseases. *Neurobiol Dis.* (2010) 37:549–57. doi: 10.1016/j.nbd.2009.11.013

68. Zhou L, Chen D, Huang XM, Long F, Cai H, Yao WX, et al. Wnt5a promotes cortical neuron survival by inhibiting cell-cycle activation. *Front Cell Neurosci.* (2017) 11:281. doi: 10.3389/fncel.2017.00281

69. Lopes JP, Oliveira CR, Agostinho P. Cell cycle re-entry in Alzheimer's disease: a major neuropathological characteristic?. *Curr Alzheimer Res.* (2009) 6:205–12. doi: 10.2174/156720509788486590

70. D'Angelo B, Astarita C, Boffo S, Massaro-Giordano M, Antonella Ianuzzi C, et al. LPS-induced inflammatory response triggers cell cycle reactivation in murine neuronal cells through retinoblastoma proteins induction. *Cell Cycle.* (2017) 16:2330–6. doi: 10.1080/15384101.2017.1363943

71. van Kruining D, Luo Q, van Echten-Deckert G, Mielke MM, Bowman A, Ellis S, et al. Sphingolipids as prognostic biomarkers of neurodegeneration, neuroinflammation, and psychiatric diseases and their emerging role in lipidomic investigation methods. *Adv Drug Deliv Rev.* (2020) 159:232–44. doi: 10.1016/j.addr.2020.04.009

72. McCall J, Weidner N, Blesch A. Neurotrophic factors in combinatorial approaches for spinal cord regeneration. *Cell Tissue Res.* (2012) 349:27–37. doi: 10.1007/s00441-012-1388-6

73. Palasz E, Wysocka A, Gasiorowska A, Chalimoniuk M, Niewiadomski W, Niewiadomska G. BDNF as a promising therapeutic agent in Parkinson's Disease. *Int J Mol Sci.* (2020) 21:1170. doi: 10.3390/ijms21031170

74. Takeda A, Sakamoto K, Tamano H, Fukura K, Inui N, Suh SW, et al. Facilitated neurogenesis in the developing hippocampus after intake of theanine, an amino acid in tea leaves, and object recognition memory. *Cell Mol Neurobiol.* (2011) 31:1079–88. doi: 10.1007/s10571-011-9707-0

75. Tamano H, Fukura K, Suzuki M, Sakamoto K, Yokogoshi H, Takeda A. Advantageous effect of theanine intake on cognition. *Nutr Neurosci.* (2014) 17:279–83. doi: 10.1179/1476830513Y.0000000094

76. Zeng L, Lin L, Chen L, Xiao W, Gong Z. l-Theanine ameliorates d-galactose-induced brain damage in rats via inhibiting AGE formation and regulating sirtuin1 and BDNF signaling pathways. *Oxid Med Cellr Longev.* (2021) 2021:8850112. doi: 10.1155/2021/8850112

77. Zhao XY, Lu MH, Yuan DJ, Xu DE, Yao PP, Ji WL, et al. Mitochondrial dysfunction in neural injury. *Front Neurosci.* (2019) 13:30. doi: 10.3389/fnins.2019.00030

78. Mandel SA, Amit T, Kalfon L, Reznichenko L, Youdim MB. Targeting multiple neurodegenerative diseases etiologies with multimodal-acting green tea catechins. *J Nutr.* (2008) 138:1578S–83S. doi: 10.1093/jn/138.8.1578S

79. Checkoway H, Powers K, Smith-Weller T, Franklin GM, Longstreth WT Jr., Swanson PD. Parkinson's disease risks associated with cigarette smoking, alcohol consumption, and caffeine intake. *Am J Epidemiol.* (2002) 155:732–8. doi: 10.1093/aje/155.8.732

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 12 August 2022
DOI 10.3389/fnut.2022.948039

Check for updates

OPEN ACCESS

EDITED BY
Andrew Scholey,
Swinburne University of Technology, Australia

REVIEWED BY
António Raposo,
CBIOS-Universidade Lusófona Research Center for Biosciences & Health Technologies, Portugal
Martin Ulrich,
Ulm University, Germany

*CORRESPONDENCE
Todd Jackson
toddjackson@um.edu.mo

SPECIALTY SECTION
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

RECEIVED 19 May 2022
ACCEPTED 25 July 2022
PUBLISHED 12 August 2022

CITATION
Xu X, Pu J, Shaw A and Jackson T (2022) Neural responsiveness to Chinese versus Western food images: An functional magnetic resonance imaging study of Chinese young adults.
Front. Nutr. 9:948039.
doi: 10.3389/fnut.2022.948039

COPYRIGHT
© 2022 Xu, Pu, Shaw and Jackson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Neural responsiveness to Chinese versus Western food images: An functional magnetic resonance imaging study of Chinese young adults

Xi Xu[1], Jiajia Pu[1], Amy Shaw[2] and Todd Jackson[2]*

[1]School of Psychology, Southwest University, Chongqing, China, [2]Department of Psychology, University of Macau, Macao, Macao SAR, China

Cross-cultural studies suggest that people typically prefer to eat familiar foods from their own culture rather than foreign foods from other cultures. On this basis, it is plausible that neural responsiveness elicited by palatable food images from one's own culture differ from those elicited by food depictions from other cultures. Toward clarifying this issue, we examined neural activation and self-report responses to indigenous (Chinese) versus Western food images among young Chinese adults. Participants (33 women, 33 men) viewed Chinese food, Western food and furniture control images during a functional magnetic resonance imaging (fMRI) scan and then rated the images on "liking," "wanting," and "difficult resisting." Analyses indicated there were no significant differences in self-report ratings of Chinese versus Western food images. However, Chinese food images elicited stronger activation in regions linked to cravings, taste perception, attention, reward, and visual processing (i.e., cerebellum crus, superior temporal gyrus, supramarginal gyrus, middle temporal gyrus, inferior parietal lobule, posterior insula, middle occipital gyrus; inferior occipital gyrus). Conversely, Western food images elicited stronger activation in areas involved in visual object recognition and visual processing (inferior temporal gyrus, middle occipital gyrus, calcarine). These findings underscored culture as a potentially important influence on neural responses to visual food cues and raised concerns about the ecological validity of using "standard" Western food images in neuroimaging studies of non-Western samples. Results also provide foundations for designing culturally informed research and intervention approaches in non-Westerns contexts guided by the use of external food cues that are most salient to the cultural group under study.

KEYWORDS

food images, neural activation, fMRI, culture, food preferences, Chinese

Introduction

Cross-cultural studies have indicated that people typically show stronger overall preferences for foods that are familiar to their own cultural group than less familiar, foreign foods (1, 2). Consequently, neural responses elicited during exposure to images of foods from one's local culture may also differ from those elicited during exposure to foods imported from other cultures. As such, the implicit assumption that "standardized" images of palatable Western foods are appropriate for use in neuroimaging studies of responses to food cues in non-Western groups warrants scrutiny. Toward testing this premise, we examined neural responses and subjective reactions of mainland Chinese young adults during exposure to images of traditional Chinese foods versus Western foods more typically depicted in neuroimaging studies of non-Western samples.

Taste is a critical factor shaping food preferences (3, 4). For example, studies of adolescents in the United States (5) and non-Western nations including China (6) indicate taste is the most important influence on food selection. Neophobia, the tendency to reject or avoid novel edibles while developing preferences for more familiar foods (3), is an important genetic predisposition that shapes preferences for particular foods (4). While neophobic tendencies are encoded in genetics, their expression is shaped by culture and can differ between countries (7, 8). Past studies have found that unfamiliar taste and appearance deter people from trying ethnic food (9) while familiarity influences food preferences and willingness to eat novel foods across cultures and socio-demographic groups (3, 10, 11).

As an illustrative example, despite considerable diversity in food preferences between its different regions, traditional culture and food tastes in China have strong roots (12). Based on Chinese traditions, good food should be excellent in terms of color, aroma and taste (2). "Proper meals" in Chinese culture comprise appropriate amounts of starches (e.g., rice, noodles), vegetables and meats, and lower amounts of dairy products and sweets (13). As well, flavors that distinguish Chinese cuisine include a soy sauce, rice wine, and ginger mixture (14). This traditional pattern provides less total fat, saturated fat, cholesterol, and calcium than the typical American dietary pattern does (13).

Studies of Chinese samples suggest food preferences are affected by culture (1, 13–17). For example, among residents of Shanghai and Xi'an, "unfamiliar," "sweet," and "greasy" were unique, negatively valenced taste descriptors of European foods while "safe" and "upscale" were endorsed as positively valenced descriptors (15). In another China-based study of adolescents, the most frequently consumed snacks included fruit, milk and instant noodles (16); underscoring possible aversions to sweet foods, these snacks were consumed at least 2–3 times more often than soft drinks, candy/gum and chocolate. Among Asian travelers to Australia, Chang et al. (14) found Chinese food was highly preferred despite immersion within a new culture. Even when participants were eager to try local delicacies, they sought Chinese foods during mealtimes because the "acceptability" and "palatability" of these dishes were guaranteed and mitigated shock experienced from eating less familiar foods. Many also sought "familiar flavors" in local Australian food and welcomed the fusion of Chinese and Australian food to foster acceptance of unfamiliar foods. Finally, in contrast to "palatability," motives for trying Australian foods included a desire to learn about the culture, increase Western culinary knowledge, develop memories about Australian holiday experiences, and assert prestige and status. Similarly, Chinese travelers to Spain preferred eating Chinese food and familiar fast food relative to unfamiliar local Spanish food (17). Finally, Li (1) found that although Chinese cruise tourists traveling abroad were initially willing to try novel Western foods, they were subsequently more reluctant due to being less accustomed to the taste, type, preparation and temperature of Western foods; these reactions were especially strong among older adults who became neophobic to Western food.

Notwithstanding evidence that Chinese samples prefer familiar Chinese foods in contrast to less familiar Western foods, concerns have emerged about diets becoming increasingly Westernized in China (18, 19). Wang et al. (19) noted the remarkable growth of U.S.-based fast-food restaurants in China during the past 30 years with McDonald's franchises expanding at a rate of nearly 10 new restaurants per week due to their perceived convenience, safety, and prestige. Given the proliferation of Western food products in China, it is also possible that reactions to Western foods do not diverge sharply from those of indigenous Chinese foods, particularly among children, adolescents, and young adults to whom Westernized diets are marketed.

Despite increased research in non-Western contexts such as China, studies on food consumption and food representations typically have comprised North American and European samples (20). Augmenting dominant North American and European perspectives on these issues with data from Asian contexts has the potential to elucidate how cultural background informs preferences for and brain responses to presentations of foods from local and non-local cultures.

In sum, numerous China-based self-report studies have underscored stronger taste preferences for familiar Chinese foods as opposed to novel foods from other cultures, albeit conflicting data highlight increasingly Westernized diets in China, especially among younger cohorts. Functional magnetic resonance imaging (fMRI) has been essential in documenting underlying neural activation patterns elicited by images of palatable foods. Though not exhaustive, food image presentations can elicit increased activity in reward/craving circuitry regions including the anterior and posterior insula (PI), orbital frontal cortex (OFC), anterior cingulate cortex

(ACC), caudate, hippocampus, amygdala, and dorsolateral prefrontal cortex (DLPFC) (21–25) as well as regions involved in taste perception, olfaction, temperature, and texture perception (e.g., insula/operculum, left OFC, pregenual cingulate cortex) (26–28). Exposure to food images can also elicit activation of regions linked to visual processing (e.g., fusiform gyrus, occipital gyrus, superior parietal lobule, cuneus) (26–28) and attention/cognitive control (e.g., precuneus, inferior parietal lobe, middle frontal gyrus) (26, 29, 30).

A majority of related fMRI studies has employed images of palatable Western foods in samples from the United States or Europe (21, 26, 31–35). Similarly, research on Chinese samples (36–38) has tended to employ standard Western food images even though these may be less preferred and/or less frequently consumed than food staples typical of Chinese diets are. Consequently, it is plausible that neural activity patterns elicited during exposure to palatable Western food images differ from patterns elicited during exposure to palatable indigenous food images that represent daily diets and/or food preferences of most mainland Chinese residents.

To test this premise, we evaluated self-reported reactions to and neural responses elicited by images of indigenous (i.e., local Chinese) versus Western food dishes among mainland Chinese young adults. Following from behavior studies of food preferences in Chinese samples reviewed above, we hypothesized that Chinese food images would elicit higher ratings of liking, wanting and difficulty resisting than Western food images would. Based on the assumption that subjective preferences for Chinese (versus Western) foods might reflect underlying differences in neural activation, we also tested an exploratory hypothesis that Chinese food images would elicit comparatively stronger neural activation responses than Western food images would in regions linked to reward/cravings circuitry, taste perception, attention and inhibitory control.

Materials and methods

Participants

The initial sample comprised 67 students (34 women, 33 men) recruited from a major university in China. On average, participants were 20.05 years of age (*SD* = 1.62, range: 18–25 years) and had an average body mass index (kg/m^2) of 25.41 (*SD* = 5.65, range: 18.12–40.14). Exclusion criteria included the presence or history of major medical conditions or psychiatric illnesses including clinical eating disorders, previous or current prescription medications, and significant visual impairments. Specific contraindications for fMRI (i.e., pacemaker, metal implants, dentures, severe claustrophobia) were other exclusion criteria.

Image sets

Images were 180 color photographs representing three image categories: 60 Chinese food items (e.g., dumplings, noodles, tofu), 60 Western food items (e.g., hamburger, French fries) and 60 furniture items (e.g., tables, cabinet, chairs) that served as control images (**Figure 1**). Chinese and Western food images were selected from the Food Image Library of the Psychology Department of the affiliated university (39). Some Western food images were selected from another food image database (40). Furniture images were obtained from freely available websites. For standardization, all images of Chinese food, Western food, and furniture were displayed in same resolution (720 × 540 pixels) and featured against a white background (41).

The final image set was selected from a pilot study in which 10 undergraduates (five women, five men), who did not participate in this study, sorted images into the three categories listed above. Participants were provided with definitions and sample images of each image category prior to the sorting task. During the task, a question was displayed below the image querying, "What kind of image do you think this is?" with A = Chinese food item, B = Western food item, and C = furniture item as the choices. Only images correctly classified within their appropriate category at rates of 90% or higher were retained in the final image set. Highlighting equivalent levels of familiarity and distinctiveness between these image sets, mean classification accuracy rates for retained Chinese food images (98%), Western food images (100%), and furniture images (100%) were near perfect and far exceeded chance alone rates (33%). In addition, rater judgments of image visual complexity did not differ between the three image categories, $F(2,177) = 0.513$, $p = 0.600$. Finally, although calorie counts of individual food images varied, Chinese versus Western food image sets did not differ significantly in terms of (i) average calories based on the operationalization of high calorie foods as those with an energy density $\geq$ 1.5 kcal/gram (42, 43) and calorie information from related databases (40, 44), percentages of high calorie food image [92% versus 85%, $\chi^2(1, N = 120) = 1.29$, $p = 0.26$], (ii) overall food content (e.g., meats, vegetables, etc.), $\chi^2(2, N = 66) = 1.31$, $p = 0.52$, based on operationalizations from other published sources (45), (iii) how food was presented (alone or in white dish against white background versus in non-white dish/container against white background), $\chi^2(1, N = 120) = 2.83$, $p = 0.093$, or (iv) flavor (i.e., sweet, savory, blandness), $\chi^2(2, N = 120) = 2.89$, $p = 0.39$.

Procedure

The study was approved by the Human Research Ethics Committee of the affiliated university. An *a priori* power analysis with the G*Power Version 3.1 (46) was used to

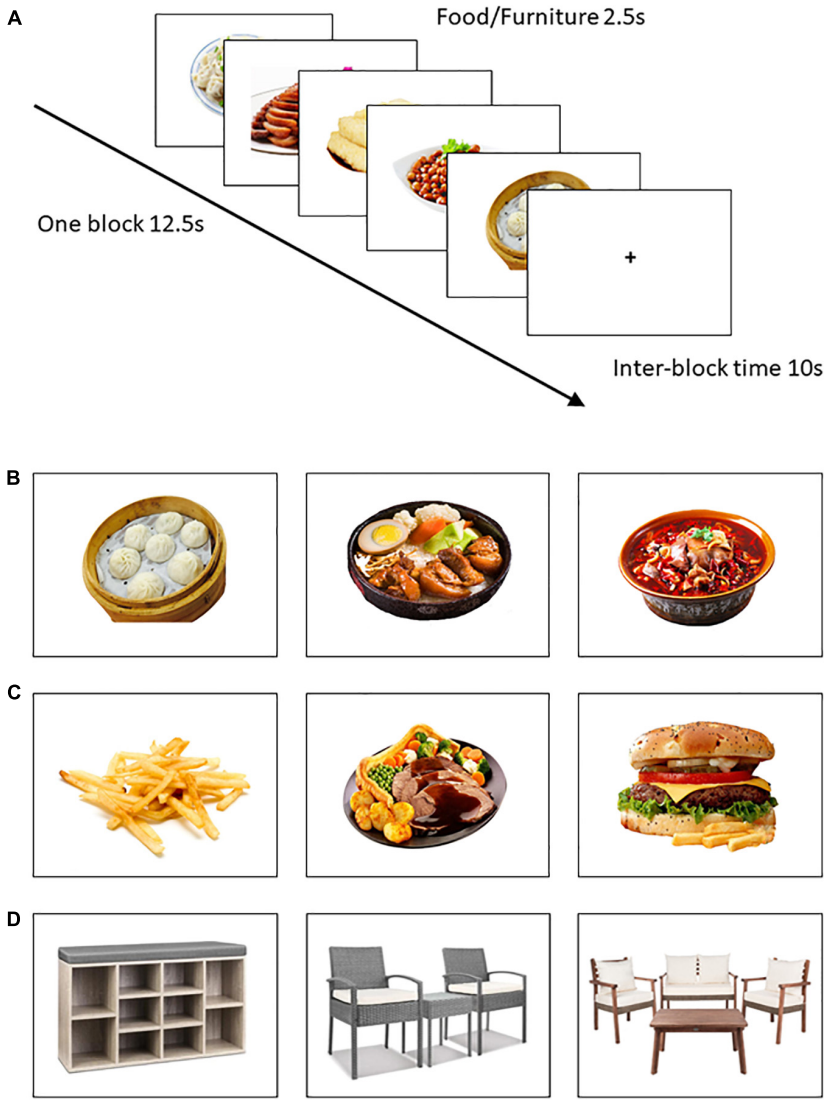


FIGURE 1
Examples of image types and the sequence of events in each task trial. **(A)** Depicted is one Chinese food block; **(B)** Chinese food (ChFd); **(C)** Western food (WeFd); **(D)** Furniture (Furn).

estimate sample size based on a repeated measures analysis of variance (ANOVA) with the following parameters: a medium effect size (Cohen's f) = 0.35, alpha = 0.05, power (1-β) = 0.95. The analysis resulted in an estimated sample N of 64, though we recruited beyond this level anticipating a 5–10% attrition rate.

Participants were solicited *via* an advertisement on the university's electronic bulletin board seeking volunteers for a study on reactions to different kinds of images. Prospective volunteers completed an online screen that included demographics (age, gender, height, weight), exclusion criteria and MRI contraindications. Appointments were made with eligible, willing volunteers. They were asked to wear light, comfortable clothing and consume their regular meals but refrain from eating or drinking (except water) for at least 2 h before their appointment (47) to better ensure they were not overly hungry or satiated during testing. Upon arrival, participants removed their shoes and objective measures of height and weight were taken to calculate body mass index (BMI). Next, measures of demographics (age, gender, Han versus minority ethnicity), parental education (high school completion or lower versus more than high school completion) were completed with single item ratings of current hunger and fullness anchored by 0 (not at all) 50 (somewhat), and 100 (extremely) (38). Time since last meal was assessed with the query, *"How long has it been since your last meal (in minutes)*?"

Prior to scanning, participants were instructed to lie still, remain awake but relaxed, think of nothing in particular while

keeping their eyes open, and focus carefully on each image presented to them. At the scan outset, written instructions were presented: "In the next task you will see food and non-food images. Please pay close attention to the images since you will be tested on their nature at the end of the MRI session." The scan then proceeded with each trial comprising one full-color image presented once for 2.5 s. Participants viewed food/control images through an adjustable mirror mounted on the scanner head coil. Following other published work (48), each block comprised five images of the same type (Chinese food, Western food, or furniture) presented with no pauses (see **Figure 1**). Blocks were separated by a 10 s inter-block interval featuring a central fixation cross. The total run comprised 36 blocks (820 s) including a 30 s rest after 18 blocks. Blocks were presented pseudo-randomly so that the same image type was never presented more than twice in a row. Immediately after the fMRI run, participants were asked if they had fallen asleep during the scan; none reported having done so. They also completed a simple memory item in which they were asked to identify two image categories from the following list that were NOT presented in the immediately preceding viewing task: cars, Chinese foods, people, Western foods, and furniture. Data from one female participant who answered this item incorrectly were excluded due to potential inattention.

After leaving the scanner, participants rated each image on computer in a separate room. Following other published research (49), respondents rated how much they (1) liked each portrayed image (anchored by *1 = dislike extremely and 5 = like extremely*), (2) wanted to eat (or use) each depicted stimulus (anchored by *0 = do not at all, 50 = want somewhat, and 100 = definitely want*), and (3) had difficulty to resist eating or using each depicted stimulus (anchored by *0 = definitely not difficult, 50 = somewhat difficult, 100 = definitely difficult*). For wanting and difficulty resisting ratings, any number between 0 and 100 could be selected. Images were presented in random order to control for potential order effects or participant "response sets." After the rating task, participants were asked to guess the main study hypotheses, debriefed, and paid 100 RMB for their time and effort. On average, this study took 35–45 min to complete.

Functional magnetic resonance imaging data acquisition and preprocessing

Images were acquired *via* a Magnatom Terra 7T MRI (Siemens Medical, Erlangen, Germany) with a 64-channel head coil. Soft foam pads were used to decrease head motion and scanner noise. Image presentations and self-report ratings were controlled by a computer outside scanner using E-prime 2.0 (50). Functional images were collected using a T1-weighted gradient echo-planar imaging (EPI) sequence [repetition time (TR) = 2000 ms; echo time (TE) = 30 ms; Flip Angle = 90°; voxel size = 2 mm × 2 mm × 2.3 mm; Field of View (FoV: AP, FH, RL) = 224 mm × 143 mm × 224 mm, slices = 62]; 410 BOLD images were collected. A high-resolution T1-weighted anatomical image was acquired for precise normalization, using a standard MPRAGE (magnetization prepared rapid acquisition gradient echo) sequence [repetition time (TR) = 2530 ms; echo time (TE) = 2.98 ms; Field of View (FoV: AP, FH, RL) = 224 × 256 × 192 mm^3; voxel size = 0.5 × 0.5 × 1 mm^3; slices = 192].

Data were preprocessed using DPABI (Data Processing & Analysis for Brain Imaging) (51) in MATLAB (version 2014a, Math Works, Natick, MA, United States). Both functional images and T1 images were converted from DICOM to NIFTI format. Slice timing was conducted to correct slice order, functional images were realigned to the first volumes, and six head-motion parameters were estimated from three translation and three rotation vectors. All images were skull stripped. T1-weighted images were co-registered to averaged functional images and segmented into constituent tissues using a DARTEL template (52). Functional images were normalized to the MNI (Montreal Neurological Institute) space with the DARTEL template. Spatially normalized echo-planar images were smoothed with Gaussian kernel of 8 × 8 × 8 mm FWHM (Full-width-at-half-maximum) (53).

Data analysis

Self-report data analyses

Descriptive statistics summarized sample characteristics, state hunger/fullness ratings, and reactions to the three image types. Repeated measures analyses of variance (ANOVAs) assessed within sample differences in ratings of (1) liking, (2) wanting, and (3) difficulty resisting Chinese food versus Western food versus control (furniture) images. Least significant difference (LSD) *post hoc* tests were run to identify specific image type differences when ANOVA values were significant. These analyses were conducted using SPSS 26 (54) with $p < 0.05$ significance cutoffs. Finally, Spearman correlation coefficients were calculated to examine intercorrelations between BMI and self-report ratings of hunger, fullness, time since last meal, image liking, wanting, and difficulty resisting ratings.

Imaging data analyses

Image preprocessing and multiple corrections were performed with DPABI. We used SPM12 (Statistical Parametric Mapping), run on MATLAB 2021, for analyses and Bspmview (55) for results presentations. In first-level analyses, task-related activity within each participant's data was detected by convolving the canonical hemodynamic response function (HRF) with a boxcar function representing onsets and durations of different conditions. Image type conditions were treated

as regressors and head motion parameters were treated covariates in a generalized linear model (GLM). For the GLM analysis, Chinese food, Western food, and furniture image type conditions were generated to model the boxcar function *via* a sustained epoch representing each stimulus duration. Main analyses featured within sample contrasts between Chinese vs. Western food images. Supplementary analyses included within sample contrasts between Chinese or Western food images vs. furniture.

In second level analyses, at a group level, contrasts from individual participants were first entered into one-sample *t*-tests to measure brain response differences regarding the within sample contrasts. Scan time served as a covariate. A family-wise error (FWE) correction based on Gaussian random field (GRF) theory was applied to control for false positives resulting from multiple comparisons at the $\alpha = 0.05$ level (56) and cluster-based inferences about findings for which there were likely to be significant activation effects (45, 57). The corrected height-extent threshold was calculated for numbers of voxels (k) in each statistical map across the whole brain. To identify significant activations for Chinese food vs. Western food, Chinese food vs. furniture and Western food vs. furniture contrasts, a $p < 0.001$ threshold was submitted to cluster detection within DPABI; cluster sizes of 178 voxels, 222 voxels and 231 voxels, respectively, resulted in corrected probabilities of $p < 0.05$. Finally, Spearman correlation coefficients were calculated to examine relations of BOLD signals in clusters from the Chinese versus Western food image contrast that included structures previously implicated as "reward" regions with measures of BMI, hunger, fullness, time since last meal, image liking, wanting, and difficulty resisting ratings. We extracted beta values in BOLD signals of these clusters using Marsbar (58) (sphere radius at 10 mm) and SPM12.

Results

Preliminary analyses

Data from one male participant whose hunger rating was nearly three standard deviations above the mean were retained in main analyses because results were very similar to those in which his data were dropped.

Description of sample characteristics and differences in reactions to image categories

Descriptive statistics for sample demographics and background measures are summarized in Table 1. The sample had significantly higher liking, wanting and difficulty resisting ratings for both Chinese and Western food images than furniture images. However, contrary to predictions, none of these ratings differed for Chinese versus Western food images (see Table 2). Finally, correlation analyses indicated BMI was not related to self-reported hunger or fullness, time since last meal, or ratings of liking, wanting, or difficulty resisting either food image set (all r's < 0.172, all p's > 0.168).

Neural activation differences between image categories

Activation differences between Chinese food versus Western food images

In the Chinese-Western food image contrast, Chinese food images elicited significantly stronger activation in clusters comprising (i) the right cerebellum crus, (ii) inferior occipital gyrus (IOG) and middle occipital gyrus (MOG), (iii), MOG and (iv) superior temporal gyrus (STG), supramarginal gyrus (SMG), middle temporal gyrus (MTG), inferior parietal lobule IPL, and PI (Table 3). PI involvement was modest based on the DPABI report (14 voxels) and SPM xjView report (20 voxels) but less evident in the anatomical map (Figure 2). Conversely, Western food images elicited significantly stronger activation in visual processing regions including two inferior temporal gyrus (ITG)/MOG clusters, a fusiform gyrus cluster, and a calcarine/MOG cluster (Table 3 and Figure 2).

Associations of self-report measures and image ratings with reward region activation in Chinese versus Western food image contrast

Analyses examining correlates of reward region activation in the Chinese versus Western food contrast indicated responsivity of the STG/SMG/MTG/IPL/PI cluster) was not related to BMI, state hunger or fullness, time since last meal or liking of Chinese or Western food images (Table 4). However, pronounced activation of this cluster had significant positive associations

TABLE 1 Demographic and background characteristics of sample ($N = 66$).

Measure	%/M ± SD
Gender (Female)	51.5%
Age	20.05 ± 1.66
Body Mass Index	25.21 ± 5.44
Ethnicity (Han majority)	90.9%
Parent Educational level (≥high school)	
Maternal	53.0%
Paternal	63.6%
Current Hunger	29.32 ± 24.13
Current Fullness	48.53 ± 21.72
Time since last meal (in minutes)	191.61 ± 155.01

TABLE 2 Sample differences in liking, wanting, and difficulty resisting food and furniture image sets (N = 66).

Reaction measure	Image type				
	Chinese food (ChFd)	Western food (WeFd)	Furniture (Furn)	F	*Post hoc* tests
Liking	3.57 ± 0.47	3.55 ± 0.52	2.91 ± 0.68	41.58***	ChFd > Furn***, WeFd > Furn***
Wanting	51.11 ± 16.81	51.63 ± 17.46	37.44 ± 17.47	41.18***	ChFd > Furn***, WeFd > Furn***
Difficulty resisting	40.18 ± 19.58	41.07 ± 20.49	26.61 ± 18.59	48.44***	ChFd > Furn***, WeFd > Furn***

***$p < 0.001$.

TABLE 3 Activation differences during exposure to Chinese vs. Western food images (N = 66).

Contrast	Anatomical label	BA	Hem	Voxels	x	y	z	Peak T-value
ChFd > WeFd	Cerebellum crus	–	R	502	42	−78	−41.4	5.39
			R		32	−60	−37	4.23
			R		22	−84	−48	3.86
	MOG/IOG	18/17	L	457	−26	−100	−9.2	5.20
	IOG	18/17	R	449	28	−100	−4.6	6.42
	STG/SMG/MTG/IPL/PI	22/13	R	293	60	−40	20.7	4.65
WeFd > ChFd	ITG/MOG	19/37	L	440	−52	−78	−6.9	5.23
	FFG	37	L		−34	−60	−2.3	4.73
	ITG/MOG	19/37	R	304	48	−70	−9.2	4.28
	Calcarine/MOG	18/17	R	234	10	−96	4.6	6.05

BA, Brodmann area; Hem, Hemisphere; x,y,z coordinates in MNI space (Montreal Neurological Institute); ChFd, Chinese food; WeFd, Western food; L and R, left and right; Reported brain activation was significant at corrected $p < 0.05$; MOG, middle occipital gyrus; IOG, inferior occipital gyrus; STG, superior temporal gyrus; SMG, supramarginal gyrus; MTG, middle temporal gyrus; IPL, inferior parietal lobule; PI, posterior insula; ITG, inferior temporal gyrus; FFG, fusiform gyrus.

with reported wanting and difficulty resisting Western food images and, especially, Chinese food images (Figure 3). Differences in strengths of relation between right PI-Chinese food versus right PI-Western food image responsiveness on measures of wanting (r = 0.36 versus r = 0.28) and difficulty resisting (r = 0.33 versus r = 0.31) were not statistically significant (p's > 0.30).

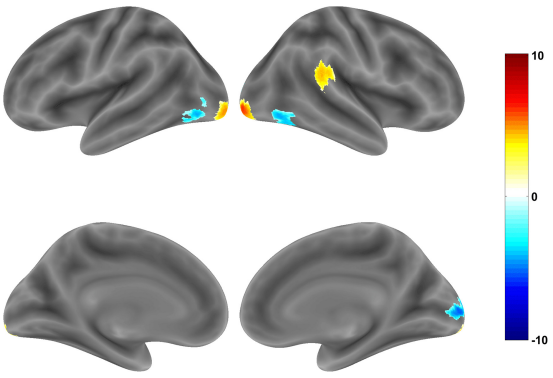


FIGURE 2
Brain regions showing significant activation in whole brain analysis of Chinese food vs. Western food images (corrected $p < 0.05$). Warm tones indicate greater activation during exposure to Chinese food images and cool tones indicate greater activation during exposure to Western food images.

Supplementary analyses of neural activation differences between food versus furniture image categories

Although no hypotheses were generated, Supplementary Table 1 summarizes activation results based on contrasts of Chinese food-furniture and Western food-furniture contrasts. Chinese food images elicited comparatively stronger activation in the left middle occipital gyrus, calcarine, and bilateral lingual gyrus and comparatively weaker activity in the right calcarine, left precuneus and cerebellum. Western

TABLE 4 Bivariate associations between self-report ratings and reward region cluster[1] differentiating Chinese versus Western food images.

Measure	Food image type	
	Chinese	Western
Body Mass Index	0.10	0.06
Current Hunger	0.06	0.05
Current Fullness	−0.17	−0.13
Time Since Last Meal	0.05	0.04
Liking of Depicted Food	0.19	0.18
Wanting of Depicted Food	0.36**	0.28*
Difficulty Resisting Depicted Food	0.33**	0.31*

*$p < 0.05$, **$p < 0.01$ (two-tailed).
[1]Cluster comprising superior temporal gyrus, supramarginal gyrus, middle temporal gyrus, inferior parietal lobule, posterior insula.

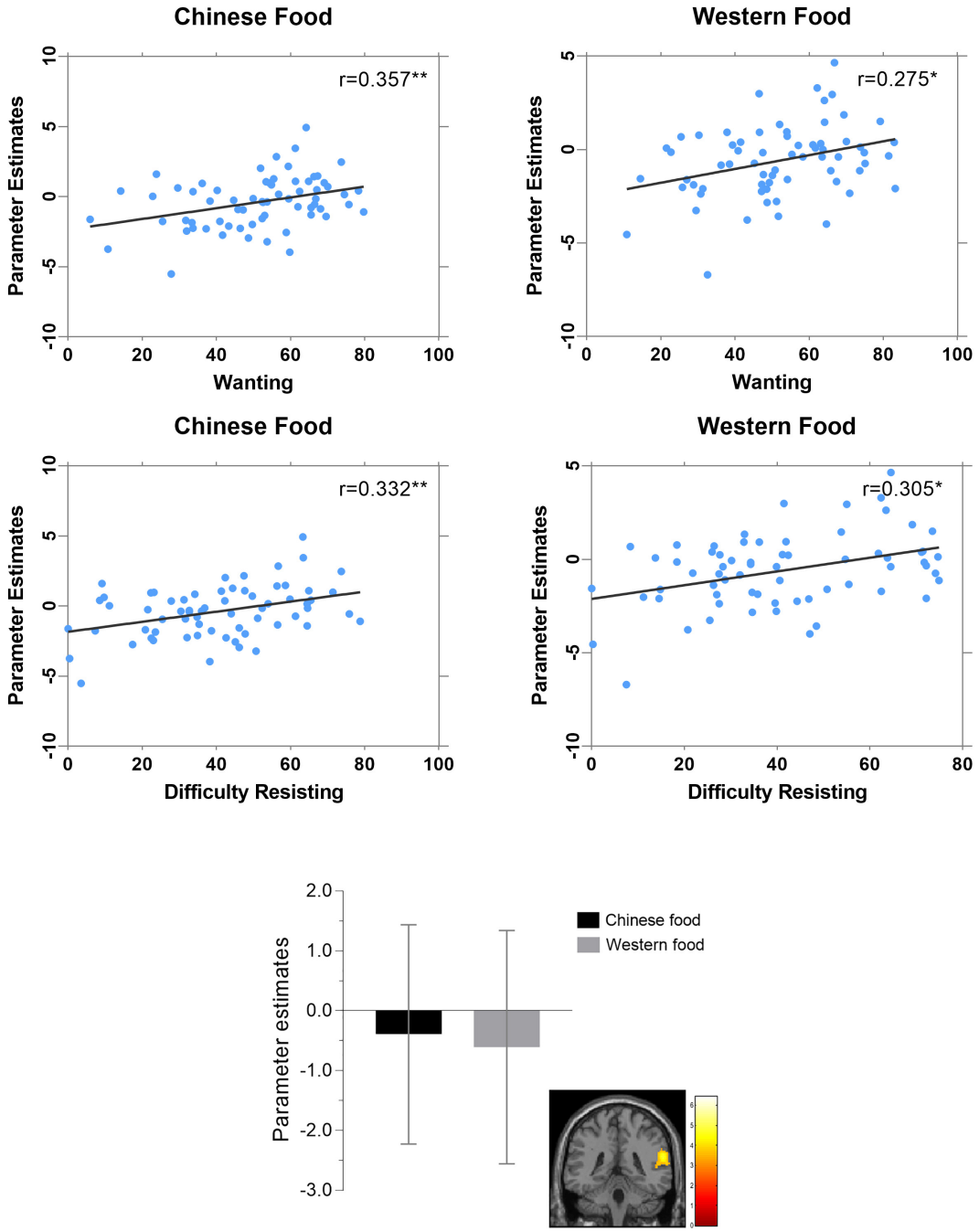


FIGURE 3
Correlations of food image ratings with BOLD responses of "reward" region cluster (superior temporal gyrus, supramarginal gyrus, middle temporal gyrus, inferior parietal lobule, posterior insula) differentiating Chinese versus Western food images (*$p < 0.05$, **$p < 0.01$).

food images elicited relatively increased responsivity in the lingual gyrus, cerebellum, and superior orbital frontal gyrus, and comparatively attenuated activity in the fusiform gyrus, precuneus, calcarine, cerebellum, frontal pole, middle frontal gyrus and angular gyrus (**Supplementary Table 1** and **Supplementary Figure 1**).

Discussion

This study is the first to evaluate differences in neural activation elicited by and judgments of Chinese versus Western food images among young Chinese adults. The hypothesis that respondents would display comparatively

stronger activation in regions associated with reward, gustatory responses, attention, and cognitive control during exposure to Chinese food images received partial support. Conversely, the hypothesis that participants would report corresponding elevations in liking, wanting, and/or difficulty resisting Chinese food images was not supported. Potential implications are elaborated in the context of related research on responses to visual food cues.

Neural activation in response to Chinese versus Western food images

Significantly stronger neural responsivity to Chinese than Western food images was found in select regions related to cravings, taste perception, and attention including the cerebellum crus, STG, SMG, MTG, IPL, and PI. Although the cerebellum is frequently overlooked in studies of neural responses to food images (59), its involvement in higher order functions such as reward-based learning, attention, emotion and executive functions (60) as well as the regulation of eating behaviors (61, 62) and olfaction (63) are widely documented. Some of this literature has underscored cerebellum involvement in processes underlying addiction including reward, motivational drive, salience, and inhibitory control (64). Briefly, the cerebellum crus is related to functional resting state networks and cerebral structures involved in executive control, drug craving, response selection and salience (65, 66). The cerebellum also modulates reward and saliency responses based on reciprocal connections with dopaminergic systems in basal ganglia (64). Previously, fMRI studies on addiction have found significantly increased cerebellum crus activation in response to images of drug use versus neutral images among recently abstinent heroin users (67, 68). Similarly, recently abstinent alcoholics have shown stronger cerebellum crus I activation during exposure to ethanol odors compared to non-patient controls (69). Although we do not claim that heightened cerebellum crus I responsiveness to Chinese versus Western food images in this study illustrates responses to addictive substances, the addiction metaphor has utility in framing fMRI results on responses to food cues (21, 70, 71). As such, our cerebellum crus I results may reflect greater intake of, stronger approach motivation toward, and/or increased automatic preferences for Chinese food images.

A cluster of structures (STG, SMG, MTG, IPL, PI) also showed significantly stronger activation during exposure to Chinese (versus Western food cues); associated functions of these structures include taste perception, reward processing, visual perception, cravings and inhibitory control. The STG is functionally related to the primary gustatory cortex (34). Links between STG responsiveness and taste perception have been found in various studies. For example, significantly stronger STG activation has been observed during presentations of food (versus non-food) images or food (versus non-food) commercials (72–74). Our results diverged somewhat from past studies (73, 74) in that we found no food versus non-food activation differences in the STG. Instead, stronger activation observed during exposure to Chinese (versus Western) food images suggested that differential STG responses are not a reflection of exposure to food *per se* but are due, instead, to indigenous versus non-indigenous culture of food images and related patterns of consumption. Aside from its involvement in taste perception, possibly due to accompanying emotional valences, the SMG has been linked to food reward processing, inhibitory control and eating behavior (59, 75–77). For example, stronger SMG disinhibition corresponds to increased food reward-related brain activation and emotional eating (75). Furthermore, compared with a satiated state, hunger elicits higher SMG functional connectivity with midbrain and limbic regions (59, 76). Increased SMG connectivity with the dorsal anterior cingulate cortex, insula, cerebellum has also been observed among binge-eaters in response to high (vs. low) calorie food cues (77). In the context of these findings, increased SMG activity during exposure to Chinese (versus Western) food images could reflect enhanced reward responsivity, reduced cognitive control, and/or increased motivation to eat.

Regarding other structures involved in this cluster, comparatively stronger activation of the IPL and MTG were also evident in response to Chinese food images. The IPL is a core attentional network node (26, 49) previously found to have enhanced responsiveness to food (versus non-food) images (78) as well as cues for addictions including gaming and smoking (79). In a recent China-based study, extreme cravers of spicy foods, showed significantly stronger activation to spicy than non-spicy food images compared to non-cravers in several regions including both the bilateral insula and right IPL. Furthermore, among extreme cravers, right IPL activation and frequency of spicy food intake had a significant positive correlation, independent of liking (80). In the context of these findings, IPL activity differences from our sample may have reflected increased attention allocation to Chinese food images and/or, more speculatively, higher intake of Chinese foods than Western foods. Differential MTG involvement may reflect increased visual perception or episodic memory elicited by Chinese food images, though one recent study reported increased MTG activation during processing of appetitive or high-calorie food cues (81).

Finally, Chinese (versus Western) food images elicited stronger activation in the PI, albeit involvement of this structure was modest, The PI has been implicated in somatosensory processing and as a food reward region (82–84). Pronounced PI activity has positive associations with subjective cravings elicited by images of addictive stimuli (73), imagination of taste and smell during visual food cue presentations (85, 86), passive visualization of food stimuli (87), consumption of highly palatable substances (88), receipt of a preferred

food/drink odors (86), internal hunger state (89), and gastric distention in the absence of actual food intake (90). In line with such data, images of Chinese foods may have elicited stronger recollections of taste properties (e.g., intensity, valence and identity of taste) and corresponding increases in PI activity compared to Western food images (91). Localization to the right PI also aligns somewhat with early support for right hemisphere dominance found for taste-related insula activation (92).

Notwithstanding the need for replications, these activation findings provide initial support for the hypothesis that, among Chinese participants, Chinese (versus Western) food images elicit stronger automatic responses in brain regions related to reward, attention, food intake and taste perception. Conversely, however, there were no activation differences in other reward circuit regions such as the nucleus accumbens, putamen, caudate, OFC, anterior insula, or amygdala (21, 23–25, 93). The absence of differences in ratings of liking, wanting, and difficulty resisting depicted Chinese versus Western food images may have contributed to the small number of reward area differences between these image sets. In addition, the use of Chinese versus Western food image sets matched for overall calories, familiarity, nutritional content, and flavors may have attenuated the number of differences in reward region responsiveness.

This explanation is not entirely sufficient, however, because supplementary analyses (**Supplementary Table 1**) revealed very few food versus furniture image differences in reward area activity compared to activation of regions involved in visual processing and attention. Research design features may have contributed to the pattern of food versus furniture activation effects. Specifically, participants did not undergo the study in a food deprived state because effects of culture, rather than hunger, were the central focus. In addition, images were presented during a passive viewing task rather than in the context of active engagement instructions (imagined taste versus imagined use). As such, it is possible that the sight of food (versus furniture) did not produce activation differences in associated brain regions (e.g., OFC) having links with reward because hunger and taste associations were attenuated (66). Future extensions evaluating activation differences in indigenous versus non-indigenous food image contrasts as well as contrasts between these food image types and non-food images under conditions of increased hunger (e.g., following an overnight fast) may provide additional insights about the role of culture as an influence on neural responses to food images.

Other activation differences were observed in regions related to vision and attention. For example, Chinese foods elicited stronger responses in middle and inferior occipital gyri. In general, these regions are involved in visual processing, yet meta-analytic evidence has linked elevations to preferred, "highly hedonic" foods relative to not-preferred, "bland/neutral" foods (93). It is not clear that our findings for these areas align with interpretations of non-conscious or conscious preferences for depictions of more hedonic foods because subjective evaluations of Chinese versus Western food images did not differ. Re-evaluations in the context of paradigms involving forced choice preferences for Chinese versus Western food items might clarify the validity of such interpretations.

Finally, Western food images elicited comparatively stronger activity in visual association regions (i.e., ITG, left middle occipital gyrus, fusiform gyrus) linked to complex visual tasks including attention to shapes, visual processing of objects and color, visual form discrimination, object recognition and object identity retrieval (94). Given that Chinese samples are more likely to eat Chinese foods than Western foods (1, 14, 15, 17), enhanced activity in these regions might reflect lower consumption rates of at least some depicted Western foods. However, extensions that include consumption frequency (e.g., food diaries) are needed to test this conjecture.

Subjective evaluations of Chinese versus Western food images

No Chinese versus Western foods image differences in liking, wanting, and difficulty resisting were reported, contrary to expectations. These null effects may have been due to equating food image sets for calorie levels, content, familiarity, and flavor characteristics. Furthermore, self-report judgments of Chinese versus Western food images may be an imprecise proxy for actual eating patterns or preferences in real world situations. Conversely, these null effects may have reflected increases in Western food outlets and gradual shift toward more Westernized diets in Chinese samples (2, 18). Relatedly, because our sample comprised young adults, differences in subjective evaluations of depicted Chinese versus Western foods may have been attenuated because neophobic attitudes toward Western food are strongest among older Chinese adults (1).

Associations of subjective evaluations with identified regions of interest

Aside from showing differential responsiveness during exposure to Chinese (versus Western) food images, the cluster comprising the STG, SMG, MTG, IPL, and PI also had significant positive correlations with wanting and difficulty resisting ratings of depicted food images following the scans; these correlations were stronger in relation to ratings of Chinese food images though associations with Western food image ratings were also statistically significant. Despite the lag between viewing and rating the food images, these correlations appear to be consistent with evidence of functions that include taste perception (34, 72), cravings (66, 79), reward processing and inhibitory control (59, 75, 88), and attention

(26, 49). While few past studies have assessed "difficulty resisting" food items or "dietary restraint" (49, 95), such research also taps evaluations of whether or not to consume depicted foods. The underlying neural system comprises the dorsolateral, inferolateral and superior prefrontal cortex, dorsal anterior cingulate cortex, basal ganglia nuclei, and cerebellum involved in attention, self-regulation and inhibitory control (49). Although preliminary, our results implicate possible involvement of a cluster comprising the right STG, SMG, MTG, IPL and PI in wanting and reported difficulty resisting visual food cues, though significantly different effect size strengths in responsiveness were not elicited by Chinese versus Western food images.

General implications for future research

Our main findings have potentially important implications for research and practice. Given that Chinese (versus Western) food images elicited significant neural activation differences in select clusters having involvement in gustation, cravings, reward processing and inhibitory control, attention, and visual processing, the implicit assumption that Western food images elicit neural responses that are universally representative of all cultural groups is not well founded. From this perspective, researchers should be cautioned against naively using "standardized" image sets of Western foods within neuroimaging studies conducted in China or other non-Western countries that have their own culinary traditions and preferences due to possible threats such images might pose for ecological validity or relevance. That is, neural responses based on exposure to images of Western foods may not always reflect typical neural responses to depictions of culturally indigenous foods that have more potential salience for actual diets of non-Western groups.

That said, the limited number of activation differences between Chinese versus Western food images and the absence of conscious subjective liking, wanting, and difficulty resisting food images from the two cultural contexts suggest that Western food products have become increasingly familiar and more popular in China, at least among young adults (2, 18). As such, hybrid sets of Chinese and Western food images may have more ecological validity than Chinese food images alone do within particular population groups. More broadly, the findings underscore the importance of considering the appropriateness of indigenous and/or standard (Western) food images in designing ecologically valid neuroimaging studies of visual food cues in non-Western groups.

In relation to possible applied implications, food stimuli can trigger significant neural, physiological and psychological changes (96). Therefore, food images can be used in research and clinical practice to detect and treat disordered eating behaviors. In light of their increased ecological validity in this research, Chinese food images from this study may aid in developing culturally informed assessments for detecting eating disturbances and stimuli for interventions (e.g., exposure therapy, imagery) designed to reduce these problems among people currently struggling with these problems within Chinese cultural contexts.

Limitations and future directions

Despite its novel focus and potential implications, the main limitations of this study should be acknowledged. First, it is not clear whether findings generalize to response difference comparisons of indigenous versus foreign food image in other non-Western cultural contexts or other age groups. In light of evidence that older Chinese adults are more prone to neophobia of Western foods (1), it is possible that both subjective preferences and neural responses of older respondents are even more distinct from those observed in our sample. Extensions should also be conducted within other age groups and samples from other cultures. Second, although food image sets were equated for calorie levels, content, familiarity, and flavor characteristics, it is possible that prior Chinese versus Western food consumption patterns reflected or affected associated preferences and neural responses. We did not assess participants' recent diets because strategies such as weekly food diaries are not well-validated in Chinese samples and are susceptible to potential biases related to a reliance upon retrospective recall. Nonetheless, as such procedures become more refined, effects of individual differences in indigenous versus non-indigenous food consumption patterns can be evaluated. Finally, because participants served as their own controls within this study, activation differences observed in this study could not be explained by numerous individual differences influences. Regardless, future studies might consider moderating effects of individual differences in biological factors (e.g., menstrual cycle stage for women, blood glucose levels), psychological influences (e.g., food neophobia, reward sensitivity), and study design features (e.g., deprivation versus satiation) on conscious reactions and neural responses to indigenous food versus Western food images.

Conclusion

To our knowledge, this study is the first to examine subjective reactions and neural responses to images of indigenous versus non-indigenous foods. Analyses indicated that even though conscious preferences did not differ between Chinese versus Western depictions of food, Chinese food images elicited comparatively stronger activation in particular regions linked to reward, higher-order cognitive control (i.e., cerebellum

crus, superior temporal gyrus inferior parietal lobule, posterior insula, occipital gyrus) as well as weaker activation in regions related to visual object recognition (ITG, occipital gyrus) and processing (cuneus). Results are preliminary but underscored culture as a potentially potent influence on neural responses to visual food cues and raised possible external validity concerns with non-critical use of Western food images in studies of non-Western samples. Consequently, in designing culturally informed research or interventions that involve visual depictions of food in non-Western contexts or distinct cultural groups in Western countries, the salience of indigenous food images versus "standard" Western food images warrants consideration.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Southwest University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TJ and JP: conceptualization. TJ, JP, and XX: methodology and resources. XX: software, investigation, and data curation. XX and TJ: validation, formal analysis, writing—original draft preparation, and visualization. TJ, XX, and AS: writing—review and editing. TJ: supervision, project administration, and funding acquisition. All author read and agreed to the published version of the manuscript.

Funding

This research was supported by a grant from the China National Natural Sciences Foundation (#31871141) and a 100 Persons Fellowship to the corresponding author.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.948039/full#supplementary-material

References

1. Li N. Food preferences of Chinese cruise tourists: an exploratory study. *J Foodserv Bus Res.* (2021) 25:1–22. doi: 10.1080/15378020.2021.1921327

2. Wang O, Gellynck X, Verbeke W. Perceptions of Chinese traditional food and European food among Chinese consumers. *Br Food J.* (2016) 118:2855–72. doi: 10.1108/BFJ-05-2016-0180

3. Mak AHN, Lumbers M, Eves A, Chang RCY. The effects of food-related personality traits on tourist food consumption motivations. *Asia Pacific J Tour Res.* (2017) 22:1–20. doi: 10.1080/10941665.2016.1175488

4. Xi Y, Liu Y, Yang Q, Liu H, Luo J, Ouyang Y, et al. Food neophobia and its association with vegetable, fruit and snack intake among 12-to 36-month toddlers in china: a cross-sectional study. *Food Qual Pref.* (2022) 98:104513. doi: 10.1016/j.foodqual.2021.104513

5. Neumark-Sztainer D, Wall M, Perry C, Story M. Correlates of fruit and vegetable intake among adolescents: findings from project eat. *Prev Med.* (2003) 37:198–208. doi: 10.1016/s0091-7435(03)00114-2

6. Veeck A, Yu FG, Yu H, Veeck G, Gentry JW. Influences on food choices of urban Chinese teenagers. *Young Consum.* (2014) 15:296.

7. Frewer LJ, van der Lans IA, Fischer AR, Reinders MJ, Menozzi D, Zhang X, et al. Public perceptions of agri-food applications of genetic modification–a systematic review and meta-analysis. *Trends Food Sci Technol.* (2013) 30:142–52. doi: 10.1016/j.tifs.2013.01.003

8. Okumus B, Dedeoğlu BB, Shi F. Gender and generation as antecedents of food neophobia and food neophilia. *Tour Manag Persp.* (2021) 37:100773. doi: 10.1016/j.tmp.2020.100773

9. Mascarello G, Pinto A, Rizzoli V, Tiozzo B, Crovato S, Ravarotto L. Ethnic food consumption in Italy: the role of food neophobia and openness to different cultures. *Foods.* (2020) 9:112. doi: 10.3390/foods9020112

10. Promsivapallop P, Prathana K. Factors influencing tourists' destination food consumption and satisfaction: a cross-cultural analysis. *Asia Pacific Soc Sc Rev.* (2020) 20:87–105.

11. La Barbera F, Verneau F, Amato M, Grunert K. Understanding westerners' disgust for the eating of insects: the role of food neophobia and implicit associations. *Food Qual Pref.* (2018) 64:120–5. doi: 10.1016/j.foodqual.2017.10.002

12. Çınar Ç, Karinen AK, Tybur JM. The multidimensional nature of food neophobia. *Appetite.* (2021) 162:105177. doi: 10.1016/j.appet.2021.105177

13. Cheung S, Wu DY. *The Globalisation of Chinese Food*. New York, NY: Routledge (2014).

14. Chang RC, Kivela J, Mak AH. Food preferences of Chinese tourists. *Ann Tour Res.* (2010) 37:989–1011. doi: 10.1016/j.annals.2010.03.007

15. Wang O, De Steur H, Gellynck X, Verbeke W. Motives for consumer choice of traditional food and European food in mainland China. *Appetite.* (2015) 87:143–51. doi: 10.1016/j.appet.2014.12.211

16. Chan K, Tse T, Tam D, Huang A. Perception of healthy and unhealthy food among Chinese adolescents. *Young Consum.* (2016) 1:32. doi: 10.1108/YC-03-2015-00520

17. Lin J, Guia Julve J, Xu H, Cui Q. Events. food habits and tourist food consumption: an exploratory study on dining behaviours of chinese outbound tourists in Spain. *J Policy Res Tour Leis Events.* (2020) 12:82–99. doi: 10.1080/19407963.2018.1506199

18. Howard AG, Attard SM, Herring AH, Wang H, Du S, Gordon-Larsen P. Socioeconomic gradients in the westernization of diet in China over 20 years. *SSM Popul Health.* (2021) 16:100943. doi: 10.1016/j.ssmph.2021.100943

19. Wang Y, Wang L, Xue H, Qu WA. Review of the growth of the fast food industry in China and its potential impact on obesity. *Int J Environ Res Public Health.* (2016) 13:1112. doi: 10.3390/ijerph13111112

20. Seo Y, Cruz AGB, Fam K-S. Food consumption and marketing: turning toward Asia. *J Int Consum Mark.* (2015) 32:501–4. doi: 10.1108/JCM-07-2015-1498

21. Pursey KM, Contreras-Rodriguez O, Collins CE, Stanwell P, Burrows TL. Food addiction symptoms and amygdala response in fasted and fed states. *Nutrients.* (2019) 11:1285. doi: 10.3390/nu11061285

22. Moreno-Padilla M, Verdejo-Román J, Fernández-Serrano MJ, Del Paso GAR, Verdejo-García A. Increased food choice-evoked brain activation in adolescents with excess weight: relationship with subjective craving and behavior. *Appetite.* (2018) 131:7–13. doi: 10.1016/j.appet.2018.08.031

23. Devoto F, Zapparoli L, Bonandrini R, Berlingeri M, Ferrulli A, Luzi L, et al. Hungry brains: a meta-analytical review of brain activation imaging studies on food perception and appetite in obese individuals. *Neurosci Biobehav Rev.* (2018) 94:271–85. doi: 10.1016/j.neubiorev.2018.07.017

24. Siep N, Roefs A, Roebroeck A, Havermans R, Bonte ML, Jansen A. Hunger is the best spice: an fMRI study of the effects of attention, hunger and calorie content on food reward processing in the amygdala and orbitofrontal cortex. *Behav Brain Res.* (2009) 198:149–58. doi: 10.1016/j.bbr.2008.10.035

25. Stice E, Yokum S. Gain in body fat is associated with increased striatal response to palatable food cues, whereas body fat stability is associated with decreased striatal response. *J Neurosci.* (2016) 36:6949–56. doi: 10.1523/JNEUROSCI.4365-15.2016

26. Murdaugh DL, Cox JE, Cook IIIEW, Weller RE. FMRI reactivity to high-calorie food pictures predicts short-and long-term outcome in a weight-loss program. *Neuroimage.* (2012) 59:2709–21. doi: 10.1016/j.neuroimage.2011.10.071

27. Charbonnier L, van Meer F, Johnstone AM, Crabtree D, Buosi W, Manios Y, et al. Effects of hunger state on the brain responses to food cues across the life span. *Neuroimage.* (2018) 171:246–55. doi: 10.1016/j.neuroimage.2018.01.012

28. Chen F, He Q, Han Y, Zhang Y, Gao X. Increased bold signals in dlPFC is associated with stronger self-control in food-related decision-making. *Front Psychiatry.* (2018) 9:689. doi: 10.3389/fpsyt.2018.00689

29. Stopyra MA, Friederich H-C, Mönning E, Lavandier N, Bendszus M, Herzog W, et al. The influence of homeostatic mechanisms on neural regulation of food craving in anorexia nervosa. *Psychol Med.* (2021) 51:1011–9. doi: 10.1017/s0033291719003970

30. Stice E, Yokum S, Veling H, Kemps E, Lawrence NS. Pilot test of a novel food response and attention training treatment for obesity: brain imaging data suggest actions shape valuation. *Behav Res Ther.* (2017) 94:60–70. doi: 10.1016/j.brat.2017.04.007

31. Yokum S, Stice E. Weight gain is associated with changes in neural response to palatable food tastes varying in sugar and fat and palatable food images: a repeated-measures fMRI study. *Am J Clin Nutr.* (2019) 110:1275–86. doi: 10.1093/ajcn/nqz204

32. Yang X, Casement M, Yokum S, Stice E. Negative affect amplifies the relation between appetitive-food-related neural responses and weight gain over three-year follow-up among adolescents. *Neuroimage Clin.* (2019) 24:102067. doi: 10.1016/j.nicl.2019.102067

33. Chin S-H, Kahathuduwa CN, Stearns MB, Davis T, Binks M. Is hunger important to model in FMRI visual food-cue reactivity paradigms in adults with obesity and how should this be done? *Appetite.* (2018) 120:388–97. doi: 10.1016/j.appet.2017.09.012

34. English LK, Fearnbach SN, Wilson SJ, Fisher JO, Savage JS, Rolls BJ, et al. Food portion size and energy density evoke different patterns of brain activation in children. *Am J Clin Nutr.* (2017) 105:295–305. doi: 10.3945/ajcn.116.136903

35. Porubská K, Veit R, Preissl H, Fritsche A, Birbaumer N. Subjective feeling of appetite modulates brain activity: an fMRI study. *Neuroimage.* (2006) 32:1273–80. doi: 10.1016/j.neuroimage.2006.04.216

36. Lyu Z, Jackson T. Acute stressors reduce neural inhibition to food cues and increase eating among binge eating disorder symptomatic women. *Front Behav Neurosci.* (2016) 10:188. doi: 10.3389/fnbeh.2016.00188

37. Liu Y, Zhang L, Jackson T, Wang J, Yang R, Chen H. Effects of negative mood state on event-related potentials of restrained eating subgroups during an inhibitory control task. *Behav Brain Res.* (2020) 377:112249. doi: 10.1016/j.bbr.2019.112249

38. Wang Y, Dong D, Jackson T, Du J, Yang Z, Lu H, et al. Neural correlates of restrained eaters'. High susceptibility to food cues: an FMRI study. *Neurosci Lett.* (2016) 631:56–62. doi: 10.1016/j.neulet.2016.08.017

39. Xinhang L. *The Establishment of Chinese Food Picture Library and Its Application in Dietary Psychology [Establishment and Application in Dieting Psychology of Chinese Food Image Library]*. Chongqing: Southwest University (2018).

40. Blechert J, Lender A, Polk S, Busch NA, Ohla K. Food-Pics_extended-an image database for experimental research on eating and appetite: additional images, normative ratings and an updated review. *Front Psychol.* (2019) 10:307. doi: 10.3389/fpsyg.2019.00307

41. Blechert J, Meule A, Busch NA, Ohla K. Food-Pics: an image database for experimental research on eating and appetite. *Front Psychol.* (2014) 5:617. doi: 10.3389/fpsyg.2014.00617

42. Samara A, Li X, Pivik RT, Badger TM, Ou X. Brain activation to high-calorie food images in healthy normal weight and obese children: a fMRI study. *BMC Obes.* (2018) 5:31. doi: 10.1186/s40608-018-0209-1

43. Mengotti P, Foroni F, Rumiati RI. Neural correlates of the energetic value of food during visual processing and response inhibition. *Neuroimage.* (2019) 184:130–9. doi: 10.1016/j.neuroimage.2018.09.017

44. Yuexin Y. *Chinese Food Composition Table*. 2nd ed. Beijing: Peking University Medical Press (2004).

45. Smeets PA, Dagher A, Hare TA, Kullmann S, van der Laan LN, Poldrack RA, et al. Good practice in food-related neuroimaging. *Am J Clin Nutr.* (2019) 109:491–503. doi: 10.1093/ajcn/nqy344

46. Erdfelder E, Faul F, Buchner A. Gpower: a general power analysis program. *Behav Res Methods Instrum Comput.* (1996) 28:1–11. doi: 10.3758/BF03203630

47. van Meer F, van der Laan LN, Eiben G, Lissner L, Wolters M, Rach S, et al. Development and body mass inversely affect children's brain activation in dorsolateral prefrontal cortex during food choice. *Neuroimage.* (2019) 201:116016. doi: 10.1016/j.neuroimage.2019.116016

48. Amaro E Jr, Barker GJ. Study design in fMRI: basic principles. *Brain Cogn.* (2006) 60:220–32. doi: 10.1016/j.bandc.2005.11.009

49. Carnell S, Benson L, Wang Z, Huo Y, Geliebter A, Peterson BS. Neural correlates of familial obesity risk and overweight in adolescence. *Neuroimage.* (2017) 159:236–47. doi: 10.1016/j.neuroimage.2017.07.052

50. Schneider W, Eschman A, Zuccolotto A. *E-Prime: User's Guide. Reference Guide. Getting Started Guide*. Pennsylvania, PA: Psychology Software Tools (2002).

51. Yan C-G, Wang X-D, Zuo X-N, Zang Y-F. Dpabi: data processing & analysis for (resting-state) brain imaging. *Neuroinformatics.* (2016) 14:339–51. doi: 10.1007/s12021-016-9299-4

52. Ashburner JA. Fast diffeomorphic image registration algorithm. *Neuroimage.* (2007) 38:95–113. doi: 10.1016/j.neuroimage.2007.07.007

53. Poldrack RA, Fletcher PC, Henson RN, Worsley KJ, Brett M, Nichols TE. Guidelines for reporting an fMRI study. *Neuroimage.* (2008) 40:409–14. doi: 10.1016/j.neuroimage.2007.11.048

54. Nie NH, Bent DH, Hull CH. *SPSS: Statistical Package for the Social Sciences*. New York, NY: McGraw-Hill (1975).

55. Spunt B. *Spunt/Bspmview: Bspmview V. 20161108*. Geneva: Zenodo (2016).

56. Eklund A, Nichols TE, Knutsson H. Cluster failure: why fMRI inferences for spatial extent have inflated false-positive rates. *Proc Natl Acad Sci U.S.A.* (2016) 113:7900–5. doi: 10.1073/pnas.1602413113

57. Nichols TE, Das S, Eickhoff SB, Evans AC, Glatard T, Hanke M, et al. Best practices in data analysis and sharing in neuroimaging using MRI. *Nat Neurosci.* (2017) 20:299–303. doi: 10.1038/nn.4500

58. Brett M, Anton JL, Valabregue R, Poline J-B. Region of interest analysis using the marsbar toolbox for spm 99. *Neuroimage.* (2002) 16:S497. doi: 10.1016/S1053-8119(02)90013-3

59. Chen EY, Zeffiro TA. Hunger and BMI modulate neural responses to sweet stimuli: fMRI meta-analysis. *Int J Obes.* (2020) 44:1636–52. doi: 10.1038/s41366-020-0608-5

60. Thoma P, Bellebaum C, Koch B, Schwarz M, Daum I. The cerebellum is involved in reward-based reversal learning. *Cerebellum.* (2008) 7:433–43. doi: 10.1007/s12311-008-0046-8

61. Mendoza J, Pévet P, Felder-Schmittbuhl M-P, Bailly Y, Challet E. The cerebellum harbors a circadian oscillator involved in food anticipation. *J Neurosci.* (2010) 30:1894–904. doi: 10.1523/JNEUROSCI.5855-09.2010

62. Zhu J-N, Wang J-J. The cerebellum in feeding control: possible function and mechanism. *Cell Mol Neurobiol.* (2008) 28:469–78. doi: 10.1007/s10571-007-9236-z

63. Ferdon S, Murphy C. The cerebellum and olfaction in the aging brain: a functional magnetic resonance imaging study. *Neuroimage.* (2003) 20:12–21. doi: 10.1016/S1053-8119(03)00276-3

64. Moulton EA, Elman I, Becerra LR, Goldstein RZ, Borsook D. The cerebellum and addiction: insights gained from neuroimaging research. *Addict Biol.* (2014) 19:317–31. doi: 10.1111/adb.12101

65. Goldstein RZ, Volkow ND. Dysfunction of the prefrontal cortex in addiction: neuroimaging findings and clinical implications. *Nat Rev Neurosci.* (2011) 12:652–69. doi: 10.1038/nrn3119

66. Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F, Baler R. Addiction: decreased reward sensitivity and increased expectation sensitivity conspire to overwhelm the brain's control circuit. *Bioessays.* (2010) 32:748–55. doi: 10.1002/bies.201000042

67. Lou M, Wang E, Shen Y, Wang J. Cue-Elicited craving in heroin addicts at different abstinent time: an fMRI pilot study. *Subst Use Misuse.* (2012) 47:631–9. doi: 10.3109/10826084.2011.646381

68. Sell L, Morris J, Bearn J, Frackowiak R, Friston K, Dolan RJ. Neural responses associated with cue evoked emotional states and heroin in opiate addicts. *Drug Alcohol Depend.* (2000) 60:207–16. doi: 10.1016/S0376-8716(99)00158-1

69. Schneider F, Habel U, Wagner M, Franke P, Salloum JB, Shah NJ, et al. Subcortical correlates of craving in recently abstinent alcoholic patients. *Am J Psychiatry.* (2001) 158:1075–83. doi: 10.1176/appi.ajp.158.7.1075

70. Dagher A. The neurobiology of appetite: hunger as addiction. *Obes Prev.* (2010) 33:S30–3. doi: 10.1016/B978-0-12-374387-9.00002-7

71. Schulte EM, Yokum S, Jahn A, Gearhardt AN. Food cue reactivity in food addiction: a functional magnetic resonance imaging study. *Physiol Behav.* (2019) 208:112574. doi: 10.1016/j.physbeh.2019.112574

72. Stapleton P, Buchan C, Mitchell I, McGrath Y, Gorton P, Carter B. An initial investigation of neural changes in overweight adults with food cravings after emotional freedom techniques. *OBM Integr Complement Med.* (2019) 4:1. doi: 10.21926/obm.icm.1901010

73. Buchsbaum BR, Hickok G, Humphries C. Role of left posterior superior temporal gyrus in phonological processing for speech perception and production. *Cogn Sci.* (2001) 25:663–78. doi: 10.1016/S0364-0213(01)00048-9

74. St-Onge M-P, Sy M, Heymsfield SB, Hirsch J. Human cortical specialization for food: a functional magnetic resonance imaging investigation. *J Nutr.* (2005) 135:1014–8. doi: 10.1093/jn/135.5.1014

75. Drummen M, Dorenbos E, Vreugdenhil AC, Raben A, Westerterp-Plantenga MS, Adam TC. Insulin resistance, weight, and behavioral variables as determinants of brain reactivity to food cues. *Amer J Clin Nutr.* (2019) 109:315–21. doi: 10.1093/ajcn/nqy252

76. Yousuf M, Heldmann M, Göttlich M, Münte TF, Doñamayor N. Neural processing of food and monetary rewards is modulated by metabolic state. *Brain Imag Behav.* (2018) 12:1379–92. doi: 10.1007/s11682-017-9811-y

77. Geliebter A, Benson L, Pantazatos SP, Hirsch J, Carnell S. Greater anterior cingulate activation and connectivity in response to visual and auditory high-calorie food cues in binge eating: preliminary findings. *Appetite.* (2016) 96:195–202. doi: 10.1016/j.appet.2015.08.009

78. Nakamura Y, Imafuku M, Nakatani H, Nishida A, Koike S. Difference in neural reactivity to taste stimuli and visual food stimuli in neural circuits of ingestive behavior. *Brain Imaging Behav.* (2020) 14:1395–405. doi: 10.1007/s11682-019-00048-0

79. Sun Y, Ying H, Seetohul RM, Xuemei W, Ya Z, Qian L, et al. Brain fMRI study of crave induced by cue pictures in online game addicts (male adolescents). *Behav Brain Res.* (2012) 233:563–76. doi: 10.1016/j.bbr.2012.05.005

80. Zhou Y, Gao X, Small DM, Chen H. Extreme spicy food cravers displayed increased brain activity in response to pictures of foods containing chili peppers: an fMRI study. *Appetite.* (2019) 142:104379. doi: 10.1016/j.appet.2019.104379

81. Han P, Roitzsch C, Horstmann A, Pössel M, Hummel T. Increased brain reward responsivity to food-related odors in obesity. *Obesity.* (2021) 29:1138–45. doi: 10.1002/oby.23170

82. Sescousse G, Caldú X, Segura B, Dreher J-C. Processing of primary and secondary rewards: a quantitative meta-analysis and review of human functional neuroimaging studies. *Neurosci Biobehav Rev.* (2013) 37:681–96. doi: 10.1016/j.neubiorev.2013.02.002

83. Le TM, Liao D-L, Ide J, Zhang S, Zhornitsky S, Wang W, et al. The interrelationship of body mass index with gray matter volume and resting-state functional connectivity of the hypothalamus. *Int J Obes.* (2020) 44:1097–107. doi: 10.1038/s41366-019-0496-8

84. Castro DC, Berridge KC. Opioid and orexin hedonic hotspots in rat orbitofrontal cortex and insula. *Proc Natl Acad Sci U.S.A.* (2017) 114:E9125–34. doi: 10.1073/pnas.1705753114

85. Siep N, Roefs A, Roebroeck A, Havermans R, Bonte M, Jansen A. Fighting food temptations: the modulating effects of short-term cognitive reappraisal, suppression and up-regulation on mesocorticolimbic activity related to appetitive motivation. *Neuroimage.* (2012) 60:213–20. doi: 10.1016/j.neuroimage.2011.12.067

86. Wright H, Li X, Fallon NB, Crookall R, Giesbrecht T, Thomas A, et al. Differential effects of hunger and satiety on insular cortex and hypothalamic functional connectivity. *Eur J Neurosci.* (2016) 43:1181–9. doi: 10.1111/ejn.13182

87. Britton JC, Phan KL, Taylor SF, Welsh RC, Berridge KC, Liberzon I. Neural correlates of social and nonsocial emotions: an fMRI Study. *Neuroimage.* (2006) 31:397–409. doi: 10.1016/j.neuroimage.2005.11.027

88. Stice E, Spoor S, Bohon C, Veldhuizen MG, Small DM. Relation of reward from food intake and anticipated food intake to obesity: a functional magnetic resonance imaging study. *J Abnorm Psychol.* (2008) 117:924.

89. Charroud C, Poulen G, Sanrey E, de Champfleur NM, Deverdun J, Coubes P, et al. Task-and rest-based functional brain connectivity in food-related reward processes among healthy adolescents. *Neuroscience.* (2021) 457:196–205. doi: 10.1016/j.neuroscience.2021.01.016

90. Wang G, Tomasi D, Volkow N, Wang R, Telang F, Caparelli E, et al. Effect of combined naltrexone and bupropion therapy on the brain's reactivity to food cues. *Int J Obes.* (2014) 38:682–8. doi: 10.1038/ijo.2013.145

91. Rolls ET. Functions of the anterior insula in taste, autonomic, and related functions. *Brain Cogn.* (2016) 110:4–19. doi: 10.1016/j.bandc.2015.07.002

92. Small DM, Gregory MD, Mak YE, Gitelman D, Mesulam MM, Parrish T. Dissociation of neural representation of intensity and affective valuation in human gustation. *Neuron.* (2003) 39:701–11. doi: 10.1016/S0896-6273(03)00467-7

93. van der Laan LN, De Ridder DT, Viergever MA, Smeets PA. The first taste is always with the eyes: a meta-analysis on the neural correlates of processing visual food cues. *Neuroimage.* (2011) 55:296–303. doi: 10.1016/j.neuroimage.2010.11.055

94. Greenlee MW, Tse PU. Functional neuroanatomy of the human visual system: a review of functional mri studies. *Pediatr Ophthalmol Neuro Ophthalmol Genet.* (2008):119–38. doi: 10.1007/978-3-540-33679-2_8

95. Alonso-Alonso M, Ziemke F, Magkos F, Barrios FA, Brinkoetter M, Boyd I, et al. Brain responses to food images during the early and late follicular phase of the menstrual cycle in healthy young women: relation to fasting and feeding. *Am J Clin Nutr.* (2011) 94:377–84. doi: 10.3945/ajcn.110.010736

96. Burmester V, Graham E, Nicholls D. Physiological, emotional and neural responses to visual stimuli in eating disorders: a review. *J Eat Disord.* (2021) 9:1–16. doi: 10.1186/s40337-021-00372-1

frontiers | Frontiers in Nutrition

TYPE Review
PUBLISHED 22 August 2022
DOI 10.3389/fnut.2022.943998

Check for updates

OPEN ACCESS

EDITED BY
Andrew Scholey,
Swinburne University of Technology, Australia

REVIEWED BY
Monica Carrera,
Spanish National Research Council (CSIC), Spain
Yu Xiao,
Hunan Agricultural University, China

*CORRESPONDENCE
Karolina Krupa-Kotara
kkrupa@sum.edu.pl

SPECIALTY SECTION
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

RECEIVED 14 May 2022
ACCEPTED 20 July 2022
PUBLISHED 22 August 2022

CITATION
Grajek M, Krupa-Kotara K, Białek-Dratwa A, Sobczyk K, Grot M, Kowalski O and Staśkiewicz W (2022) Nutrition and mental health: A review of current knowledge about the impact of diet on mental health. *Front. Nutr.* 9:943998. doi: 10.3389/fnut.2022.943998

COPYRIGHT
© 2022 Grajek, Krupa-Kotara, Białek-Dratwa, Sobczyk, Grot, Kowalski and Staśkiewicz. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Nutrition and mental health: A review of current knowledge about the impact of diet on mental health

Mateusz Grajek[1], Karolina Krupa-Kotara[2]*, Agnieszka Białek-Dratwa[3], Karolina Sobczyk[4], Martina Grot[1], Oskar Kowalski[3] and Wiktoria Staśkiewicz[5]

[1]Department of Public Health, Department of Public Health Policy, Faculty of Health Sciences in Bytom, Medical University of Silesia in Katowice, Katowice, Poland, [2]Department of Epidemiology, Faculty of Health Sciences in Bytom, Medical University of Silesia in Katowice, Katowice, Poland, [3]Department of Human Nutrition, Department of Dietetics, Faculty of Health Sciences in Bytom, Medical University of Silesia in Katowice, Katowice, Poland, [4]Department of Economics and Health Care Management, Faculty of Health Sciences in Bytom, Medical University of Silesia in Katowice, Katowice, Poland, [5]Department of Technology and Food Quality Evaluation, Department of Dietetics, Faculty of Health Sciences in Bytom, Medical University of Silesia in Katowice, Katowice, Poland

Applied psychopharmacotherapy and psychotherapy do not always bring the expected results in the treatment of mental disorders. As a result, other interventions are receiving increasing attention. In recent years, there has been a surge in research on the effects of nutrition on mental status, which may be an important aspect of the prevention of many mental disorders and, at the same time, may lead to a reduction in the proportion of people with mental disorders. This review aims to answer whether and to what extent lifestyle and related nutrition affect mental health and whether there is scientific evidence supporting a link between diet and mental health. A review of the scientific evidence was conducted based on the available literature by typing in phrases related to nutrition and mental health using the methodological tool of the PubMed database. The literature search yielded 3,473 records, from which 356 sources directly related to the topic of the study were selected, and then those with the highest scientific value were selected according to bibliometric impact factors. In the context of current changes, urbanization, globalization, including the food industry, and changes in people's lifestyles and eating habits, the correlations between these phenomena and their impact on mental state become important. Knowledge of these correlations creates potential opportunities to implement new effective dietary, pharmacological, therapeutic, and above all preventive interventions. The highest therapeutic potential is seen in the rational diet, physical activity, use of psychobiotics, and consumption of antioxidants. Research also shows that there are nutritional interventions that have psychoprotective potential.

KEYWORDS

nutrition, mental health, diet, psychology of food, eating behavior

Background

Inherent in urbanization and the accompanying technological and cultural development, the rush of life, the pursuit of self-actualization, and the resulting overstimulation and lack of time, affect the change in eating habits and the consumption of high-calorie and processed foods (1). We can consider them as factors influencing the development of civilization diseases, important from the point of view of public health. Among them, we cannot forget about depressive and anxiety disorders that are becoming a global epidemic (2). The number of people requiring assistance from a mental health professional is steadily increasing in Poland and worldwide. According to the International Health Metrics Evaluation (IHME), at the end of 2017, 13% of the world population suffered from mental disorders (3). The Wittchen et al. study shows that mental disorders affect 38% of the European population (4). By the end of 2019, about 1.6 million people in Poland had received psychiatric treatment (5). The situation was not improved by the COVID-19 pandemic and related sanitary restrictions, which led to the isolation of many people, with feelings of insecurity, sadness, anxiety, and misinformation (6). All this has made psychological and psychiatric help the most sought-after form of health support today. There are only about 4,300 practicing psychiatrists in Poland (7). Even fewer, only 455, are practicing child psychiatry specialists (8). Statistics are believed to be better in the psychological and psychotherapeutic support sector, although public opinion is still divided about this form of support. Moreover, registers of psychologists and psychotherapists are not common. The described phenomena lead to a transformation of the psychiatric care model and mental health support. The number of people receiving psychiatric treatment is expected to increase over the next decades. The applied psychopharmacotherapy and psychotherapy do not always bring the expected treatment result (9). As a result, other interventions are receiving increasing attention. In recent years, there has been a dramatic increase in research on the effects of nutrition on mental status, which may be an important aspect of the prevention of many mental disorders, and at the same time may lead to a reduction in the proportion of people with mental disorders.

Thus, this review aims to answer the question of whether and to what extent lifestyle and related nutrition affect mental health and whether there is scientific evidence supporting the diet and mental health relationship.

The question posed in the objective can be divided into specific questions according to which this review was divided.

Q1: Are there correlations between nutrition and mental health?
Q2: Are there psychoprotective food ingredients?
Q3: Are there nutritional interventions with proven preventive potential for mental disorders?

Review methodology

Methodology background

The main aspect that guided the review works conducted was to look for nutritional recommendations in the cited works regarding nutrition as psychoprophylaxis and dietary management of psychiatric disorders. Unfortunately, the current state of knowledge on this topic, despite many studies, is still poor, so the authors decided to conduct a broad review of the most current knowledge in this area to identify those sources that address the described topic and gather in one place the available knowledge.

Review procedure

The review was conducted following good practices associated with conducting similar reviews. Literature items were searched by a team of researchers (authors) along with a library staff member trained in literature searching and EBM (evidence-based medicine) and HTA (health technology assessment). A preliminary search for items consistent with the topic and purpose of the review was conducted to identify the research field. After reviewing existing data, a keyword package was selected that seemed most relevant and consistent with the review topic.

Eligibility criteria

The primary eligibility criteria were the language of publication, years of research or review, publication status, and whether the authors were specialists in their field (or had other publications in a similar field). Regarding language, English-language articles were selected because this language seems to be universal in the scientific community. In addition, articles that were published after 2005 were included to make sure that the topic addressed was not a completely new field of research, but also to avoid very old data, because as is known from common practice, dietetics, as well as mental health expertise, are two of the most rapidly developing scientific fields. Additionally, articles were selected that were available in full-text on an open-access basis and had impact factor values.

Search strategy

A review of the scientific evidence was conducted based on the available literature by entering sample phrases (consistent with the MeSh dictionary) with Boole operators, logical operators (and, or, not), and special characters,: "psychodietetics," "nutripsychiatry," "diet," "mental health,"

"lifestyle," "body weight," "obesity," "depression," "mental disorders" (and various combinations thereof) using the methodological tool of the PubMed database. The PubMed database in this regard seems most appropriate because it is a methodological tool that allows searching for articles available in multiple scientific databases (such as Medline or Embase). Its use provides the opportunity to meet all expectations from the review (transparency, clarity, comprehensiveness, focus, uniformity, accessibility, coverage of the entire topic).

Sources selection

The literature search yielded 3,473 records, from which 356 sources directly related to the topic of the study were selected, and then those with the highest scientific value were selected according to eligibility criteria.

The accuracy, objectivity, validity, and relevance of the evidence were tested using questions consistent with the GRADE scale: Is the information reliable? Is the information free of mistakes? Has the information been properly substantiated? Is it possible to verify the information against other reliable sources? Who are the authors? Are they qualified to present information on the topic? Are they affiliated with reputable institutions working on the issue? Is the data source peer-reviewed? For what purpose was the information? Is the information an evidenced-based fact or constitutes an opinion? Is the information subject to risk? Can this risk be estimated? When was the information published? Is the information current or outdated? Is the timeliness relevant to the issue at hand? Does the information cover the entire issue? Does the information contain background data or does it explore the issue in depth? The final literature review was based on 110 sources, representing mainly scientific output after 2005 and important multicenter studies performed after 2015. The data obtained from the review are presented in descriptive and tabular form. In addition, 11 additional sources were used in preparing the background of the research problem and the theoretical introduction.

Critical appraisal

In critically evaluating the sources, attention was paid to whether the articles appeared in peer-reviewed journals (by at least two reviewers) and whether they had an impact factor. As described above, 110 sources were eligible for final review. A limitation of the method adopted was primarily the exclusion of sources written in a language other than English. In addition, IF has many well-documented drawbacks as a research assessment tool and therefore is not the best way to evaluate the quality of individual research articles. Nevertheless, it was chosen because it is a synthetic indicator of a source's impact on the field of science, and a journal that has it can more likely claim to be publishing credible scientific evidence. The review did not include so-called "grey literature", i.e., literature that has not gone through the review process or that is internal to the university (theses, conference reports, government leaflets, newsletters, etc.). Despite their multiple values, these sources are characterized by a high risk of containing outdated knowledge (**Figure 1**).

Q1: Are there correlations between nutrition and mental health?

Excess body weight is certainly an important social problem today. More than 0.7 billion people worldwide are obese, this is about 30% of the total population, and the number of obesity-related deaths is constantly increasing (10). We consume more and more processed, high-energy, and nutrient-poor foods. Consequently, we face problems of overweight and obesity with concomitant nutrient deficiencies (quantitative malnutrition) (11). Although the level of calories consumed is increasing, we are not taking in the recommended values of micro- and macroelements that play a significant role in the proper functioning of our nervous system – B vitamins, zinc, and magnesium. Additionally, we consume less fiber- and nutrient-rich vegetables and cereal products than recommended (10, 11). Superimposing smoking, limited physical activity, and harmful alcohol consumption to the above dietary patterns, adversely affect health and development of mental disorders, including depression (10). Whose nutritional prevention is well documented in the literature (12).

The antioxidant system, which has been implicated in the development of psychiatric disorders, is relevant here (13) and its proper functioning depends on the presence of nutrients in food. In addition, the concentration of brain-derived neurotrophic factor (BDNF), which is involved in plasticity and neurodegenerative processes, depends on nutrients (14). Findings indicate a reduction in the incidence of depression and suicide with a healthy eating pattern (15, 16). Randomized trials are emerging that evaluate the efficacy of dietary change as a form of treatment for depression (15–17). Selective food supplementation can be beneficial in the treatment of psychiatric disorders. Among them, compounds such as S-adenosylmethionine, *N*-acetylcysteine, zinc, and B vitamins including folic acid, and vitamin D are mentioned. Also, omega-3 unsaturated fatty acids have a wide range of effects. They participate in synaptogenesis by influencing receptor degradation and synthesis. They have an anti-inflammatory effect and inhibit apoptosis. They affect cell membrane function, BDNF action, and neurotransmitter reuptake (18). S-adenosylmethionine (SAM) is a compound formed from adenosine and methionine, which plays a key role in methylation processes. The results of studies show its antidepressant effects (19). The use of *N*-acetylcysteine influenced the effectiveness of therapy in schizophrenia, bipolar

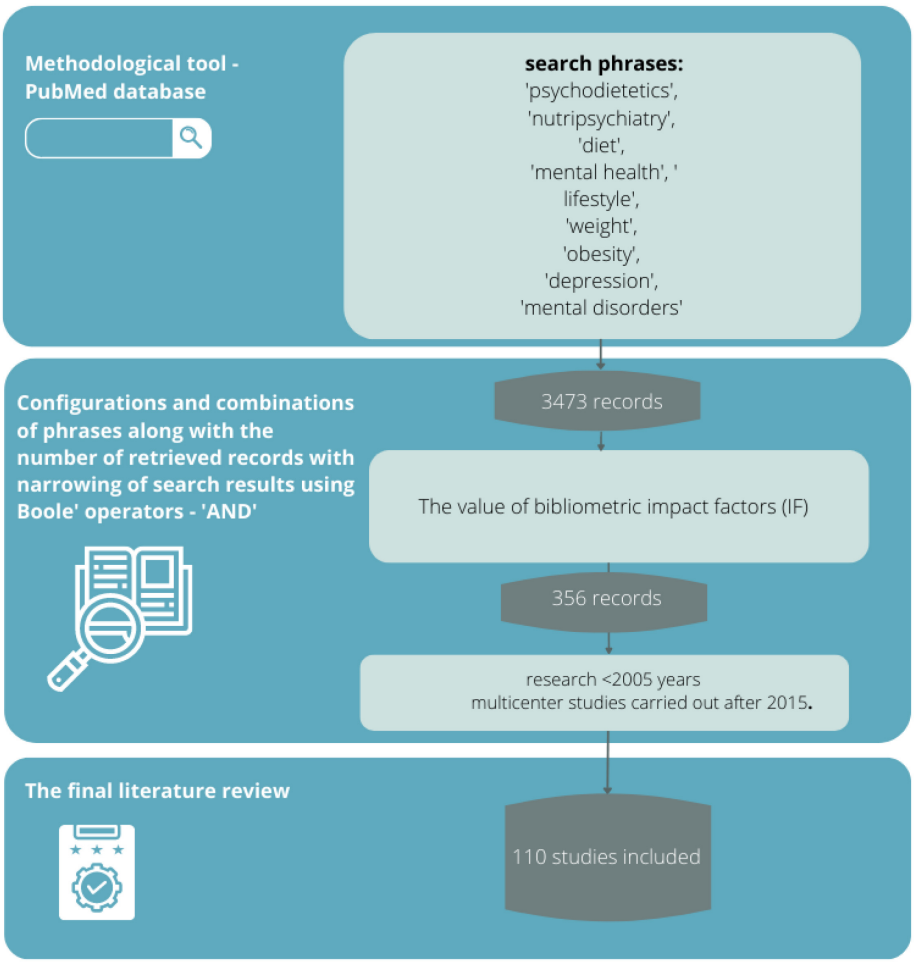


FIGURE 1
A flowchart of how to proceed in selecting bibliographic sources.

affective disorder, or trichotillomania. It has anti-inflammatory, antioxidant, and neuroprotective effects (20). Zinc deficiency, in turn, has been linked to the severity of depressive symptoms, and its supplementation included with antidepressants plays a role in mood stabilization. Zinc modulates cytokine activity and influences neurogenesis by affecting brain-derived neurotrophic factor levels (21). B vitamins play a role in the proper functioning of the nervous tissue. Folic acid (vitamin B9) deficiency has been associated with depressive symptoms and determined in subjects with mediocre responses to antidepressants (22). Low vitamin D levels were associated with a higher risk of schizophrenia and depression (23). It has been proven that vitamin D supplementation for a period of 3 months (4,000 IU/day for 1 month and 2,000 IU/day for 2 months) significantly reduced the severity of depression, irritability, fatigue, mood swings, sleep difficulties, weakness, and ability to concentrate in adolescents diagnosed with depression. This effect is supported by studies on animal models – vitamin D contributes to the plasticity of synapses, has a neuroprotective effect, supports the production of neurotrophic factors such as nerve growth factor (NGF) and regulates the function of the dopaminergic system. (24).

For the review, the results of the most important studies on the psychoprotective effects of bioactive components contained in foods (vitamins, minerals, omega-3, and more). have been collected in tabular form (**Table 1**).

Q2: Are there psychoprotective food ingredients?

The gut microbiota is estimated to form a complex ecosystem containing 1,014 microorganisms. It contains 3.3 million genes and outnumbers the human genome by about 150-fold. At the same time, it is built by more than a thousand different species of microorganisms (25). The gut-brain axis describing the bidirectional relationship between the gastrointestinal tract and the central nervous system uses several

TABLE 1 Review of selected studies on the psychoprotective effect of probiotics.

Source	Sample	Bioactive ingredient	Results or conclusions
Gazerani (96)	Review article – group struggling with migraine headaches	Folate in the form of folic acid – B_9	Addition of a methyl group to DNA methyltransferase during the DNA methylation process and adequate serum homocysteine secretion levels prevent migraine headaches
Cater et al. (97); Parikh et al. (98)	Review article – neurotherapeutic properties among healthy human populations and newborns	Docosahexaenoic acid – omega-3	Stimulates neurotransmission and development of the cerebral cortex and visual organ through the blood-brain barrier. Increased neuro-efficiency of non-verbal and verbal communication processes
Parikh et al. (98)	Review article – among a population of people with nervous system conditions – depression and newborns with encephalopathy	Alpha-lipoic acid, lignans, soluble fiber, phytoestrogen – secoisolariciresinol diglucoside	Development and size of cortical cells in the prenatal and postnatal periods . Neuromodulates cognitive-behavioral behavior. Prevention against depressive symptoms in offspring and hypoxic-ischemic encephalopathy among newborns. Reduced oxidative stress parameters in the oxidation process reducing inflammation within the nervous system
Park et al. (99); Mulati et al. (100)	Depressed patients. Blinded randomized study. $N = 40$, duration – 8 weeks An obese mouse model of neuronal impairment. Blinded, randomized study. Duration – 14 weeks	Flavonoids	Improving brain-derived neurotrophic parameter (BDNF), reducing symptoms in the pathomechanism of depression. PSD-95 protein expression affects dysfunction within synapses and neurons
Mittal et al. (101)	Review article – reduction in symptoms and progression of Parkinson's disease	Exogenous amino acid – tryptophan	Metabolic transformations to the starting compound serotonin allow to achieve regulation of the diurnal rhythm, emotional state. Participation in the metabolism of catecholamines regulating processes at the level of the brain-gut axis. Prevention in the pathomechanism of Parkinson's disease
Fernández et al. (102); Dogan-Sander et al. (103); Godos et al. (104)	Review studies, meta-analysis of studies. Improvement of neuronal and cognitive impairment in patients with Parkinson's disease, schizophrenia, depression	Magnesium calcium, selenium, zinc, manganese, copper, antioxidants – vitamin D, E, C, carotenoids	Reducing the mechanism of oxidative stress achieving systemic balance consequently the absence of chronic inflammation along with a decrease in CRP, IL-6, WBC indices and somato-psychological symptoms in a depressed state.
Godos et al. (104); Burton-Freeman et al. (105)	Review studies, meta-analysis of studies Improving neuronal and cognitive impairment in patients with Alzheimer's disease, inflammation within neurons	Complex carbohydrates, eicosapentaenoic acid, amino acid – glycine, polyphenols, anthocyanins	Regulation of neuromodulator and neurotransmitter expression. Reduced activation of the hypothalamic-pituitary-adrenal axis under the influence of lower levels of endogenous stress – lower corticosterone concentrations. Proper insulin secretion and glucose ejection into cells – adequate GLUT receptor functionality. Modulation of the processes of neurogenesis, synaptic plasticity and activation of microglia in the central nervous system. Prevention of inflammation, neurodegenerative changes through inactivation of the process of oxidation of the LDL fraction, lipid peroxidation and activation of the enzymes catalase and superoxide dismutase

Source: Own compilation based on literature review.

10.3389/fnut.2022.943998

communication mechanisms. Mutual exchange of information can occur *via* the autonomic nervous system and the vagus nerve (26). Many of the effects of probiotics on mental status are associated with information transmission *via* the vagus nerve (27). Results from germ-free (GF) mice cultured under sterile conditions, devoid of detectable microorganisms, demonstrate the involvement of the gut microbiota in the proper formation and function of the endocrine system by influencing the development of the hypothalamic-pituitary-adrenal axis. The response to a stress stimulus as measured by glucocorticosteroid and adrenocorticotropin levels was significantly elevated in GF mice. It was normalized after gastrointestinal colonization with the *Bifidobacterium infantis* strain (28). Additionally, stress affects the formation and diversity of intestinal microflora (29). Another link of communication is the immune system. The microbiota is involved in the proper development of the gastrointestinal mucosal immune system (30). Bacterial antigens such as polysaccharide A, lipopolysaccharides, and thymic acids shape its proper functioning (31). The microbiota also produces neurotransmitters: gamma-aminobutyric acid, butyric acid, serotonin, dopamine, and short-chain fatty acids, which can directly affect the nervous system (32).

So, can the psychoprotective effect of strains be used in nutritional intervention? It seems reasonable here to consider the possibility of implementing treatment with probiotic preparations containing selected bacterial strains that show positive effects on the human psyche. In this approach, "probiotic" is defined as living organisms that, when consumed in adequate amounts, have a beneficial effect on the functioning of the body (33). Ilya Metchnikov was awarded the Nobel Prize in 1908 for his research on probiotics. Among them, lactic acid bacteria are the most popular. Probiotics are mainly found in fermented dairy products, or pickled products (34). Prebiotics are non-digested food components whose fermentation in the gastrointestinal tract stimulates either bacterial growth or activity or affects both, leading to the development of beneficial intestinal microflora (35). Prebiotics can include ingredients such as inulin or fructooligosaccharides. Prebiotics may also have a beneficial effect by inhibiting the growth of pathogenic bacteria. Moreover, some research results show that prebiotics can reduce inflammation by modifying the composition of the microbiota (36). Synbiotics are ingredients that contain both prebiotics and probiotics. Such a constellation allows the use of synergistic effects of these preparations. In turn, psychobiotics are defined as microorganisms that are probiotics, that show positive effects in patients treated for mental disorders (37). They can often achieve their effect through the production of neurotransmitters such as gamma-aminobutyric acid, serotonin, or other substances with an effect on the cells of the nervous system such as short-chain organic acids: acetic, propionic, or butyric (36). Oral substitution of such probiotics as *Lactobacillus helveticus* and *Bifidobacterium longum* over a period of 1 month was associated with a reduction in symptoms of anxiety and depressive disorders and a reduction in stress levels as measured by the determination of cortisol levels in animal models (38). Currently, the most effective treatment of psychiatric disorders is achieved through the use of antidepressants, or antipsychotics. However, the additional use of psychobiotics to treat anxiety or depressive disorders may prove effective in the future. It is also worth noting that popular antidepressants and antipsychotics can affect the quality of gut flora and change the composition of the microbiome to a disadvantage by killing the cultures of bacteria living in the gastrointestinal tract (39).

For the review, the results of the most important studies on the psychoprotective effect of probiotics were collected in tabular form – **Table 2**.

Factors such as genotype, intrauterine infections, developmental disorders, later traumatizing events, use of harmful psychoactive substances, and many others will influence the onset of psychiatric disorders. These factors influence not only the onset of the disorder but also its progression. Treating early conditions in psychiatry can result in a much better response to the treatment given and better functioning of patients. This fact can be particularly observed in studies on the early detection of psychotic disorders (40). Prevention in medicine, including psychiatry, requires knowledge of appropriate and useful tools that would allow detection of increased risk of mental illness and monitoring of the developing psychopathology of the disorder. McGorry et al. (41) proposed a four-stage model of the development of mental disorders. According to this model, serious mental disorders develop from high-risk states: grade 0 means the development of undifferentiated, general symptoms, such as slight anxiety, restlessness, depressive symptoms, or somatic symptoms lead to grade 1, in which types 1A and 1B can be distinguished according to their severity. Further progression of the disease results in the development of a first episode of the disorder and here we speak of stage 2, which is accompanied by persistent 7ncludims and frequent relapses. Grade 3 includes incomplete remission and regular and repeated relapses. Grade 4 in this context means treatment-resistant disorder. The worsening of a psychiatric disorder is determined by genetic and environmental factors, and it is the latter that seems to be the main target for preventive interventions in psychiatry. Some biomarkers in psychiatry are directly related to nutrition. The first of these is the hypothalamic-pituitary-adrenal axis (HPA). Reduced ability to cope with stress plays a role in the development of psychiatric disorders (42). It is known that traumatizing experiences in early childhood shape vulnerability to stress in later life (43). The normal functioning of the HPA axis is often altered in psychiatric disorders, and increased cortisol secretion is observed in affective and psychotic disorders. Additionally, antipsychotic drugs appear to decrease HPA

TABLE 2 Review of selected studies on the psychoprotective effects of substances contained in food.

Source	Sample	Preparation (Bacterial strain)	Results or conclusions
Healthy persons			
Diop et al. (106)	Healthy adults. Blinded, randomized study. Duration – 12 weeks	Lactobacillus acidophilus Rosell-52, Bifidobacterium longum Rosell-175 (3×10^9 CFU/day)	Probiotic therapy has been shown for the first time to reduce gastrointestinal complaints in people under stress: • Significant reduction in gastrointestinal symptoms compared to the placebo group; • Significant reduction in the severity of stress-induced nausea and abdominal pain.
Messaoudi et al. (37)	Healthy adults. Double-blind, randomized study. *N* = 55, duration – 30 days	Lactobacillus helveticus R0052, Bifidobacterium longum R0175 (3×10^9 CFU/day)	The first study to show that administration of a psychobiotic alleviates stress-induced psychiatric symptoms: • Reduction in anxiety symptoms on the HSCL-90 scale; • Significant reduction in anxiety and depressive symptoms; • Confirmed reduction of the stress hormone cortisol in urine; • In the group of people with lower cortisol levels (less stressed), improvements in depression and anxiety scores on the PSS, HADS, and HSCL-90 scales.
Depression			
Wallace et al. (107)	Depressed patients who were not taking antidepressants. Blinded, randomized study. *N* = 108, duration – 16 weeks	Lactobacillus helveticus R0052, Bifidobacterium longum R0175 (6×10^9 CFU/day)	After 4 weeks of taking the psychobiotic, there was a reduction in scores on the assessment scales: • Poor mood (MADRS – Montgomery-Asberg Depression Scale, QUIDS-SR16 – Quick List of Depressive Symptoms); • Stress intensity (PSQI – Sleep Quality Questionnaire); • Anhedonia (SHAPS – Scale of Perceived Pleasure); Level of anxiety (GAD-7 – Generalized Anxiety Questionnaire, STAI – State and Trait Anxiety Inventory).
Kazemi et al. (108)	Depressed patients who were taking antidepressants (sertaline, escitalopram, fluixetine, or amitriptyline). RCT study. *N* = 81, duration – 8 weeks	Lactobacillus helveticus, Bifidobacterium longum	• Decreased scores on the Beck Depression Scale (compared to the group taking placebo or the prebiotic galactooligosaccharide). • Increase serotonin production from tryptophan (decrease in kynurenine/tryptophan ratio)
Rudzki et al. (109)	Patients with depression. Double-blind RCT study. *N* = 60, duration – 8 weeks	SSRI + Lactobacillus plantarum 299v (10×10^9 CFU/day)	Augmenting SSRI treatment with probiotic bacteria Lactobacillus Plantarum 299v improved cognitive performance and reduced KYN levels in MDD patients. Reduced KYN levels may have contributed to cognitive improvement in the LP299v group compared to the placebo group
Wallace et al. (107)	Patients with depression. Double-blind RCT study. *N* = 10, duration – 8 weeks	Lactobacillus helveticus Rosell-52, Bifidobacterium Longum Rosell-175 (3×10^9 CFU)	Probiotics have a role in alleviating symptoms of depression
Heidarzadeh-Rad et al. (110)	Patients with depression. RCT *post hoc* analysis. *N* = 78, duration – 8 weeks	Lactobacillus helveticus Rosell-52, Bifidobacterium Longum Rosell-175 ($\geq 10 \times 10^9$ CFU)	Eight-week supplementation in depressed patients improved depressive symptoms, likely by increasing BDNF levels

(Continued)

10.3389/fnut.2022.943998

TABLE 2 Continued

Source	Sample	Preparation (Bacterial strain)	Results or conclusions
Alzheimer's disease			
Agahi et al. (111)	Alzheimer's patients. Double-blind RCT study. $N = 48$, duration – 12 weeks	Lactobacillus fermentum, Lactobacillus plantarum, Bifidobacterium lactis, Lactobacillus acidophilus, Bifidobacterium bifidum, Bifidobacterium longum (3×10^9 CFU/day)	Cognitive and biochemical indications in patients with severe AD are insensitive to probiotic supplementation. Therefore, in addition to the composition and dose of probiotic bacteria, the severity of the disease and the timing of administration profoundly affect treatment outcomes.
Akbari et al. (112)	Alzheimer's patients. Double-blind RCT study. $N = 52$, duration – 12 weeks	200 mL/day of milk product containing Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, Lactobacillus fermentum (2×10^9 CFU/day)	Probiotic treatment had no significant effect on biomarkers of oxidative stress and inflammation, fasting glucose and other lipid profiles. The study showed that probiotic consumption for 12 weeks had a positive effect on cognitive function and some metabolic statuses in AD patients
Tamtaji et al. (113)	Alzheimer's patients. Double-blind RCT study. $N = 79$, duration – 12 weeks	Lactobacillus acidophilus, Bifidobacterium bifidum, Bifidobacterium longum (6×10^9 CFU/day) + 200 mcg selenium	Co-supplementation of probiotics and selenium for 12 weeks in AD patients improved cognitive function and some metabolic profiles
Chronic fatigue syndrome			
Wallis et al. (114)	Patients with Chronic Fatigue Syndrome. Open-label study. $N = 44$, duration – 6 weeks	Alternating antibiotic and probiotic therapy: Erythromycin + Lactobacillus rhamnosus (2.5×10^{10} CFU/day), Bifidobacterium lactis (1.5×10^{10} CFU/day), Bifidobacterium breve (5×10^6 CFU/day), Bifidobacterium longum (5×10^6 CFU/day)	Specific microorganisms interact with some ME/CFS symptoms and offer the promise of therapeutic potential targeting intestinal dysbiosis in this population
Cognitive dysfunction			
Hwang et al. (115)	Patients with mild cognitive impairment. Double-blind RCT study. $N = 92$, duration – 12 weeks	Lactobacillus plantarum C29 (1.25×10^{10} CFU/day) + powdered fermented soybeans (DW2009)	DW2009 can be safely administered to improve cognitive function in people with MCI
Kobayashi et al. (116)	Patients with mild cognitive impairment. Open-label study. $N = 27$, duration – 6 months	Bifidobacterium breve A1 (2×10^{10} CFU/day)	Oral supplementation of B. breve A1 in participants with MCI improved cognitive function, thus suggesting the potential of B. breve A1 for improving cognitive function and maintaining quality of life in the elderly
Kobayashi et al. (117)	Patients with mild cognitive impairment. Double-blind RCT study. $N = 117$, duration – 12 weeks	Bifidobacterium breve A1 (2×10^{10} CFU/day)	The results of the present study suggest the safety of B. breve A1 supplementation and its potential in maintaining cognitive function in elderly people with memory impairment

Source: Own compilation based on literature review.

10.3389/fnut.2022.943998

axis activity (44–47). Furthermore, healthy individuals who were first-degree relatives of individuals with psychotic disorders were found to have HPA axis dysfunction with elevated cortisol levels (48). These studies show that the HPA axis appears to be an important biological marker of susceptibility to developing psychiatric disorders. In this context, its association with gut microbiota is not insignificant. Other potential biomarkers involved in the pathophysiology of psychiatric disorders are inflammation and oxidative stress (49). The inflammatory theory of depression development is gaining increasing attention, and elevated levels of proinflammatory cytokines are observed in depressive, psychotic, and manic states (50, 51). Elevated levels of proinflammatory cytokines occur before the onset of *de novo* disorders, suggesting their role in the genesis of these disorders (52). An increase in oxidative stress in psychotic disorders with a decrease in glutathione and antioxidant enzymes has also been observed (53). The potential effectiveness of selective cyclooxygenase-2 antagonists in the treatment of bipolar affective disorder and schizophrenia has been demonstrated (51, 54). The use of statins, which have anti-inflammatory and antioxidant properties, reduced the risk of depressive disorders (55). Polyunsaturated fatty acids are further potential biomarkers that may have applications in psychiatry. Omega-3 polyunsaturated fatty acids may play a role in the pathogenesis of affective and psychotic disorders (56, 57). Their deficiency may be present in the early stages of psychotic disorders – stage 1b. Supplementation with omega-3 polyunsaturated fatty acids reduced the risk of psychotic disorders among individuals at high risk of developing them (58).

The intestinal barrier is composed of several layers, including the intestinal microflora, mucus layer, intestinal epithelium, and elements of the circulatory, immune, nervous, and lymphatic systems. The layer of epithelial cells, mainly enterocytes connected by tight junctions, is the most important for the intestinal barrier (59). Its main function is to regulate the absorption of nutrients, electrolytes, and water from the gastrointestinal lumen into the blood or lymphatic system and prevent the penetration of pathogens from the gastrointestinal lumen. Factors such as stress, pro-inflammatory factors, dysbacteriosis of the intestinal microflora, alcohol, or antibiotics may cause excessive permeability of the intestinal barrier (60–62). Currently, the microbiota and its diversity as a trigger for generalized inflammation are gaining great importance (61) Under the influence of the impaired functioning of the barrier, the migration of bacteria from the lumen of the gastrointestinal tract occurs, which activates the cells of the immune system affecting the functioning of the immune, endocrine and nervous systems (62). It has been observed that patients with depression have elevated IgA and IgM immunoglobulins against lipopolysaccharides of the bacterial microbiome (63). The current study indicates the use of a dietary inflammatory index, which assesses the effect of the entire diet or individual dietary components on the concentration of inflammatory markers. The results of a systematic review by Chen et al. (64) indicate that a higher dietary inflammatory index is associated with an increased risk of common psychiatric disorders, including symptoms of depression, anxiety, distress, and schizophrenia. Of particular importance is the novel finding from the dose-response analysis that a 1 unit increase in the dietary inflammatory index was associated with a 6% higher risk of depressive symptoms. Similar relationships have been observed by Firth et al. (63), particularly in schizophrenia – individuals who consume more pro-inflammatory foods and less anti-inflammatory foods are more predisposed to psychiatric disorders. At this point, it is important to look at the relationship between diet and the proper functioning of the intestinal barrier. It turns out that it is not without significance in maintaining homeostasis. A diet consisting of fast food and highly processed foods is associated with increased intestinal barrier permeability (65, 66).

Q3: Are there nutritional interventions with proven preventive potential for mental disorders?

Epidemiological studies have shown that diet impacts mental health, and intervention studies confirm this relationship (17). The challenge for "nutritional psychiatry" is to produce comprehensive, consistent, and scientifically rigorous evidence-based studies that define the role of diet and nutrients in different aspects of mental health (67–70). Overall, few randomized trials investigate the effectiveness of dietary change in mental health treatment. One intervention study to date involved a 12-week Mediterranean diet. This study reported significant improvements in mood and reduced anxiety in adults with major depression (71) More recent RCTs – HELFIMED (72) and PREDI_DEP (73) have confirmed the benefits of a Mediterranean-style diet for mental health in depression. In contrast to these studies, in the MooDFOOD RCT, multiple nutrient supplementation did not reduce episodes of major depression in overweight or obese adults with subsyndromal depressive symptoms. This study found that multinutrient supplements containing omega-3 PUFAs, vitamin D, folic acid, and selenium neither reduced depressive symptoms, anxiety symptoms nor improved health utility indices (74). Similar results regarding the lack of effect on mental state improvement were obtained in a review of the literature in the context of vitamin D (75). For omega-3 PACs, one RCT including people with mild to moderate depression found no beneficial effect of omega-3 PACs on depressive symptoms (76). No effect of folic acid supplementation in combination

with vitamin B 6 and B 12 on the onset of depression was found in older men (77) and older women (78). Furthermore, Rayman et al. (79) found no effect of selenium supplementation on mood in older people. Overall, the studies available to date, do not support the use of nutritional supplementation to prevent depression.

However, many studies confirm that higher dietary quality in adulthood is associated with a reduced risk of cognitive decline (17). Additionally, the intake of antioxidant polyphenols in older adults is associated with improved cognitive ability (80–82). Another study showed that a Mediterranean diet supplemented with olive oil and nuts was associated with improved cognitive function in an older population (83).

Therefore, we undertook an analysis of diets that could potentially affect mental health such as the MIND diet, the Mediterranean diet, and the ketogenic diet.

The MIND diet is a dietary recommendation to counteract neurodegenerative brain changes and improve nervous system function. This diet is beneficial for cognitive decline in the aging process, as well as for the prevention and progression of neurodegenerative diseases, including Alzheimer's disease (84). The MIND diet combines the principles of the Mediterranean diet and the DASH diet, which are based on a high intake of vegetables, fruits, nuts, whole grain cereal products, olive oil, fish, and seafood, and moderate consumption of dry red wine with meals (85). Studies prove the positive effects of the DASH and Mediterranean diets on other diseases such as diabetes, cancer, and obesity (86–89).

Long-term observations confirm that adherence to the Mediterranean diet reduces the risk of developing neurological disorders by up to 28% compared to the use of other diets (83). Adherence to the MIND diet was significantly associated with a lower chance of depression and psychological distress, but not anxiety, in the entire study population (90). Like the Mediterranean diet and the DASH diet, the MIND diet emphasizes natural plant-based foods and limited intake of animal and high-fat foods, especially of animal origin. However, there are some differences between the MIND diet and the DASH diet, and the Mediterranean diet. For example, leafy green vegetables and especially berries are unique components of the MIND diet that are not included in the Mediterranean and DASH diets (90). The MIND diet does not focus on a high intake of fruit, dairy products, and potatoes. Another difference between MIND and the DASH and Mediterranean diets concerns fish consumption. In MIND, individuals consuming as little as 1 portion of fish per week receive a positive result, whereas, in the Mediterranean and DASH diets, larger amounts of fish would need to be consumed to achieve a result (91). The MIND diet significantly slows cognitive decline with age (92). The Mediterranean diet has also been shown to have a protective effect on anxiety and mental stress (93).

Mental illnesses are associated with numerous metabolic disorders in the brain and co-occur with many other metabolic disorders such as obesity, diabetes, and CVD. The ketogenic diet is an evidence-based treatment for epilepsy that has been shown to have profound effects on brain metabolism and

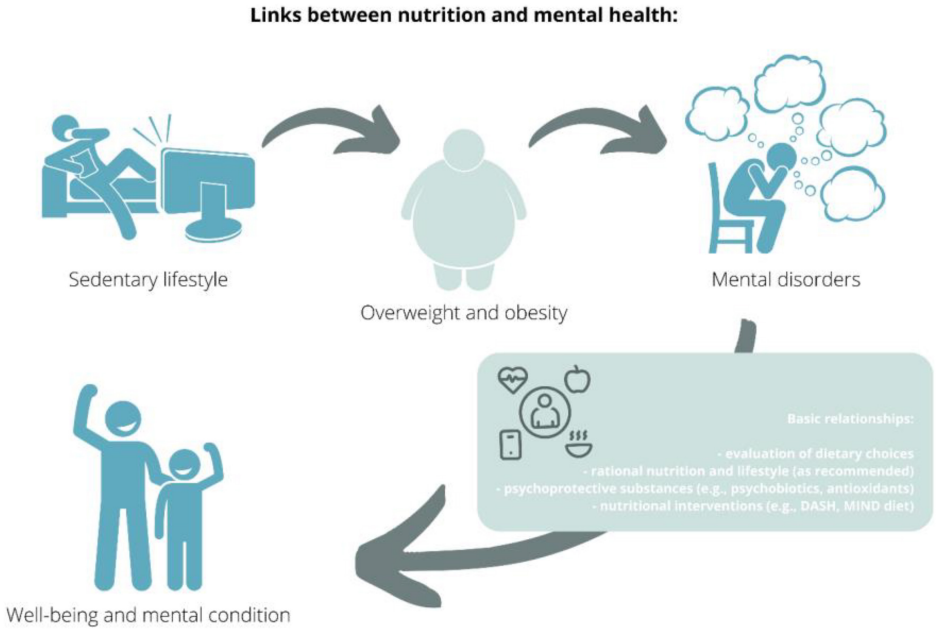


FIGURE 2
Links between nutrition and mental health.

neurotransmitter function. In a ketogenic diet, as much as 80 percent of energy can come from fat. This proportion sounds like a deal-breaker for healthy eating, but it turns out that ketones formed from fats can alleviate epileptic seizures unresponsive to anticonvulsant drug therapy (83). In the case of mitochondrial epilepsy, reports on the effects of the ketogenic diet are conflicting. In a study by El Sabbagh et al. (94), no patients on a ketogenic diet achieved no significant reduction in seizure frequency epileptic seizures. In contrast, a study by Kang et al. (95) involving 14 patients showed that the use of a ketogenic diet in 10 of them reduced the frequency of epileptic seizures by more than 50%, and in 7 patients, epileptic seizures ceased. In the analysis, there were improvements in symptoms including mood, cognitive function, communication skills, energy, anxiety, and auditory and visual hallucinations (90). Other reported benefits included positive biometric changes such as improvements in lipid profile, weight reduction, positive change in blood glucose, and reduction in HbA1c. These benefits may facilitate the management of comorbidities and improve overall health and well-being (93). This highlights that advances in nutritional psychiatry are important and it will be important to replicate, refine and scale up dietary intervention studies targeting the prevention and treatment of common mental health disorders. In addition, there is an unmet need for more randomized, controlled clinical trials (118–121).

Strengths and limitations

There is still little work in the scientific space that summarizes the major findings related to the impact of nutrition on mental health, especially, as this review does, highlighting the importance of nutrition in psychoprevention and pointing to the psychoprotective effects of nutrients. The primary limitation of the presented review of research on the relationship between diet and mental health is the plethora of studies on the topic. The plethora of studies here does not mean that they all address the issue presented in this manuscript. Much of the work that was searched and queried assumes a relationship between nutrition and the psyche, but these tend to be very superficial opinions that are not scientifically grounded. The authors are aware that in the face of such a large body of research, important reports may have been overlooked, but it should be noted that every effort was made to ensure that this review was conducted fairly, taking into account large, multi-center research projects and highlighting the major research streams in psychodietetics and nutripsychiatry.

Additionally, it was observed that in the current state of scientific knowledge, few large meta-analyses are treating the effects of food and diet on mental health. Therefore, it is difficult to discuss the effectiveness of introducing nutritional interventions among people with mental disorders or treating nutrition as the only means of prevention. Furthermore, the primary threat to interventions of this type is the difficulty in monitoring dietary patterns or intake of specific components. In addition, their absorption and metabolism are also dependent on many factors that rarely have a consistent course. Therefore, it is postulated that further research should be directed toward the creation of unambiguous dietary recommendations for mental health problems.

Conclusion

In recent decades, the relationship between nutrition and patients' mental status has been underappreciated, as evidenced by the lack of research conducted before the 21st century in this area of knowledge – cited in this review. In recent years, this trend has been reversed, with research in psychodietetics and nutripsychiatry gaining popularity. In the context of current changes, urbanization, globalization, including the food industry, and changes in people's lifestyles and eating habits, correlations between these phenomena and their impact on psychological status are becoming important. Exploring these correlations creates potential opportunities to implement new effective dietary, pharmacological, therapeutic, and above all preventive interventions (**Figure 2**).

Author contributions

MATG: conceptualization. MATG and KK-K: investigation and methodology. KS and AB-D: data curation. MATG: writing – original draft preparation. MATG, KK-K, MARG, and AB-D: writing – review and editing. KS and AB-D: supervision. KK-K: project administration. WS: conducting an additional literature review, creating tables summarizing current knowledge of psychobiotics and psychoprotective food ingredients, and revising the work. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Hidaka BH. Depression as a disease of modernity: Explanations for increasing prevalence. *J Affect Disord.* (2012) 140:205–14. doi: 10.1016/j.jad.2011.12.036

2. Baxter AJ, Patton G, Scott KM, Degenhardt L, Whiteford HA. Global epidemiology of mental disorders: what are we missing? *PLoS One.* (2013) 8:e65514. doi: 10.1371/journal.pone.0065514

3. IHME. *Data visualizations.* Seattle, WA: IHME (2020)

4. Wittchen HU, Fuetsch M, Sonntag H, Müller N, Liebowitz M. Disability and quality of life in pure and comorbid social phobia. Findings from a controlled study. *Eur Psychiatry.* (2000) 15:46–58. doi: 10.1016/s0924-9338(00)00211-x

5. NPOZP. *Narodowy program ochrony zdrowia psychicznego na lata 2017-2022. Rozporządzenie rady ministrów z dnia 8 lutego 2017 roku.* Warsaw: Ministerstwo Zdrowia (2017)

6. Hawes, MT, Szenczy AK, Klein DN, Hajcak G, Nelson BD. Increases in depression and anxiety symptoms in adolescents and young adults during the COVID-19 pandemic. *Psychol Med.* (2021) 5:1–9. doi: 10.1017/S0033291720005358

7. Notes From Poland. *Stigmatisation and medication: Poland's outdated approach to mental health.* New York, NY: NFP (2020).

8. Pawłowski T, Kiejna A. Pathways to psychiatric care and reform of the public health care system in Poland. *Eur Psychiatry.* (2004) 19:168–71. doi: 10.1016/j.eurpsy.2003.09.009

9. Kirsch I, Deacon BJ, Huedo-Medina TB, Scoboria A, Moore TJ, Johnson BT. Initial severity and antidepressant benefits: a meta-analysis of data submitted to the Food and Drug Administration. *PLoS Med.* (2008) 5:e45. doi: 10.1371/journal.pmed.0050045

10. Parker E, Goldman J, Moshfegh A. America's nutrition report card: comparing WWEIA, NHANES 2007-2010 Usual nu-trient intakes to dietary reference intakes. *FASEB J.* (2014) 28:384.2.

11. Opie RS, Itsiopoulos C, Parletta N, Sanchez-Villegas A, Akbaraly TN, Ruusunen A, et al. Dietary recommendations for the prevention of depression. *Nutr Neurosci.* (2017) 20:161–71. doi: 10.1179/1476830515Y.0000000043

12. Berk M, Malhi GS, Gray LJ, Dean OM. The promise of N-acetylcysteine in neuropsychiatry. *Trends Pharmacol Sci.* (2013) 34:167–77. doi: 10.1016/j.tips.2013.01.001

13. Diniz BS, Mendes-Silva AP, Silva LB, Bertola L, Vieira MC, Ferreira JD, et al. Oxidative stress markers imbalance in late-life depression. *J Psychiatr Res.* (2018) 102:29–33. doi: 10.1016/j.jpsychires.2018.02.023

14. Guimarães LR, Jacka FN, Gama CS, Berk M, Leitão-Azevedo CL, Belmonte de Abreu MG, et al. Serum levels of brain-derived neurotrophic factor in schizophrenia on a hypocaloric diet. *Prog Neuropsychopharmacol Biol Psychiatry.* (2008) 32:1595–8. doi: 10.1016/j.pnpbp.2008.06.004

15. Lai JS, Hiles S, Bisquera A, Hure AJ, McEvoy M, Attia J. A systematic review and meta-analysis of dietary patterns and depression in community-dwelling adults. *Am J Clin Nutr.* (2014) 99:181–97. doi: 10.3945/ajcn.113.069880

16. Akter K, Lanza EA, Martin SA, Myronyuk N, Rua M, Raffa RB. Diabetes mellitus and Alzheimer's disease: Shared pathology and treatment? *Br. J. Clin. Pharmacol.* (2011) 71:365–76. doi: 10.1111/j.1365-2125.2010.03830.x

17. Jacka FN, O'Neil A, Opie R, Itsiopoulos C, Cotton S, Mohebbi M, et al. A randomised controlled trial of dietary improvement for adults with major depression (the "SMILES" trial). *BMC Med.* (2017) 15:23. doi: 10.1186/s12916-017-0791-y

18. Mischoulon D, Freeman MP. Omega-3 fatty acids in psychiatry. *Psychiatr Clin North Am.* (2013) 36:15–23. doi: 10.1016/j.psc.2012.12.002

19. Sarris J, Papakostas GI, Vitolo O, Fava M, Mischoulon D. S-adenosyl methionine (SAMe) versus escitalopram and placebo in major depression RCT: efficacy and effects of histamine and carnitine as moderators of response. *J Affect Disord.* (2014) 164:76–81. doi: 10.1016/j.jad.2014.03.041

20. Berk M, Williams LJ, Jacka FN, O'Neil A, Pasco JA, Moylan A, et al. So depression is an inflammatory disease, but where does the inflammation come from? *BMC Med.* (2013) 11:200. doi: 10.1186/1741-7015-11-200

21. Lai J, Moxey A, Nowak G, Vashum K, Bailey K, McEvoy M. The efficacy of zinc supplementation in depression: systematic review of randomised controlled trials. *J Affect Disord.* (2012) 136:e31–9. doi: 10.1016/j.jad.2011.06.022

22. Fava M, Mischoulon D. Folate in depression: efficacy, safety, differences in formulations, and clinical issues. *J Clin Psychiatry.* (2009) 70:12–7. doi: 10.4088/JCP.8157su1c.03

23. Eyles DW, Burne TH, McGrath JJ. Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. *Front Neuroendocrinol.* (2013) 34:47–64. doi: 10.1016/j.yfrne.2012.07.001

24. Brouwer-Brolsma EM, Dhonukshe-Rutten RA, van Wijngaarden JP, van der Zwaluw NL, Sohl E, In't Veld PH, et al. Low vitamin D status is associated with more depressive symptoms in dutch older adults. *Eur J Nutr.* (2016) 55:1525–34. doi: 10.1007/s00394-015-0970-6

25. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature.* (2012) 489:220–30. doi: 10.1038/nature11550

26. Schemann M, Neunlist M. The human enteric nervous system. *Neurogastroenterol Motil.* (2004) 16:55–9. doi: 10.1111/j.1743-3150.2004.00476.x

27. Forsythe P, Bienenstock J, Kunze WA. Vagal pathways for microbiome-brain-gut axis communication. *Adv Exp Med Biol.* (2014) 817:115–33. doi: 10.1007/978-1-4939-0897-4_5

28. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalam-ic-pituitary-adrenal system for stress response in mice. *J Physiol.* (2004) 558:263–75. doi: 10.1113/jphysiol.2004.063388

29. Bangsgaard Bendtsen KM, Krych L, Sørensen DB, Pang W, Nielsen DS, Josefsen K, et al. Gut microbiota composition is correlated to grid floor induced stress and behavior in the BALB/c mouse. *PLoS One.* (2012) 7:e46231. doi: 10.1371/journal.pone.0046231

30. Mayer L. Mucosal immunity. *Pediatrics.* (2003) 111:1595–600.

31. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell.* (2004) 118:229–41. doi: 10.1016/j.cell.2004.07.002

32. Lyte M. Microbial endocrinology: Host-microbiota neuroendocrine interactions influencing brain and behavior. *Gut Microbes.* (2014) 5:381–9. doi: 10.4161/gmic.28682

33. Grot M, Krupa-Kotara K, Wypych-Ślusarska A, Grajek M, Białek-Dratwa A. The concept of intrauterine programming and the development of the neonatal microbiome in the prevention of SARS-CoV-2 infection. *Nutrients.* (2022) 14:1702. doi: 10.3390/nu14091702

34. Mackowiak PA. Recycling Metchnikoff: probiotics, the intestinal microbiome and the quest for long life. *Front Public Health.* (2013) 1:52. doi: 10.3389/fpubh.2013.00052

35. Durchschein F, Petritsch W, Hammer HF. Diet therapy for inflammatory bowel diseases: The established and the new. *World J Gastroenterol.* (2016) 22:2179–94. doi: 10.3748/wjg.v22.i7.2179

36. Bindels LB, Delzenne NM, Cani PD, Walter J. Towards a more comprehensive concept for prebiotics. *Nat Rev Gastroenterol Hepatol.* (2015) 12:303–10. doi: 10.1038/nrgastro.2015.47

37. Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A, et al. Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. *Br J Nutr.* (2011) 105:755–64. doi: 10.1017/S0007114510004319

38. Messaoudi M, Violle N, Bisson JF, Desor D, Javelot H, Rougeot C. Beneficial psychological effects of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in healthy human volunteers. *Gut Microbes.* (2011) 2:256–61. doi: 10.4161/gmic.2.4.16108

39. Cussotto S, Clarke G, Dinan TG, Cryan JF. Psychotropics and the microbiome: A chamber of secrets. *Psychopharmacology.* (2019) 236:1411–32. doi: 10.1007/s00213-019-5185-8

40. Hegelstad WT, Larsen TK, Auestad B, Evensen J, Haahr U, Joa I, et al. Long-term follow-up of the TIPS early detection in psychosis study: effects on 10-year outcome. *Am J Psychiatry.* (2012) 169:374–80. doi: 10.1176/appi.ajp.2011.11030459

41. McGorry PD, Nelson B, Goldstone S, Yung AR. Clinical staging: a heuristic and practical strategy for new research and better health and social outcomes for psychotic and related mood disorders. *Can J Psychiatry.* (2010) 55:486–97. doi: 10.1177/070674371005500803

42. Phillips LJ, McGorry PD, Garner B, Thompson KN, Pantelis C, Wood SJ, et al. Stress, the hippocampus and the hypothalamic-pituitary-adrenal axis: implications for the development of psychotic disorders. *Aust N Z J Psychiatry.* (2006) 40:725–41. doi: 10.1080/j.1440-1614.2006.01877.x

43. Zhang TY, Labonté B, Wen XL, Turecki G, Meaney MJ. Epigenetic mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans. *Neuropsychopharmacology.* (2013) 38:111–23. doi: 10.1038/npp.2012.149

44. Belvederi Murri M, Prestia D, Mondelli V, Pariante C, Patti S, Olivieri B, et al. The HPA axis in bipolar disorder: Systematic review and meta-analysis. *Psychoneuroendocrinology.* (2016) 63:327–42. doi: 10.1016/j.psyneuen.2015.10.014

45. Pariante CM, Dazzan P, Danese A, Morgan KD, Brudaglio F, Morgan C, et al. Increased pituitary volume in antipsychotic-free and antipsychotic-treated patients of the AEsop first-onset psychosis study. *Neuropsychopharmacology.* (2005) 30:1923–31. doi: 10.1038/sj.npp.1300766

46. Cohrs S, Röher C, Jordan W, Meier A, Huether G, Wuttke W, et al. The atypical antipsychotics olanzapine and quetiapine, but not haloperidol, reduce ACTH and cortisol secretion in healthy subjects. *Psychopharmacology.* (2006) 185:11–8. doi: 10.1007/s00213-005-0279-x

47. Collip D, Nicolson NA, Lardinois M, Lataster T, van Os J, Myin-Germeys I. Daily cortisol, stress reactivity and psychotic experiences in individuals at above average genetic risk for psychosis. *Psychol Med.* (2011) 41:2305–15. doi: 10.1017/S0033291711000602

48. Miller BJ, Buckley P, Seabolt W, Mellor A, Kirkpatrick B. Meta-analysis of cytokine alterations in schizophrenia: Clinical status and antipsychotic effects. *Biol Psychiatry.* (2011) 70:663–71. doi: 10.1016/j.biopsych.2011.04.013

49. Raedler TJ. Inflammatory mechanisms in major depressive disorder. *Curr Opin Psychiatry.* (2011) 24:519–25. YCO.0b013e32834b9db6 doi: 10.1097/

50. Wadee AA, Kuschke RH, Wood LA, Berk M, Ichim L, Maes M. Serological observations in patients suffering from acute manic episodes. *Hum Psychopharmacol.* (2002) 17:175–9. doi: 10.1002/hup.390

51. Pasco JA, Jacka FN, Williams LJ, Henry MJ, Nicholson GC, Kotowicz MA, et al. Clinical implications of the cytokine hy-pothesis of depression: the association between use of statins and aspirin and the risk of major depression. *Psychother Psychosom.* (2010) 79:323–5. doi: 10.1159/000319530

52. Do KQ, Cabungcal JH, Frank A, Steullet P, Cuenod M. Redox dysregulation, neurodevelopment, and schizophrenia. *Curr Opin Neurobiol.* (2009) 19:220–30. doi: 10.1016/j.conb.2009.05.001

53. Nery FG, Monkul ES, Hatch JP, Fonseca M, Zunta-Soares GB, Frey BN, et al. Celecoxib as an adjunct in the treatment of depressive or mixed episodes of bipolar disorder: a double-blind, randomized, placebo-controlled study. *Hum Psycho-Pharmacol.* (2008) 23:87–94. doi: 10.1002/hup.912

54. Müller N, Krause D, Dehning S, Musil R, Schennach-Wolff R, Obermeier M, et al. Celecoxib treatment in an early stage of schizophrenia: Results of a randomized, double-blind, placebo-controlled trial of celecoxib augmentation of amisulpride treatment. *Schizophr Res.* (2010) 121:118–24. doi: 10.1016/j.schres.2010.04.015

55. Parker G, Gibson NA, Brotchie H, Heruc G, Rees AM, Hadzi-Pavlovic D. Omega-3 fatty acids and mood disorders. *Am J Psychiatry.* (2006) 163:969–78. doi: 10.1176/ajp.2006.163.6.969

56. Horrobin DF. The membrane phospholipid hypothesis as a biochemical basis for the neurodevelopmental concept of schizophrenia. *Schizophr Res.* (1998) 30:193–208. doi: 10.1016/s0920-9964(97)00151-5

57. Amminger GP, Schäfer MR, Papageorgiou K, Klier CM, Cotton SM, Harrigan SM, et al. Long-chain omega-3 fatty acids for indicated prevention of psychotic disorders: a randomized, placebo-controlled trial. *Arch Gen Psychiatry.* (2010) 67:146–54. doi: 10.1001/archgenpsychiatry.2009.192

58. Lambert GP. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *J Anim Sci.* (2009) 87:101–8. doi: 10.2527/jas.2008-1339

59. Schmitz H, Fromm M, Bentzel CJ, Scholz P, Detjen K, Mankertz J, et al. Tumor necrosis factor-alpha (TNFalpha) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. *J Cell Sci.* (1999) 112:137–46. doi: 10.1242/jcs.112.1.137

60. Leclercq S, Matamoros S, Cani PD, Neyrinck AM, Jamar F, Stärkel P, et al. Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. *Proc Natl Acad Sci USA.* (2014) 111:E4485–93. doi: 10.1073/pnas.1415174111

61. Tulstrup MV, Christensen EG, Carvalho V, Linninge C, Ahrné S, Højberg O, et al. Antibiotic treatment affects intestinal permeability and gut microbial composition in wistar rats dependent on antibiotic class. *PLoS One.* (2015) 10:e0144854. doi: 10.1371/journal.pone.0144854

62. Maes M, Kubera M, Leunis JC. The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. *Neuro Endocrinol Lett.* (2008) 29:117–24.

63. Firth J, Veronese N, Cotter J, Shivappa N, Hebert JR, Ee C, et al. What is the role of dietary inflammation in severe mental illness? A review of observational and experimental findings. *Front Psychiatry.* (2019) 10:350. doi: 10.3389/fpsyt.2019.00350

64. Chen GQ, Peng CL, Lian Y, Wang BW, Chen PY, Wang GP. Association between dietary inflammatory index and mental health: A systematic review and dose-response meta-analysis. *Front Nutr.* (2021) 5:662357. doi: 10.3389/fnut.2021.662357

65. Sánchez-Villegas A, Toledo E, de Irala J, Ruiz-Canela M, Pla-Vidal J, Martínez-González MA. Fast-food and commercial baked goods consumption and the risk of depression. *Public Health Nutr.* (2012) 15:424–32. doi: 10.1017/S1368980011001856

66. Adan RAH, van der Beek EM, Buitelaar JK, Cryan JF, Hebebrand J, Higgs S, et al. Nutritional psychiatry: Towards im-proving mental health by what you eat. *Eur Neuropsychopharmacol.* (2019) 29:1321–32. doi: 10.1016/j.euroneuro.2019.10.011

67. Marx W, Moseley G, Berk M, Jacka F. Nutritional psychiatry: the present state of the evidence. *Proc Nutr Soc.* (2017) 76:427–36. doi: 10.1017/S0029665117002026

68. Sarris J, Logan AC, Akbaraly TN, Amminger GP, Balanzá-Martínez V, Freeman MP, et al. Nutritional medicine as main-stream in psychiatry. *Lancet Psychiatry.* (2015) 2:271–4. doi: 10.1016/S2215-0366(14)00051-0

69. Sarris J, Logan AC, Akbaraly TN, Amminger GP, Balanzá-Martínez V, Freeman MP, et al. International society for nutri-tional psychiatry research consensus position statement: nutritional medicine in modern psychiatry. *World Psychiatry.* (2015) 14:370–1. doi: 10.1002/wps.20223

70. Jacka FN, O'Neil A, Itsiopoulos C, Opie R, Mohebbi M, Castle D, et al. The SMILES trial: An important first step. *BMC Med.* (2018) 16:237. doi: 10.1186/s12916-018-1228-y

71. Parletta N, Zarnowiecki D, Cho J, Wilson A, Bogomolova S, Villani A, et al. A Mediterranean-style dietary intervention supplemented with fish oil improves diet quality and mental health in people with depression: A randomized controlled trial (HELFIMED). *Nutr Neurosci.* (2019) 22:474–87. doi: 10.1080/1028415X.2017.1411320

72. Sánchez-Villegas A, Cabrera-Suárez B, Molero P, González-Pinto A, Chiclana-Actis C, Cabrera C, et al. Preventing the recurrence of depression with a Mediterranean diet supplemented with extra-virgin olive oil. The PREDI-DEP trial: study protocol. *BMC Psychiatry.* (2019) 19:63. doi: 10.1186/s12888-019-2036-4

73. Berk M, Jacka FN. Diet and depression-from confirmation to implementation. *JAMA.* (2019) 321:842–3. doi: 10.1001/jama.2019.0273

74. Bot M, Brouwer IA, Roca M, Kohls E, Penninx BWJH, Watkins E, et al. Effect of multinutrient supplementation and food-related behavioral activation therapy on prevention of major depressive disorder among overweight or obese adults with subsyndromal depressive symptoms: The MooDFOOD randomized clinical trial. *JAMA.* (2019) 321:858–68. doi: 10.1001/jama.2019.0556

75. Okereke OI, Singh A. The role of vitamin D in the prevention of late-life depression. *J Affect Disord.* (2016) 198:1–14. doi: 10.1016/j.jad.2016.03.022

76. Ford AH, Flicker L, Thomas J, Norman P, Jamrozik K, Almeida OP. Vitamins B12, B6, and folic acid for onset of depressive symptoms in older men: results from a 2-year placebo-controlled randomized trial. *J Clin Psychiatry.* (2008) 69:1203–9. doi: 10.4088/JCP.v69n0801

77. Okereke OI, Cook NR, Albert CM, Van Denburgh M, Buring JE, Manson JE. Effect of long-term supplementation with folic acid and B vitamins on risk of depression in older women. *Br J Psychiatry.* (2015) 206:324–31. doi: 10.1192/bjp.bp.114.148361

78. Valls-Pedret C, Lamuela-Raventós RM, Medina-Remón A, Quintana M, Corella D, Pintó X, et al. Polyphenol-rich foods in the Mediterranean diet are associated with better cognitive function in elderly subjects at high cardiovascular risk. *J Alzheimers Dis.* (2012) 29:773–82. doi: 10.3233/JAD-2012-111799

79. Rayman M, Thompson A, Warren-Perry M, Galassini R, Catterick J, Hall E, et al. Impact of selenium on mood and quality of life: a randomized, controlled trial. *Biol Psychiatry.* (2006) 59:147–54. doi: 10.1016/j.biopsych.2005.06.019

80. Witte AV, Kerti L, Margulies DS, Flöel A. Effects of resveratrol on memory performance, hippocampal functional connectivity, and glucose metabolism in healthy older adults. *J Neurosci.* (2014) 34:7862–70. doi: 10.1523/JNEUROSCI.0385-14.2014

81. Anton SD, Embry C, Marsiske M, Lu X, Doss H, Leeuwenburgh C, et al. Safety and metabolic outcomes of resveratrol supplementation in older adults: results of a twelve-week, placebo-controlled pilot study. *Exp Gerontol.* (2014) 57:181–7. doi: 10.1016/j.exger.2014.05.015

82. Valls-Pedret C, Sala-Vila A, Serra-Mir M, Corella D, de la Torre R, Martínez-González MÁ, et al. Mediterranean Diet and age-related cognitive decline: A randomized clinical trial. *JAMA Intern Med.* (2015) 175:1094–103. doi: 10.1001/jamainternmed.2015.1668

83. Morris MC, Tangney CC, Wang Y, Sacks FM, Barnes LL, Bennett DA, et al. MIND diet slows cognitive decline with aging. *Alzheimers Dement.* (2015) 11:1015–22. doi: 10.1016/j.jalz.2015.04.011

84. Dutta R. Role of MIND diet in preventing dementia and Alzheimer's disease. *ARC J Neurosci.* (2019) 4:1–8. doi: 10.20431/2456-057X.0403001

85. Said MS, El Sayed IT, Ibrahim EE, Khafagy GM. Effect of DASH Diet Versus healthy dietary advice on the estimated atherosclerotic cardiovascular disease risk. *J Prim Care Community Health.* (2021) 12:2150132720980952. doi: 10.1177/2150132720980952

86. Chiavaroli L, Viguiliouk E, Nishi SK, Blanco Mejia S, Rahelić D, Kahleová H, et al. DASH dietary pattern and cardiometabolic outcomes: an umbrella review of systematic reviews and meta-analyses. *Nutrients.* (2019) 11:338. doi: 10.3390/nu11020338

87. Martínez-González MA, Gea A, Ruiz-Canela M. The Mediterranean diet and cardiovascular health. *Circ Res.* (2019) 124:779–98. doi: 10.1161/CIRCRESAHA.118.313348

88. Salas-Salvadó J, Becerra-Tomás N, García-Gavilán JF, Bulló M, Barrubés L. Mediterranean diet and cardiovascular disease prevention: What do we know? *Prog Cardiovasc Dis.* (2018) 61:62–7. doi: 10.1016/j.pcad.2018.04.006

89. Salari-Moghaddam A, Keshteli AH, Mousavi SM, Afshar H, Esmaillzadeh A, Adibi P. Adherence to the MIND diet and prevalence of psychological disorders in adults. *J Affect Disord.* (2019) 256:96–102. doi: 10.1016/j.jad.2019.05.056

90. Hodge A, Almeida OP, English DR, Giles GG, Flicker L. Patterns of dietary intake and psychological distress in older Australians: benefits not just from a Mediterranean diet. *Int Psychogeriatr.* (2013) 25:456–66. doi: 10.1017/S1041610212001986

91. Olveira C, Olveira G, Espildora F, Girón RM, Vendrell M, Dorado A, et al. Mediterranean diet is associated on symptoms of depression and anxiety in patients with bronchiectasis. *Gen Hosp Psychiatry.* (2014) 36:277–83. doi: 10.1016/j.genhosppsych.2014.01.010

92. Norwitz NG, Dalai SS, Palmer CM. Ketogenic diet as a metabolic treatment for mental illness. *Curr Opin Endocrinol Diabetes Obes.* (2020) 27:269–74. doi: 10.1097/MED.0000000000000564

93. Tillery EE, Ellis KD, Threatt TB, Reyes HA, Plummer CS, Barney LR. The use of the ketogenic diet in the treatment of psychiatric disorders. *Ment Health Clin.* (2021) 11:211–9. doi: 10.9740/mhc.2021.05.211

94. El Sabbagh S, Lebre AS, Bahi-Buisson N, Delonlay P, Soufflet C, Boddaert N, et al. Epileptic phenotypes in children with respiratory chain disorders. *Epilepsia.* (2010) 51:1225–35. doi: 10.1111/j.1528-1167.2009.02504.x

95. Kang HC, Lee YM, Kim HD. Mitochondrial disease and epilepsy. *Brain Dev.* (2013) 35:757–61. doi: 10.1016/j.braindev.2013.01.006

96. Gazerani P. Migraine and diet. *Nutrients.* (2020) 12:1658. doi: 10.3390/nu12061658

97. Cater RJ, Chua GL, Erramilli SK, Keener JE, Choy BC, Tokarz P, et al. Structural basis of omega-3 fatty acid transport across the blood-brain barrier. *Nature.* (2021) 595:315–9. doi: 10.1038/s41586-021-03650-9

98. Parikh M, Maddaford TG, Austria JA, Aliani M, Netticadan T, Pierce GN. Dietary flaxseed as a strategy for improving human health. *Nutrients.* (2019) 11:1171. doi: 10.3390/nu11051171

99. Park M, Choi J, Lee HJ. Flavonoid-rich orange juice intake and altered gut microbiome in young adults with depressive symptom: A randomized controlled study. *Nutrients.* (2020) 12:1815. doi: 10.3390/nu12061815

100. Mulati A, Ma S, Zhang H, Ren B, Zhao B, Wang L, et al. Sea-buckthorn flavonoids alleviate high-fat and high-fructose diet-induced cognitive impairment by inhibiting insulin resistance and neuroinflammation. *J Agric Food Chem.* (2020) 68:5835–46. doi: 10.1021/acs.jafc.0c00876

101. Mittal R, Debs LH, Patel AP, Nguyen D, Patel K, O'Connor G, et al. Neurotransmitters: The critical modulators regulating gut-brain axis. *J Cell Physiol.* (2017) 232:2359–72. doi: 10.1002/jcp.25518

102. Fernández MJF, Valero-Cases E, Rincon-Frutos L. Food components with the potential to be used in the therapeutic approach of mental diseases. *Curr Pharm Biotechnol.* (2019) 20:100–13. doi: 10.2174/1389201019666180925120657

103. Dogan-Sander E, Mergl R, Willenberg A, Baber R, Wirkner K, Riedel-Heller SG, et al. Inflammation and the association of vitamin D and depressive symptomatology. *Nutrients.* (2021) 13:1972. doi: 10.3390/nu13061972

104. Godos J, Currenti W, Angelino D, Mena P, Castellano S, Caraci F, et al. Diet and Mental health: review of the recent updates on molecular mechanisms. *Antioxidants Basel.* (2020) 9:346. doi: 10.3390/antiox9040346

105. Burton-Freeman BM, Sandhu AK, Edirisinghe I. Red raspberries and their bioactive polyphenols: cardiometabolic and neuronal health links. *Adv Nutr.* (2016) 7:44–65. doi: 10.3945/an.115.009639

106. Diop L, Guillou S, Durand H. Probiotic food supplement reduces stress-induced gastrointestinal symptoms in volunteers: a double-blind, placebo-controlled, randomized trial. *Nutr Res.* (2008) 28:1–5. doi: 10.1016/j.nutres.2007.10.001

107. Wallace CJK, Foster JA, Soares CN, Milev RV. The effects of probiotics on symptoms of depression: protocol for a Dou-ble-blind randomized placebo-controlled trial. *Neuropsychobiology.* (2020) 79:108–16. doi: 10.1159/000496406

108. Kazemi A, Noorbala AA, Azam K, Eskandari MH, Djafarian K. Effect of probiotic and prebiotic *vs* placebo on psychological outcomes in patients with major depressive disorder: A randomized clinical trial. *Clin Nutr.* (2019) 38:522–8. doi: 10.1016/j.clnu.2018.04.010

109. Rudzki L, Ostrowska L, Pawlak D, Malus A, Pawlak K, Waszkiewicz N, et al. Probiotic *Lactobacillus plantarum* 299v de-creases kynurenine concentration and improves cognitive functions in patients with major depression: A double-blind, randomized, placebo controlled study. *Psychoneuroendocrinology.* (2019) 100:213–22. doi: 10.1016/j.psyneuen.2018.10.010

110. Heidarzadeh-Rad N, Gökmen-Özel H, Kazemi A, Almasi N, Djafarian K. Effects of a psychobiotic supplement on serum brain-derived neurotrophic factor levels in depressive patients: A post hoc analysis of a randomized clinical trial. *J Neurogastroenterol Motil.* (2020) 26:486–95. doi: 10.5056/jnm20079

111. Agahi A, Hamidi GA, Daneshvar R, Hamdieh M, Soheili M, Alinaghipour A, et al. Does severity of Alzheimer's disease contribute to its responsiveness to modifying gut microbiota? A double blind clinical trial. *Front Neurol.* (2018) 15:662. doi: 10.3389/fneur.2018.00662

112. Akbari E, Asemi Z, Daneshvar Kakhaki R, Bahmani F, Kouchaki E, Tamtaji OR, et al. Effect of probiotic supplementation on cognitive function and metabolic status in Alzheimer's disease: A randomized, double-blind and controlled trial. *Front Aging Neurosci.* (2016) 10:256. doi: 10.3389/fnagi.2016.00256

113. Tamtaji OR, Heidari-Soureshjani R, Mirhosseini N, Kouchaki E, Bahmani F, Aghadavod E, et al. Probiotic and selenium co-supplementation, and the effects on clinical, metabolic and genetic status in Alzheimer's disease: A randomized, double-blind, controlled trial. *Clin Nutr.* (2019) 38:2569–75. doi: 10.1016/j.clnu.2018.11.034

114. Wallis A, Ball M, Butt H, Lewis DP, McKechnie S, Paull P, et al. Open-label pilot for treatment targeting gut dysbiosis in myalgic encephalomyelitis/chronic fatigue syndrome: neuropsychological symptoms and sex comparisons. *J Transl Med.* (2018) 16:24. doi: 10.1186/s12967-018-1392-z

115. Hwang YH, Park S, Paik JW, Chae SW, Kim DH, Jeong DG, et al. Efficacy and Safety of *Lactobacillus plantarum* C29-fermented soybean (DW2009) in individuals with mild cognitive impairment: A 12-week, multi-center, randomized, double-blind, placebo-controlled clinical trial. *Nutrients.* (2019) 11:305. doi: 10.3390/nu11020305

116. Kobayashi Y, Kinoshita T, Matsumoto A, Yoshino K, Saito I, Xiao JZ. *Bifidobacterium breve* A1 supplementation improved cognitive decline in older adults with mild cognitive impairment: An open-label, single-arm study. *J Prev Alzheimers Dis.* (2019) 6:70–5. doi: 10.14283/jpad.2018.32

117. Kobayashi Y, Kuhara T, Oki M, Xiao JZ. Effects of *Bifidobacterium breve* A1 on the cognitive function of older adults with memory complaints: a randomised, double-blind, placebo-controlled trial. *Benef Microbes.* (2019) 10:511–20. doi: 10.3920/BM2018.0170

118. Nanri, A, Mizoue T, Noda M, Takahashi Y, Kirii K, Inoue M, et al. Magnesium intake and type II diabetes in Japanese men and women: the Japan public health center-based prospective study. *Eur J Clin Nutr.* (2010) 64:1244–7. doi: 10.1038/ejcn.2010.138

119. Ibsen DB, Levitan EB, Åkesson A, Gigante B, Wolk A. The dietary approach to stop hypertension (DASH) diet is associated with a lower risk of heart failure: A cohort study. *Eur J Prev Cardiol.* (2022) 29:1114–23. doi: 10.1093/eurjpc/zwac003

120. Chatterton ML, Mihalopoulos C, O'Neil A, Itsiopoulos C, Opie R, Castle D, et al. Economic evaluation of a dietary inter-vention for adults with major depression (the "SMILES" trial). *BMC Public Health.* (2018) 18:599. doi: 10.1186/s12889-018-5504-8

121. Rogers PJ, Appleton KM, Kessler D, Peters TJ, Gunnell D, Hayward RC, et al. No effect of n-3 long-chain polyunsaturated fatty acid (EPA and DHA) supplementation on depressed mood and cognitive function: A randomised controlled trial. *Br J Nutr.* (2008) 99:421–31. doi: 10.1017/S0007114507801097

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 24 August 2022
DOI 10.3389/fnut.2022.919769

Check for updates

OPEN ACCESS

EDITED BY
Andrew Scholey,
Swinburne University of Technology, Australia

REVIEWED BY
Sanjay Basak,
National Institute of Nutrition (ICMR), India
Eglantine Balland,
Monash University, Australia

*CORRESPONDENCE
Weili Lin
weili_lin@med.unc.edu

SPECIALTY SECTION
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

RECEIVED 13 April 2022
ACCEPTED 29 July 2022
PUBLISHED 24 August 2022

CITATION
Li T, Samuel TM, Zhu Z, Howell B, Cho S, Baluyot K, Hazlett H, Elison JT, Wu D, Hauser J, Sprenger N, Zhu H and Lin W (2022) Joint analyses of human milk fatty acids, phospholipids, and choline in association with cognition and temperament traits during the first 6 months of life. *Front. Nutr.* 9:919769. doi: 10.3389/fnut.2022.919769

COPYRIGHT
© 2022 Li, Samuel, Zhu, Howell, Cho, Baluyot, Hazlett, Elison, Wu, Hauser, Sprenger, Zhu and Lin. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Joint analyses of human milk fatty acids, phospholipids, and choline in association with cognition and temperament traits during the first 6 months of life

Tengfei Li[1,2], Tinu M. Samuel[3], Ziliang Zhu[4], Brittany Howell[5], Seoyoon Cho[4], Kristine Baluyot[2], Heather Hazlett[6], Jed T. Elison[7], Di Wu[8], Jonas Hauser[9], Norbert Sprenger[9], Hongtu Zhu[2,4] and Weili Lin[1,2]*

[1]Department of Radiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [2]Biomedical Research Imaging Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [3]Nestlé Product Technology Center-Nutrition, Société des Produits Nestlé S.A., Vevey, Switzerland, [4]Department of Biostatistics, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [5]Department of Human Development and Family Science, Fralin Biomedical Research Institute at Virginia Tech Carilion, Virginia Polytechnic Institute and State University, Roanoke, VA, United States, [6]Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [7]Institute of Child Development, University of Minnesota, Minneapolis, MN, United States, [8]Division of Oral and Craniofacial Health Science, Adams School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [9]Nestlé Institute of Health Sciences, Société des Produits Nestlé SA, Lausanne, Switzerland

Early dietary exposure *via* human milk nutrients offers a window of opportunity to support cognitive and temperament development. While several studies have focused on associations of few pre-selected human milk nutrients with cognition and temperament, it is highly plausible that human milk nutrients synergistically and jointly support cognitive and behavioral development in early life. We aimed to discern the combined associations of three major classes of human milk nutrients with cognition and temperament during the first 6 months of life when human milk is the primary source of an infant's nutrition and explore whether there were persistent effects up to 18 months old. The Mullen Scales of Early Learning and Infant Behavior Questionnaires—Revised were used to assess cognition and temperament, respectively, of 54 exclusively/predominantly breastfed infants in the first 6 months of life, whose follow-ups were conducted at 6–9, 9–12, and 12–18 months old. Human milk samples were obtained from the mothers of the participants at less than 6 months of age and analyzed for fatty acids [total monounsaturated fatty acids, polyunsaturated fatty acid, total saturated fatty acid (TSFA), arachidonic acid (ARA), docosahexaenoic acid (DHA), ARA/DHA, omega-6/omega-3 polyunsaturated fatty acids ratio (n-6/n-3)], phospholipids [phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylinositol

(PI), sphingomyelin], and choline [free choline, phosphocholine (PCho), glycerophosphocholine]. Feature selection was performed to select nutrients associated with cognition and temperament. The combined effects of selected nutrients were analyzed using multiple regression. A positive association between the arachidonic acid (ARA) and surgency was observed (p = 0.024). A significant effect of DHA, n-6/n-3, PE, and TSFA concentrations on receptive language (R^2 = 0.39, p = 0.025) and the elevated ARA, PCho, and PI with increased surgency (R^2 = 0.43, p = 0.003) was identified, suggesting that DHA and ARA may have distinct roles for temperament and language functions. Furthermore, the exploratory association analyses suggest that the effects of human milk nutrients on R.L. and surgency may persist beyond the first 6 months of life, particularly surgency at 12–18 months (p = 0.002). Our study highlighted that various human milk nutrients work together to support the development of cognition and temperament traits during early infancy.

KEYWORDS

human milk, cognition and temperament, Mullen Scales of Early Learning, IBQ, receptive language, surgency, ARA, DHA

Introduction

Human milk has been considered the best and primary source of nutrients for infants, particularly during the first 6 months of life. Human milk contains a wide variety of nutrients, bioactive components, immunological compounds, and commensal bacteria, which are vital for infants' survival, health, immunity, brain maturation, and cognitive and temperament development (1, 2). Associations between breastfeeding (BF) and improved child cognitive functions have been widely documented (3); exclusive BF promotes an infant's receptive language (4) and executive function (5) later in life. BF also has a significant effect on temperament development. Children who were exclusively breastfed had higher surgency and regulation, a trait reflecting an inclination toward high levels of positive affectivity (6) and lower negative affectivity than those who were formula-fed (7). BF duration might be negatively associated with infant fussiness and positively associated with infant unpredictability (8) although these influences on the infant may be mediated by maternal sensitivity (9).

Preclinical and human studies thus far have largely focused on three main classes of nutrients in human milk, namely, the fatty acids, phospholipids, and choline/choline metabolites, separately regarding their effects on early cognitive development. Two long-chain polyunsaturated fatty acids (LCPUFAs), namely, docosahexaenoic acid (DHA) and arachidonic acid (ARA), are believed to be involved in infant brain structural and cognitive development (10–13). It was reported that DHA and ARA supplementation is vital for early cognitive development (14–17) and can improve memory and problem-solving scores of preterm infants (18). In addition, Carlson et al. showed that plasma ARA status was strongly correlated with weights and lengths of preterm infants in the first 12 months of life, suggesting that dietary ARA could lead to an improvement in growth in preterm infants (19). In contrast, the potential effects of total saturated fatty acid (TSFA) are controversial. Some argued that increased saturated fat intake was associated with an impaired ability to maintain multiple task sets in working memory and to flexibly modulate cognitive operations, particularly when faced with greater cognitive challenges (20). Phospholipids have also long been shown to be positively associated with brain cognitive processes (21). As major components of biological membranes and particularly abundant in the nervous system (22), phospholipids could act on the hypothalamic–pituitary–adrenal axis (21) and the microbiota–gut–brain axis (23) to exert their beneficial effects on the brain. Choline provides substrates for phosphatidylcholine and sphingomyelin formation, which are essential for neuronal and other cellular membranes, potentially improving signal transduction and brain development (24). Regarding how aforementioned human milk nutrients may be associated with temperament traits, few significant findings have been reported. For example, the total omega-3 LCPUFA was reported to be associated with infant negative affectivity, but this was not significant at an individual level for DHA, eicosapentaenoic acid (EPA), or eicosatetraenoic acid (ETA), three components of the omega-3 LCPUFA (25). Endocannabinoids, a class of ARA derivatives, were shown to be critically important for

motivational processes, emotion, stress responses, pain, and energy balance (26).

Despite the well-recognized importance of human milk on infant health, most of the above studies focused only on the effects of an individual human milk nutrient or a class of nutrients. Nutrients in human milk form a biological system (27); single nutrient supplementation is usually overly simplistic, ignoring the existence of other nutrients and their interplays within such a system. While a joint analysis of the micronutrient effects such as vitamins and iron was performed decades ago (28), this has rarely been investigated for the human milk nutrients described above. To this end, we aimed first to examine the combined effects of three major classes of human milk nutrients, namely, fatty acids, phospholipids, and choline, rather than separately evaluating their individual associations with cognition and temperament traits during the first 6 months of life and second to determine whether the effects persist up to 18 months of age.

Materials and methods

A subset of infants (n = 54) enrolled in the Baby Connectome Project—Enriched (BCP-E) study (29) who were exclusively/predominantly breastfed [fed less than four teaspoons or 20 g per day of non-formula and complementary foods/liquids (water, apple juice, etc.)] and younger than 6 months (chronological mean age: 4.43 ± 0.83 months) were included. The rationale for only including exclusively/predominantly breastfed infants was to minimize nutritional contributions from sources other than human milk, i.e., cow's milk or formula. Children were returned for follow-up visits up to 18 months of age.

Subject recruitment and data collection were conducted by two institutions (University of North Carolina at Chapel Hill, Chapel Hill, NC, and University of Minnesota, Twin Cities, MN). All study activities were approved by the institutional review boards of the two universities. Informed consent was obtained from parents prior to enrolling in the study. The inclusion criteria were as follows: birth at gestational age 37–42 weeks; birth weight appropriate for gestational age; and absence of major pregnancy and delivery complications. The exclusion criteria were as follows: adopted infant; birth weight < 2,000 g; abnormal MR in previous MR imaging; contraindication for MRI; neonatal hypoxia (10-min Apgar score < 5); chromosomal or major congenital abnormality; illness requiring NICU stay > 2 days; significant medical illness or developmental delay, or significant medical and/or genetic conditions affecting growth, development, or cognition (including visual/hearing impairment); the presence of a first-degree relative with autism, intellectual disability, schizophrenia, or bipolar disorder; or maternal preeclampsia, placental abruption, HIV status, and alcohol or illicit drug use during pregnancy.

We used R version 3.6.3 for all the following statistical analyses. We fixed a significance level $\alpha = 0.05$ and corrected for regression model significance with false discovery rate (FDR) adjustment.

Human milk collection and macronutrient analyses

Human milk samples were collected at each visit from the second feed of the day whenever possible and from the right breast using a hospital-grade, electric Medela Symphony breast pump. Mothers were instructed to completely express the contents of their right breast to ensure that the collected human milk was representative of nutrients received by the infant across a full feed. The collected human milk samples were vortexed at a maximum speed for 2 min, whose volume and weight were measured and recorded with special attention to avoid bubbles. Subsequently, 3 ml of human milk was used for mid-infrared spectroscopic analyses using the MIRIS Human Milk Analyzer. This step was to ensure that the total fat content (as an indicator of the quality of milk sampling) fell within the expected range. Finally, an aliquot of the minimum 30 ml of volume was transferred from the collection bottle to a 50-ml glass beaker. Using a repeating pipette and an appropriate tip, 11 aliquots of 1 ml of volume were made in 1-ml Eppendorf tubes and nine aliquots of 2 ml of volume were made in 2 ml Eppendorf tubes for storage in a –80°C freezer within 30 min from the end of the time of collection.

Fatty acids, phospholipids, and choline analyses

A representative 1 ml aliquot of collected human milk was shipped on dry ice to the Nestle Research Center (Switzerland) for analyses of fatty acids (FAs) and phospholipids, whereas choline was analyzed at UNC-Chapel Hill. Specifically, direct quantifications of FAs were accomplished using gas chromatography as detailed in Cruz-Hernandez et al. (30), and the total monounsaturated fatty acids (TMUFA), total polyunsaturated fatty acids (TPUFA), TSFA, ARA, DHA, ARA-to-DHA ratio (ARA/DHA), and omega-6/omega-3 polyunsaturated fatty acids ratio (n-6/n-3) were obtained. In contrast, analyses of phospholipids were accomplished using high-performance liquid chromatography coupled with a mass spectrometer detector (31), which yielded phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and sphingomyelin (SPH). Finally, quantification of choline and choline metabolites was performed using liquid chromatography–stable isotope dilution–multiple reaction monitoring/mass spectrometry (LC–SID–MRM/MS). Chromatographic separations were performed on an Acquity HILIC 1.6 μm 2.1 × 50 mm column (Waters Corp., Milford, CT,

United States) using a Waters ACQUITY UPLC system, and free choline, phosphocholine (PCho), and glycerophosphocholine (GPC) were obtained.

Assessments of cognition and temperament

Two measures, namely, the Mullen Scales of Early Learning (MSEL) and Infant Behavior Questionnaires—Revised (IBQ-R) (32), were employed to assess cognition and temperament, respectively. The MSEL includes five subscales, namely, fine motor (F.M.), gross motor (G.M.), visual reception (V.R.), receptive language (R.L.), and expressive language (E.L.). An early learning composite (E.L.C) score considered as the developmental quotient of infants was calculated by summing the T-scores of all subdomains excluding G.M. The MSEL was administered by trained staff at every visit. In contrast, the IBQ-R is a widely used 14-scale parent report measure designed to assess infant temperament. To reduce the number of variables, three factors were extracted by using three linear weighted averages of the 14 subscales. Specifically, we incorporated the weights of three latent factors reported in the exploratory factor analysis results of Gartstein and Rothbart (32) and obtained three personality traits, namely, surgency/extraversion (SUR), negative affectivity (NEG), and orienting/regulation (REG). The subscale items included in the above three factors and loading scales are provided in **Supplementary Table 1**. In our study, the MSEL or IBQ-R assessments in the first 6 months were obtained within 30 days from the collection of human milk samples.

Statistical modeling for associations analyses in the first 6 months of life

All human milk nutrient concentrations were first normalized with mean zero and unit variance. Marginal association analyses between each individual nutrient and each subdomain of MSEL and IBQ-R scores were carried out by linear regression of each subdomain score on each nutrient. As nutrients could vary with postpartum duration, to ensure that age does not contribute to our analyses, age was included as a controlled variable. Other confounding factors including sex, data collection site, and household income (if $<$ 75k) were also controlled in the above linear model, and the standardized regression coefficient was obtained between each nutrient and each MSEL/IBQ-R score. Household incomes of two infants were missing and imputed with the overall average. Specifically, we used the linear model $M0:\ \text{MSEL or IBQ}\ \sim\ 1+\sum_{k=1}^{4} C_k + X_j, j=1,\ldots,\ p,$ where $C_k,\ k=1,\ldots,\ 4,$ are the four controlled confounders for each nutrient X_j.

To evaluate the potential combined and conditional effects of human milk nutrients in association with MSEL and IBQ-R and to deal with the collinearity among the human milk nutrients, the correlation matrix among all nutrients was calculated. Subsequently, nutrients were clustered into several subgroups based on their correlation using the single linkage clustering analysis (SLCA) (33). The SLCA clustering, also known as the nearest-neighbor clustering, is one of the widely used approaches of hierarchical clustering. The SLCA approach was chosen as it offers intuitive interpretations of the results by using the minimum spanning tree; all pairs of nutrients exhibiting correlation coefficients greater than a predefined threshold T_c were combined into one sub-group. The optimal number of clusters was determined by maximizing the Dunn index (34). A higher Dunn's index indicates a better clustering with either an increased minimal intercluster distance or a decreased maximal cluster diameter. Furthermore, in order to minimize redundancy and collinearity (35), the best subset selection model (36–38) was employed for nutrient selection. To reduce multi-collinearity, we only consider possible combinations from those with at most a single nutrient from each cluster. To formularize the model, we denote $\mathcal{T}$ as the set of all the nutrients, and $\mathcal{T}_k,\ k=1,\ldots,\ p,$ as p clusters obtained based on the SLCA clustering approach. The nutrient selection model is $M1:\ \text{MSEL or IBQ}\ \sim\ 1+\sum_{k=1}^{4} C_k+\sum_{j=1}^{q} X_j,\ \ X_j\in\ \mathcal{T}_{k_j}$, where q is the number of selected nutrients with $0\le q\le p$ and X_j is the selected nutrient from the k_jth cluster $\mathcal{T}_{k_j}$ with $k_j\in\{1,\ldots,\ p\}$ and $k_j\neq k_l$ if $k\neq l$. Different combinations of nutrients X_j were fed into model $M1$, and the final selected nutrients will be determined by maximizing the adjusted R-squared of model $M1$ to reduce overfitting. The final selected nutrients were then used as the covariates and MSEL or IBQ-R obtained within 30 days before or after the collection of human milk samples as the outcomes for the regression model to uncover the potential associations. The overall significance was reported using the ANOVA F-statistics in comparison with the reduced baseline model $M2:\ \text{MSEL or IBQ}\ \sim\ 1+\sum_{k=1}^{q} C_k$. The FDR adjustment was adopted for controlling type I errors of multiple comparisons. To assess whether the identified nutrients predict cognitive and temperament scores of new subjects, multiple regression models with the above-selected nutrients were evaluated by squared cross-validation (CV) errors and prediction correlations through 100 repetitions of 5-fold CV.

Model evaluation on follow-up visits beyond 6 months

To evaluate whether the identified associations of human milk nutrients and cognition/temperament persisted beyond

the first 6 months of life in the same subjects, the subjects with follow-up visits were binned into three age groups: 6–9 months, 9–12 months, and 12–18 months. Each observation within each age group corresponded to a unique subject. The regression models trained above were used to predict MSEL and IBQ-R scores (predicted scores). The correlation and p-value based on Pearson's t-test between the predicted and observed scores were evaluated.

Results

Of the 54 subjects, cognition was assessed using MSEL in 38 subjects (4.64 ± 0.89 months; 12 male subjects), whereas temperament was assessed using IBQ-R in 42 subjects (4.48 ± 0.73 months; 16 male subjects) and both were available in 26 subjects (4.81 ± 0.71 months; 9 male subjects). After the initial assessments, the follow-up assessments of MSEL were available in 27, 34, and 25 subjects at 6–9 (7.63 ± 0.87 months; 9 male subjects), 9–12 (10.5 ± 0.97 months; 10 male subjects), and 12–18 months old (14.02 ± 1.08 months; 8 male subjects), respectively. In contrast, IBQ-R was obtained from 7, 7, and 27 subjects between 6 and 9 (mean: 243.9 ± 12.3 days), 9 and 12 (mean: 341.1 ± 18.8 days), and 12 and 18 (mean: 13.34 ± 0.79 months) months, respectively (Figure 1 and Table 1). Detailed demographic information on infants and their mothers including infant age, sex, anthropometrics, measured breast milk gross composition such as fat, carbohydrates, proteins, and energy, as well as household income and mother's education, is summarized in Table 1.

Figure 2 shows how the 14 human milk nutrients vary with age during the first six postpartum months, while the summary statistics of their individual concentrations are provided in Table 2. Among these nutrients, DHA decreases ($p < 0.002$),

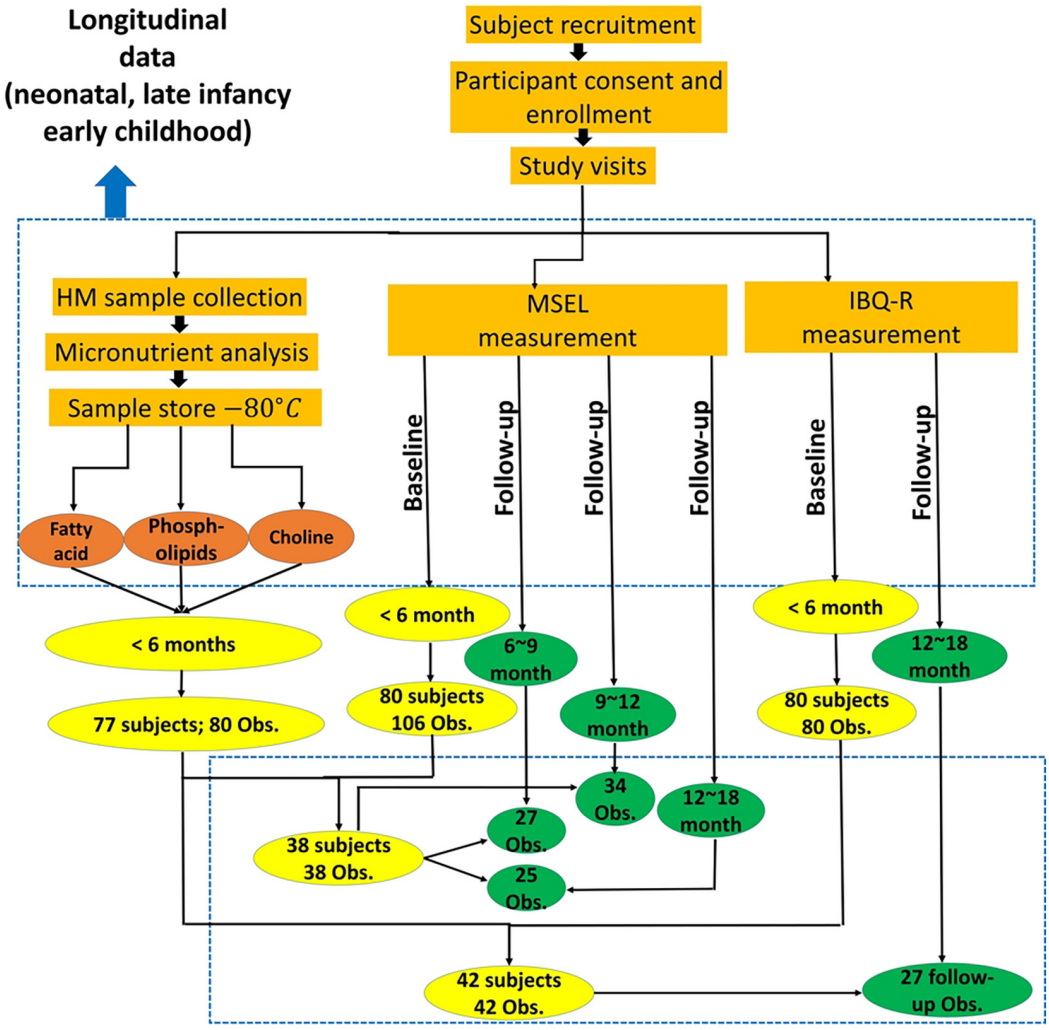


FIGURE 1
Study flowchart provides the overall experimental details.

TABLE 1 Characteristics of the participants.[a]

	All subjects	MSEL[b] (<6 m)	MSEL (6–9 m)	MSEL (9–12 m)	MSEL (12–18 m)	IBQ-R[c] (<6 m)	IBQ-R (12–18 m)
Numbers of subjects	54	38	27	34	25	42	27
Sex (male)	19	12	9	10	8	16	4
Age (months)	4.43 (0.83)	4.64 (0.89)	7.63 (0.87)	10.5 (0.97)	14.02 (1.08)	4.48 (0.73)	13.34 (0.79)
Birth length (cm)	52.35 (2.50)	52.29 (2.35)	51.85 (2.18)	52.26 (2.49)	52.27 (2.20)	52.32 (2.45)	52.51 (2.47)
Gestation ages (days)	280.6 (6.0)[d]	279.3 (6.2)	279.1 (5.5)	279.3 (6.2)	278.4 (6.5)	280.2 (5.9)	280.8 (5.3)
Birth weight (g)	3630 (420)	3548 (399)	3469 (396)	3534 (419)	3523 (415)	3619 (428)	3611 (388)
Weight at visit (g)	6818 (1081)	6872 (1244)	8649 (1658)	9396 (1379)	10115 (1160)	6936 (824)	1028 (1284)
Height at visit (cm)	63.81 (4.54)	64.14 (5.15)	68.47 (6.10)	72.98 (4.70)	75.90 (6.64)	64.62 (4.27)	76.38 (8.24)
Household incomes (n)							
<75k	18	13	7	12	8	14	9
75k–150k	28	18	15	17	13	23	16
150k<	6	5	4	3	3	5	2
Not answered	2	2	1	2	1	0	0
Maternal education							
≥ Graduate[e]	26	15	9	14	12	17	23
<Graduate	27	22	18	19	13	10	19
Unavailable	1	1	0	1	0	0	0

[a]Provided are the means with standard deviations in parentheses.
[b]MSEL, Subjects with Mullen Scales of Early Learning scores taken.
[c]IBQ-R, Subjects with Infant Behavior Questionnaires—Revised scores taken.
[d]The gestation age range for all subjects is 268–292 days.
[e]≥ Graduate: subjects with a graduate degree; < Graduate: subjects without a graduate degree.

while ARA/DHA ratio ($p < 0.03$) increases with postpartum age. As some of the subjects only had MSEL, but not IBQ-R, or *vice versa* during the first 6 months of life, the mean values of each nutrient from the MSEL dataset and the IBQ-R dataset were compared using the two-sided two-sample *t*-test. No significant differences were observed, suggesting that there is no selection bias in the mean nutrient concentrations. In addition, the MSEL and IBQ-R scores are provided in **Supplementary Tables 2, 3**, respectively, which are within the normal ranges of both scales.

While the focus of our study was to jointly analyze a wide array of human milk nutrients in association with cognition and temperament in typically developing children, the marginal associations of each nutrient with MSEL and IBQ-R scores are given in **Tables 3**, **4**, respectively. A significant association between ARA and SUR ($r = 0.54$, $p = 0.0006$, adjusted $p = 0.024$, 95% CI = [0.25, 0.83]) was observed. Several other nutrients including TSFA ($r = 0.35$, $p = 0.035$, 95% CI = [0.03, 0.68]), TPUFA ($r = 0.35$, $p = 0.031$, 95% CI = [0.03, 0.67]), and PCho ($r = 0.46$, $p = 0.003$, 95% CI = [0.17, 0.76]) were positively associated with SUR; TPUFA ($r = 0.32$, $p = 0.046$, 95% CI = [0.007, 0.64]) and ARA ($r = 0.39$, $p = 0.015$, 95% CI = [0.08, 0.70]) were associated with REG. In addition, marginal negative associations between G.M. and TSFA (the standardized regression coefficient $r = -0.40$, $p = 0.01$, and 95% confidence interval (CI) = [−0.71, −0.08]) and TMUFA ($r = -0.38$, $p = 0.02$ and 95% CI = [−0.70, −0.05]) and a positive association between DHA and R.L. ($r = 0.38$, $p = 0.049$ and 95% CI = [0.002, 0.76]) were observed, although all of these associations did not pass the FDR control due to limited sample size.

We further tested whether human milk nutrients are correlated. Evidently, many of the human milk nutrients were highly correlated (**Figure 3A**). In particular, the nutrients in the phospholipid family were highly correlated. As outlined above, to minimize collinearity, the single linkage clustering analysis was used to cluster highly correlated nutrients into one group and the Dunn index was used to determine the optimal number of clustered nutrient groups. The rationale here is to keep highly correlated nutrients in the same group while splitting those less correlated nutrients into different groups. The optimal number of clusters was 7, which exhibited the largest Dunn index (**Table 5**). Note that the optimal clustering result was stable for $0.53 < T_c \leq 0.69$. In addition, the memberships of groups 1–3 were stable independent of the T_c for $0.53 < T_c \leq 0.77$. The minimum spanning tree representing the smallest sum of distances to touch all vertices of the graph of the optimal clustering results is shown in **Figure 3B**.

Significant combined associations of human milk nutrients were observed for R.L. (MSEL) and SUR (IBQ-R) summarized in **Figure 4A**. Specifically, for receptive language, the final linear regression model includes DHA, n-6/n-3 ratio, PE and TSFA (-), and R-squared = 0.39 (adjusted $p = 0.025$), where the information provided in the parentheses indicates the sign of the coefficients and positive otherwise. In contrast, for SUR,

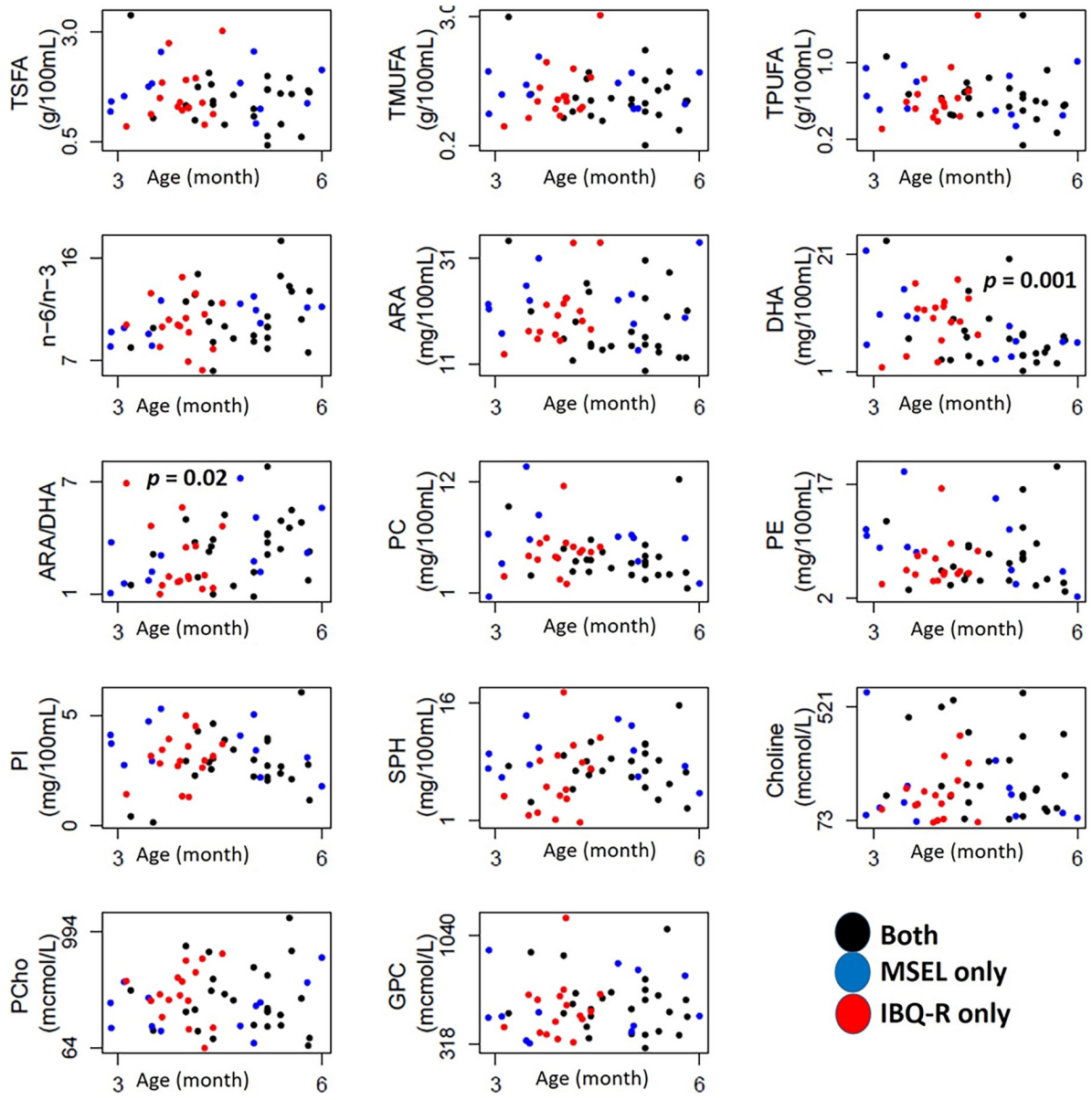


FIGURE 2
Scatterplots of the 14 nutrients with age in months (x-axis) for subjects assessed with MSEL (blue circles), IBQ-R (red circles), and both (black circles). TMUFA, total monounsaturated fatty acids; TPUFA, polyunsaturated fatty acid; TSFA, saturated fatty acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; ARA/DHA, the ARA-to-DHA ratio; n-6/n-3, omega-6/omega-3 polyunsaturated fatty acids ratio; PC, phospholipids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SPH, sphingomyelin; Choline, free choline; PCho, phosphocholine; GPC, glycerophosphocholine; SUR, surgency; NEG, negative affectivity; REG, regulation; mcmol, micromoles.

the model includes ARA, PI, and PCho with R-squared = 0.43 and adjusted p = 0.003. Finally, a positive association between ARA and REG with R-squared = 0.23 and adjusted p = 0.03 was observed. The scatterplots of the combined associations between the experimentally obtained and fitted R.L. and SUR using the identified association model are shown in Figures 4B,C, respectively. More detailed coefficients, confidence intervals, and p-values for each model are given in Table 6. In addition, the scatterplots of the associations between each of the selected nutrients for R.L., SUR, and REG are shown in Figure 5. Finally, a 5-fold CV between the observed and predicted MSEL and IBQ-R scores was conducted. The boxplots of Pearson's correlations between the observed and predicted MSEL and IBQ-R scores from 100 random splits of 5-fold CV are shown in Figure 6A. The boxplots imply that in 99% and 91% cases of the 100 random splits of CV repetitions, the correlation between the observed and predicted R.L. scores and SUR was significant, respectively, whereas in most cases the association with the observed REG

TABLE 2 Composition of fatty acids, phospholipids, and choline metabolites in human milk in the first six postpartum months.

Nutrients	Mean (SD)	Median	(Lower) 5%	(Upper) 95%
TSFA (g/100ml)	1.54 (0.59)	1.43	0.76	2.64
TMUFA (g/100ml)	1.32 (0.53)	1.21	0.69	2.19
TPUFA (g/100ml)	0.64 (0.26)	0.6	0.33	1.03
n-6/n-3	10.57 (2.27)	10.14	7.39	14.37
ARA (mg/100ml)	20.07 (6.32)	19.15	12.21	33.99
DHA (mg/100ml)	8.35 (5.14)	7.09	2.58	18.14
ARA (% of total fatty acids)	0.60 (0.15)	0.60	0.40	0.80
DHA (% of total fatty acids)	0.24 (0.14)	0.19	0.11	0.52
ARA/DHA	3.24 (1.73)	3.2	1.01	6.15
PC (mg/100ml)	4.99 (2.58)	4.6	1.93	10.51
PE (mg/100ml)	7.53 (3.98)	6.4	3.45	16.39
PI (mg/100ml)	3.06 (1.19)	2.94	1.24	5
SPH (mg/100ml)	7.7 (3.61)	7.66	1.86	14.19
Choline (mcmol/l)	216.49 (140.47)	173.5	72.5	530.45
PCho (mcmol/l)	448.85 (235.64)	439.56	120.85	834.8
GPC (mcmol/l)	568.53 (192.07)	528.62	335.08	931.9

TABLE 3 Standardized regression coefficients of the human milk nutrients with the Mullen Scales of Early Learning scores.[a]

Nutrients	Composite score	Gross motor	Visual reception	Fine motor	Receptive language	Expressive language
TSFA	−0.25	***−0.40 (0.01)***[b]	−0.07	−0.21	−0.24	−0.19
TMUFA	−0.15	***−0.38 (0.02)***	−0.07	−0.15	−0.12	−0.08
TPUFA	−0.22	−0.27	−0.18	−0.11	−0.19	−0.09
n-6/n-3	0.10	0.14	0.23	−0.06	0.02	0.05
ARA	−0.06	−0.25	−0.08	0.00	−0.11	0.06
DHA	0.25	−0.08	0.02	0.14	***0.38 (0.049)***	0.07
ARA/DHA	−0.13	0.20	−0.14	−0.15	−0.09	0.06
PC	0.05	−0.28	−0.03	−0.11	0.22	−0.03
PE	0.04	−0.22	−0.14	−0.26	0.17	0.26
PI	−0.16	−0.19	−0.05	−0.21	−0.13	−0.11
SPH	−0.30	−0.26	−0.37	−0.31	−0.13	−0.10
Choline	0.03	0.09	0.03	−0.07	−0.10	0.29
PCho	−0.02	−0.03	0.03	−0.23	0.20	−0.09
GPC	−0.05	0.07	0.21	−0.10	−0.23	0.06

[a]Confounding factors: age, sex, site, and household income (if < 75k) were controlled.
[b]Statistics in bold italics are standardized coefficients with raw *p*-values (in parentheses) of < 0.05.

was not significant. These results showed a stronger prediction power of R.L. and SUR, but not REG. The observed (X-axis) versus the predicted R.L. (Figure 6B) and SUR (Figure 6C) of the 100 times' predictions are also provided. The mean and the 95% confidence interval of prediction correlations (based on the random splits in CV) were 0.434 and [0.349, 0.501] for R.L. and 0.409 and [0.281, 0.509] for SUR, respectively. These results showed model robustness of predictions against training data perturbations.

Finally, we performed exploratory analyses for predicting the two outcome measures at the follow-up observations using results obtained < 6 months old. With the limited sample sizes for IBQ-R at the 6–9- and 9–12-month follow-up visits (Table 1 and Figure 1), the IBQ-R analyses were only performed for the 12–18-month age group, whereas, MSEL was conducted for all three age bins. The correlation coefficients (*p*-values) for MSEL were 0.35 ($p = 0.07$), 0.14 ($p = 0.43$), and −0.21 ($p = 0.32$) at 6–9, 9–12, and 12–18 months, respectively; the correlation coefficient (*p*-value) for SUR at 12–18 months was 0.58 ($p = 0.002$). Evidently, the associations between the predicted and observed R.L. decrease with age with the best performance at 6–9 months ($p = 0.07$; Figure 7A). In contrast, a significant association between the predicted and observed SUR at 12–18 months was observed ($p = 0.002$; Figure 7B).

Discussion

The human milk produced by each mother for her infant has a unique composition and contains a myriad of different lipids, vitamins, minerals, bioactive carbohydrates, proteins, and immune factors that evolve in tandem with the growth and developmental needs of the infant (39). In addition, maternal diet and lifestyle can also affect human milk composition, especially the lipid components (40). While several studies have shown positive associations of human milk bioactive components and nutrients with brain development (29, 41), most of them have largely considered one or only few nutrients. Christian et al. (27) recently pointed out that these approaches may be overly simplistic and failed to consider the complex synergetic effects of human milk nutrients on infant development and health. They advocated the need for a paradigm shift by considering human milk nutrients as a biological system in future studies aiming to discern the benefits of human milk nutrients. To this end, we collected human milk samples, concurrently assessed the cognition and temperament of BF infants, utilized advanced statistical models to jointly consider 14 widely evaluated human milk nutrients spanning the three families, namely, choline, fatty acids, and phospholipids, and reported combined associations between these nutrients and cognition/temperament during the first 6 months of life. Furthermore, whether the effects of identified nutrients during the first 6 months persist through to 1.5 years old was also evaluated.

An analysis of the temporal trends in human milk nutrients over the first six postnatal months revealed that DHA exhibited a significant negative age effect and choline remained relatively stable, consistent with those reported previously (42). In contrast, while the phospholipids were stable with age, several studies have reported an increase in lactation (42, 43). Human milk DHA content was highly variable (ranging from 0.08% to 0.71%) with a median concentration of 0.19%, which is below the worldwide average (0.37%) and those reported from Southeast Asian regions (42, 44). Nevertheless, lower levels of human milk DHA have been reported in the North American region (45), possibly due to low habitual intake of seafood in the inland areas, and those levels are strongly impacted by maternal DHA intake (46). In addition, the median human milk ARA of 0.60% in our cohort was higher compared with

TABLE 4 Standardized regression coefficients of the human milk nutrients with the Infant Behavior Questionnaires—Revised factors.[a]

Nutrients	Surgency	Negative affectivity	Regulation
TSFA	***0.35 (0.035)***[b]	0.24	0.09
TMUFA	0.28	0.19	0.28
TPUFA	***0.35 (0.031)***	0.18	***0.32 (0.046)***
n-6/n-3	0.00	−0.12	0.06
ARA	***0.54 (0.0006)****	0.30	***0.39 (0.015)***
DHA	0.15	0.24	0.12
ARA/DHA	0.02	−0.12	−0.07
PC	0.24	0.17	0.23
PE	0.38	0.23	0.23
PI	0.26	0.23	0.09
SPH	0.09	0.12	0.07
Choline	−0.19	−0.13	−0.07
PCho	***0.46 (0.003)***	0.05	0.18
GPC	0.09	0.22	0.11

[a]Confounding factors: age, sex, site, and household income (if < 75k) were controlled.
[b]Statistics in bold italics are standardized coefficients with raw p-values (in parentheses) of < 0.05.
*Adjusted $p < 0.05$ after FDR correction.

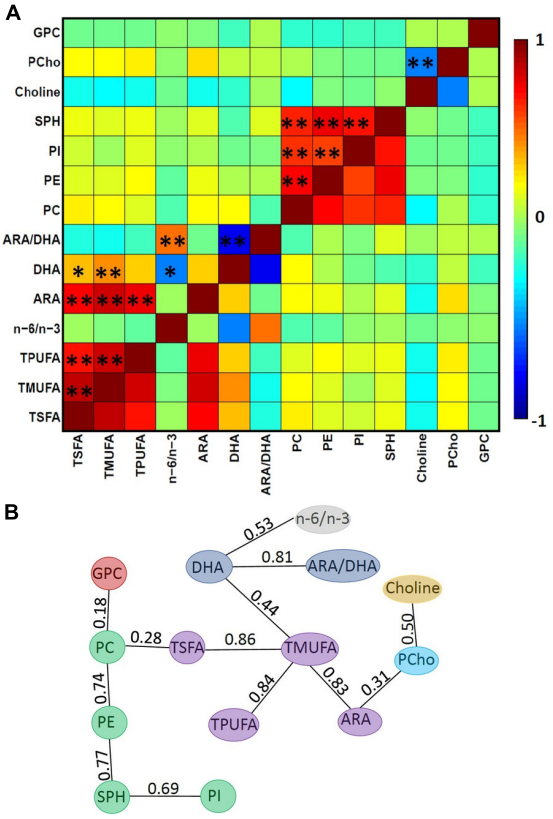


FIGURE 3
Relations between nutrients. **(A)** Pearson's correlation between each pair of the 14 nutrients, where single asterisks (*) and double asterisks (**) represent a raw p-value of <0.05 and adjusted $p < 0.05$, respectively, using the standard Pearson correlation test and the FDR correction. **(B)** The minimum spanning tree, where colors represent different clusters and numbers represent the correlation coefficients between a given pair of nutrients. Note the optimal clustering result is stable for $0.53 < T_c \leq 0.69$. In addition, the minimum spanning tree shows that the strongest correlation between the clusters (PC, PI, PE, and SPH) and (TSFA, TMUPA, TPUFA, and ARA) is between the pair of TSFA and PC (0.28). It implies that only when $T_c \leq 0.28$, these two clusters will merge together, while PCho and choline are closer in the sense that these two will merge when $T_c \leq 0.50$.

TABLE 5 Correlation and grouping of the 14 nutrients.

	Clustering result 1	Clustering result 2	Clustering result 3
	$0.53 < T_c$[a] ≤ 0.69	$0.69 < T_c \leq 0.74$	$0.74 < T_c \leq 0.77$
Group 1	TSFA, TMUFA, TPUFA, ARA	TSFA, TMUFA, TPUFA, ARA	TSFA, TMUFA, TPUFA, ARA
Group 2	n-6/n-3	n-6/n-3	n-6/n-3
Group 3	DHA, ARA/DHA	DHA, ARA/DHA	DHA, ARA/DHA
Group 4	PC,PE,PI,SPH	PC, PE, SPH	PE, SPH
Group 5	GPC	Choline	Choline
Group 6	PCho	PCho	PCho
Group 7	Choline	GPC	GPC
Group 8	-	PI	PC
Group 9	-	-	PI
Dunn index	0.64	0.44	0.41

[a]T_c, The threshold values for the correlation coefficients.

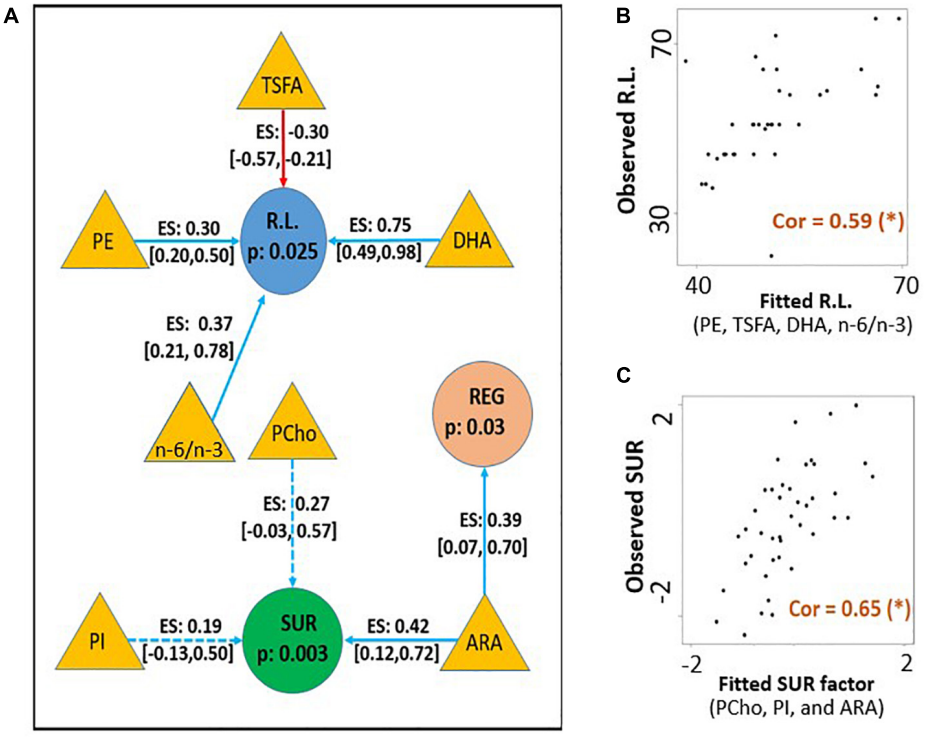


FIGURE 4
(A) The identified conditional effects of human milk nutrients (filled triangles) on receptive language score (R.L., filled blue circles) and surgency (SUR, filled green circles) using linear regression. The blue and red arrows represent the positive and negative associations, respectively. The corresponding effect size (ES), confidence interval of coefficients for each association, and FDR-corrected p-values for the regression model are provided. Dotted lines indicate the regression coefficient of the covariate is not significant, but the inclusion of the corresponding nutrient improves model fitting with a higher adjusted R^2 value. **(B)** The association between the experimentally obtained R.L. and the combined effect of selected human milk nutrients DHA, n-6/n-3, PC, and TSFA (fitted R.L.). **(C)** The association between the experimentally obtained SUR and the combined effect of selected human milk nutrients ARA, PCho, and PI (fitted SUR). Pearson's correlation between the fitted and observed measurements is included in panels **(B,C)** with (*) indicating adjusted $p < 0.05$.

the global average (0.55 %), leading to a higher ARA/DHA ratio (3.24) when compared with that reported in the literature (1.5–2) (47). The human milk n-6/n-3 fatty acid ratio in our study (mean 10.57), while comparing some geographies, was higher than others (48), suggesting differences in either body fat composition and mobilization of fat stores or dietary habits such as consumption of LA-rich vegetable oils (49). The levels of most PLs (PC and PE) in our study were

TABLE 6 Multiple regression results for MSEL scores/IBQ-R factors with the selected nutrients.

	Composite score	Gross motor	Receptive language	Surgency	Regulation
R-squared	0.31	0.42	0.36	0.43	0.23
Adjusted *R*-squared	0.12	0.26	0.18	0.31	0.12
Raw *p*-value[a]	0.05	0.05	0.005	0.0008	0.017
Adjusted *p*-value[b,c]	0.05	0.11	**0.025**	**0.003**	**0.03**
TSFA[d,e,f]	−0.42 (0.03)	-	−0.30 (0.05)	-	-
TPUFA	-	-	-	-	-
TMUFA	-	−0.51	-	-	-
ARA	-	-	-	0.42 (0.008)	0.39 (0.02)
n-6/n-3	0.26 (0.19)	0.19	0.37 (0.006)	-	-
DHA	0.55 (0.02)	0.30	0.75 (0.002)	-	-
ARA/DHA	-	-	-	-	-
PC	-	-	-	-	-
PE	-	-	0.30 (0.07)	-	-
PI	-	-	-	0.19 (0.24)	-
SPH	−0.22 (0.23)	−0.24	-	-	-
Choline	-	-	-	-	-
PCho	-	-	-	0.27 (0.08)	-
GPC	-	-	-	-	-
Age	−0.006 (0.98)	0.28 (0.12)	0.30 (0.03)	0.18 (0.26)	0.18 (0.31)
Sex (if Male)	0.03 (0.87)	0.08 (0.61)	−0.01 (0.79)	−0.03 (0.83)	−0.004 (0.98)
Site (if UNC)	0.20 (0.29)	0.44 (0.01)	0.10 (0.63)	−0.32 (0.05)	0.22 (0.21)
Income (if < 75k)	−0.13 (0.44)	0.14 (0.35)	−0.15 (0.21)	−0.15 (0.34)	0.06 (0.71)

[a]This row shows ANOVA *F*-test *p*-values comparing the full and reduced models (confounders only); the results with raw *p*-values of > 0.05 are not shown.
[b]*P*-values in this line indicate the *p*-values after FDR correction for multiple comparisons.
[c]Adjusted *p*-values less than 0.05 are shown in bold.
[d]Numbers in parentheses show the *p*-values of the coefficients using the *t*-test statistic.
[e]Hyphen "-" indicates that this variable is not selected in this model based on the best subset selection feature selection algorithm.
[f]Coefficients here are standardized linear regression coefficients.

comparable to those reported from Singapore (42). We observed higher concentrations of free choline and GPC, but lower levels of PCho, compared with reports from Canada and Cambodia (50), possibly due to differences in dietary choline intake and amounts of choline available from the maternal circulation. Taken together and compared with other studies, our findings highlight that several human milk nutrients in the choline, fatty acids, and phospholipids families may show geographical differences across regions reflecting differences in diet and lifestyle.

The potential interplay between nutrients and early brain development has been widely recognized (51). Our brains undergo rapid development during the first years of life by establishing new synapses (synaptogenesis), removing excessive synapses (pruning), myelination (52), and forming highly complex yet efficient brain functional networks enabling the performance of cognitive tasks and social behaviors (53). During these dynamic and highly energy-demanding brain developmental processes, an appropriate nutrient supply is key to healthy neurodevelopment in infancy. Our results show that while the individual content of PE, DHA, n-6/n-3, or TSFA does not show a significant association on its own, a combined effect is observed on receptive language (R.L.). In contrast, ARA by itself is significantly associated with SUR (adjusted p = 0.024), whereas neither PCho nor PI is associated. Nevertheless, three nutrients, namely, ARA, PCho, and PI, jointly exhibit an improved association and predictive performance with SUR in terms of elevated model significance, decreased AIC, and CV errors. Importantly, we also observed a significant association between ARA and REG. This finding differs from the marginal association analyses where no associations were observed between REG and ARA after FDR correction. Largely, this finding is not surprising. Specifically, FDR correction was employed for the marginal associations to control for 14 nutrients and subscales of scores. In contrast, as multiple regression was employed with features selected using approaches outlined above, the FDR correction was only employed to control for subscales of scores, but not for 14 nutrients. Instead, 5-fold cross-validation was used to evaluate the possibility of overfitting. Indeed, CV results showed that the identified association between (PE, DHA, n-6/n-3, TSFA) and R.L. and (ARA, PCho, and PI) and SUR was robust, but not between ARA and REG. Therefore, caution should be taken when interpreting the association between ARA and REG.

Future studies with a larger sample size are warranted to further evaluate the association between ARA and REG.

Several key findings of our study deserve additional discussion. First, while MSEL assesses five domains of cognition, significant joint associations were only observed between PE, DHA, n-6/n-3, TSFA, and receptive language in our study. Several studies have previously evaluated the potential associations between individual human milk nutrients and infants' language ability. However, thus far the results are inconsistent and sometimes controversial. Although the effect of PE on language abilities has not been specifically reported in the literature, the milk fat globule membrane (MFGM), which is a diverse mixture of bioactive components including phospholipids, has been shown to improve visual function, language, and motor domains in both term and preterm infants (16, 54, 55). In contrast, DHA has been reported to improve language ability. However, a recent comprehensive review by Gawlik et al. (56) has concluded that the current evidence of DHA supplementation on language development is limited and non-conclusive. Ramos et al. (57) reported that a higher ratio of the linoleic (n-6 fatty acid) to the alpha-linolenic acid (n-3 fatty acid) could exert beneficial effects for R.L. in human milk-fed preterm infants. Nevertheless, their results are contrary to the body of literature showing that n-6/n-3 ratio in the maternal blood and diet is negatively associated with the vocabulary and verbal fluency of their infants (58, 59). Although the use of the n-6/n-3 ratio to assess potential health benefits is debatable due to the observed positive correlation between ARA and DHA concentrations in maternal plasma phospholipids (60–62), an appropriate balance between the two has been shown as an important factor contributing to cognitive development during infancy (63, 64). Nonetheless, inconsistent results are reported in the literature. The reasons may be found in differences in methodology, infant ages at assessment, and length of supplementation among these studies. Alternatively, failure to consider possible joint effects of multiple nutrients could also contribute to the observed inconsistent results reported in the literature, underscoring the importance of considering the strong dependency between human milk nutrients and the need to jointly analyze multiple nutrients.

Second, the general consensus is that temperament traits emerge early in life and have a strong genetic and neurobiological basis (65). However, what is less understood is the role of BF and human milk nutrients in shaping these offspring behavioral traits. Our study highlights that human milk ARA (adjusted p = 0.024) alone (but not DHA) and the combined effects of ARA, PCho, and PI exhibited a significant association with SUR, which includes high-intensity pleasure, smiling and laugher, perceptual sensitivity, vocal reactivity, and activity. Tallima and Ridi reported that the downstream metabolites of ARA such as eicosanoids or endocannabinoids play a critical role in brain reward signaling, motivational processes, emotion, stress responses, and pain (26), which may

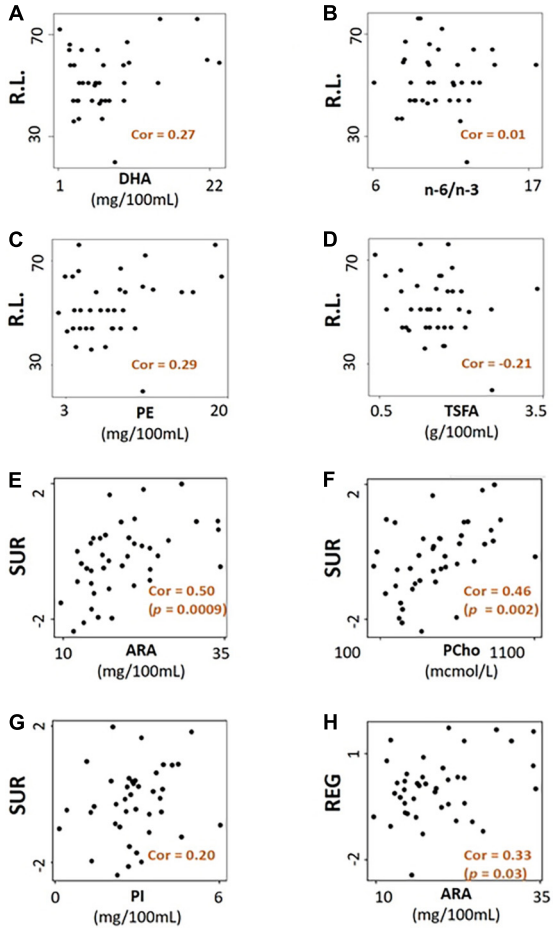


FIGURE 5
The scatterplots of selected nutrients for receptive language score (R.L.) are shown in panels **(A–D)**, surgency (SUR) in panels **(E–G)**, and regulation (REG) in panel **(H)** mcmol, micromoles.

further shape infants' behavioral traits. Our results differ from the recent findings of Hahn-Holbrook et al. (25), who showed that higher n-3 PUFAs in human milk (more specifically ALA which is a precursor of DHA), but not any of the n-6 PUFAs, were associated with significantly less sadness and distress to limitations (25). It is plausible that differences in experimental design (varying ages among subjects in our cohort vs. all assessed at 3 months old by Hahn-Holbrook) and the limited sample size in our study may have contributed to the different findings. Nevertheless, compared with the human milk nutrients associated with receptive language and SUR in our study, it appears that there may be distinct LCPUFA roles (DHA vs. ARA) for temperament and language functions, respectively. This finding could be of importance particularly in light of the legislation adopted by the European Commission in 2016 stipulating that all infant and follow-up formulas marketed in Europe must contain DHA in the amount of 20–50 mg/100 kcal, but without any requirement for ARA starting 2020 (66). Our

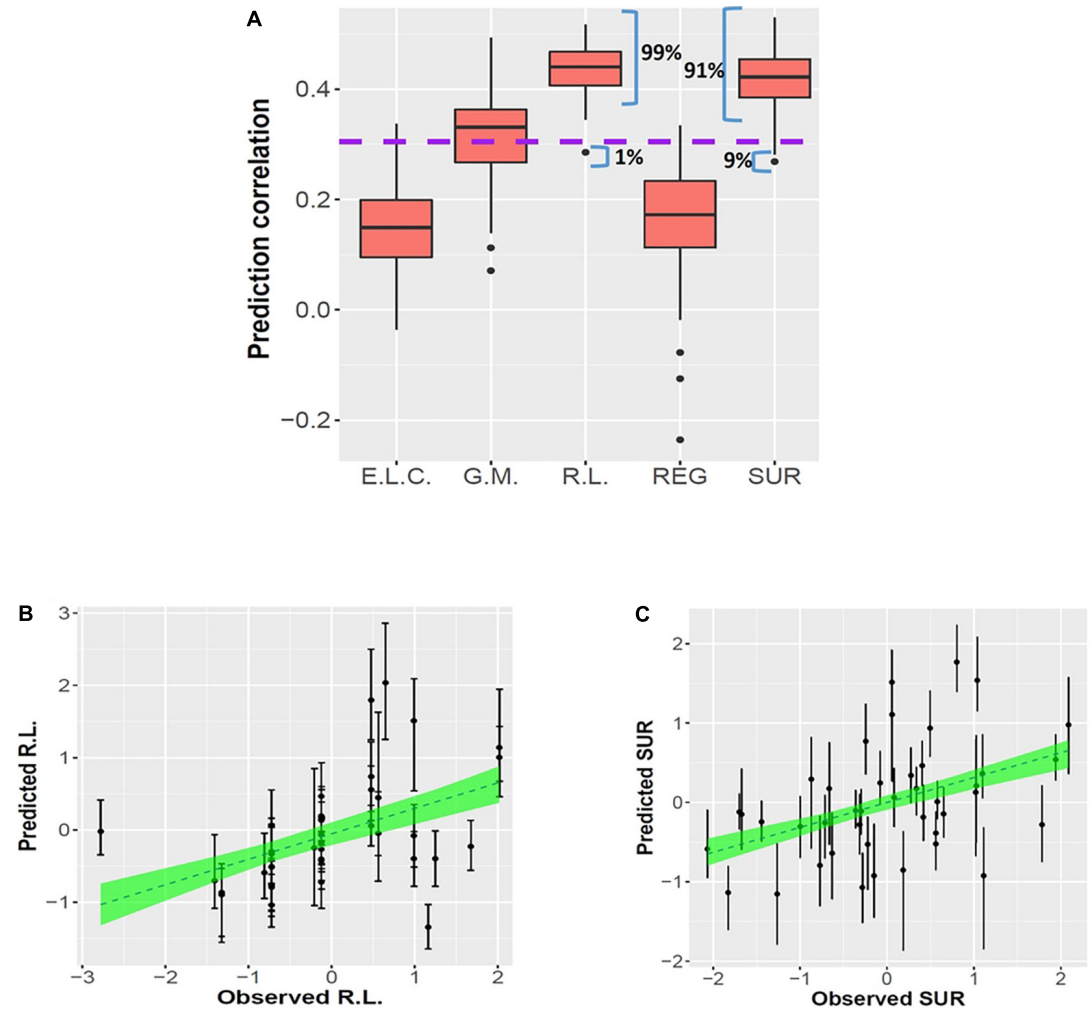


FIGURE 6
Prediction results for linear regression models from 100 random splits of 5-fold CV. Panel **(A)** shows the boxplots of 100 Pearson's correlations between the observed and predicted MSEL scores, including the early learning composite score (E.L.C.), gross motor (G.M.), and receptive language score (R.L.) and IBQ-R factors including the regulation (REG) and surgency (SUR), respectively, based on 100 repetitions of CVs. The purple dashed line represents the significance threshold of the correlation at $\alpha = 0.05$ with $n = 38$ subjects. Panels **(B,C)** show the observed R.L. (left) and SUR (right) vs. the predicted R.L. and SUR of the 100 times' predictions, respectively. The dashed lines and the green areas show the mean and the 95% confidence intervals of the fitted linear slopes between the observed and predicted observations, respectively.

study highlights the importance of ARA, which by itself and jointly with PCho and PI exhibit a significant association with SUR. Nevertheless, future studies with a larger sample size are warranted to determine whether ARA or its metabolites have a distinct role from that of DHA on behavioral development.

Finally, our findings also reinforce the importance of choline (PCho) during the BF period in supporting the developmental needs of the infant (67). While no study has specifically looked at HM choline levels and infant behavior, phosphatidylcholine supplementation during pregnancy has shown to result in fewer attention problems and less social withdrawal at infant age of 40 months by increasing activation of the α7-nicotinic acetylcholine receptor (68), and newborns of mothers with higher choline levels during gestation (and concurrent infection) had improved inhibition of auditory cerebral response, while their infants at 1 year of age had improved development of self-regulation, approaching the level of children of mothers without infection at 1 year of age (69). Equally, phospholipid supplementation in early life (*via* milk fat globule membrane-supplemented formula) was shown to be associated with fewer parent-reported behavioral problems in their children and improved behavioral regulation (70).

The regression models elucidating potential relations between the observed human milk nutrients and cognition/temperament during the first 6 months of life were evaluated on subjects whose follow-up MSEL and IBQ-R were available at 6–18 months of age. Using association analyses, the correlation between the predicted and observed

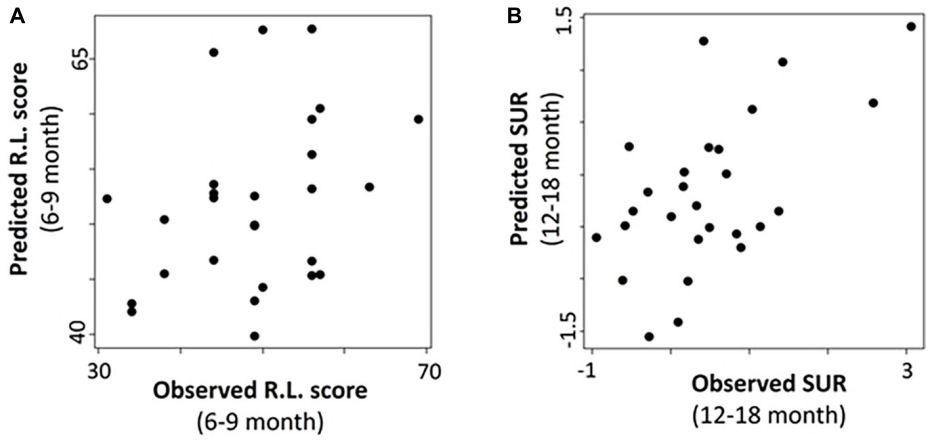


FIGURE 7
Association of the predicted and observed receptive language score (R.L.) and surgency (SUR) at the follow-up ages. **(A)** Predicted vs. observed R.L. at 6–9 months. **(B)** Predicted vs. observed SUR at 12–18 months.

SUR scores was significant at 12–18 months of age (r = 0.58, p = 0.002), and there is a trend toward significance (p = 0.07) between the predicted and observed R.L. at 6–9 months. These results suggest that the MSEL or SUR scores beyond 6 months can be possibly predicted using the derived regression models encompassing the combined effects of human milk nutrients from the first 6 months of life. Although the limited sample sizes for SUR at 6–9 and 9–12 months made it difficult to determine whether the findings observed at 12–18 months are also present during the two age periods, our results appear to suggest that the effects of human milk on temperament persist longer than those for R.L. Specifically, the association of SUR is quite strong at 12–18 months, while the association of R.L. exhibits a trend toward significance at 6–9 months (p = 0.07) but continues decreasing at 9–12 and 12–18 months. It is, however, worth noting that other unobserved confounders could affect the future cognition and temperament, such as type of confounding factors (amount and variety), solid food intakes, and environmental stimulations. As a result, the effects of human milk nutrients that infants received < 6 months on cognition/temperament could diminish with age. Nevertheless, our results suggest that the SUR is less affected by unobserved confounders and thus may be more predictable from baseline. Future studies need to carry out an in-depth investigation to confirm our results.

It should be noted that our studies consisted of two main limitations. First, the limited sample size could lead to biases, including collinearity among the human milk nutrients, overfitting, and instability due to potential outliers. To this end, we carefully carried out several analysis steps to mitigate potential biases. To reduce the multi-collinearity caused by the limited sample size and potential correlations among the nutrients, we used a data-driven approach to classify human milk nutrients into six clusters. Nutrients in different clusters are significantly less correlated compared with those within the same cluster. By selecting only one nutrient from each cluster for the optimal model, we could minimize the collinearity. To reduce potential overfitting caused by the limited sample size, we evaluated the prediction performance using cross-validation. Our results indicated that the predicted and the true MSEL and IBQ-R measures on the test set were significantly correlated. Finally, our results showed that the prediction performance with random alterations between the training and test data based on 100 repetitions of cross-validation was stable and consistently significant, suggesting the robustness against outliers. Second, it was reported that the gestational length could influence DHA and ARA levels among the milk samples, especially for preterm infants (71). In our study, the potential effects of gestational lengths (280.6 ± 6.0 days) on DHA and ARA levels were not considered. Future studies including the effects of gestational lengths on DHA and ARA should be considered.

Conclusion

The development of cognitive and behavioral functioning is complex and likely involves the interplay of social, psychological, and biological factors. Nevertheless, human milk nutrients such as LCPUFAs, choline, and phospholipids may be modifiable contributors to cognitive and behavioral development, especially during the early BF period. Our results provide evidence that specific nutrients in human milk may act together to support cognitive and behavioral traits. However, these findings warrant replication in larger cohorts of BF infants with longitudinal follow-up for more definitive behavioral

phenotyping, by controlling maternal diet and lifestyle, by an in-depth understanding of the mechanisms by which human milk nutrients jointly affect developmental trajectories, and by careful examination of the synergistic effects, if any, of these nutrients on functional outcomes.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, and further inquiries can be directed to the corresponding author.

Ethics statement

All study activities were approved by the Institutional Review Boards of the University of North Carolina at Chapel Hill, Chapel Hill, NC and University of Minnesota, Twin Cities, MN. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

BH, JE, and WL designed the research. KB, BH, HH, JE, and WL conducted the research. TL, TS, ZZ, SC, DW, and WL analyzed the data or performed the statistical analysis. TL, TS, and WL wrote the manuscript. All authors had primary responsibility for the final content and read and approved the final version of the manuscript.

Funding

The sources of support include grants, fellowships, and gifts of materials. This study was supported in part by NIH grants [U01MH110274 (WL and JE); R01MH116527 (TL); R01MH086633 (HZ); and MH104324-03S1 (JE)], MH015755 (BH), and a grant (WL) from Nestlé Product Technology Center-Nutrition, Société des Produits Nestlé S.A., Switzerland. Analysis of choline and choline metabolites was performed by the Metabolism and Metabolomics Core at UNC-Chapel Hill (NIDDK P30DK056350-21). BH was an iTHRIV Scholar. The iTHRIV Scholars Program was supported in part by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Numbers UL1TR003015 and KL2TR003016.

Acknowledgments

We acknowledge Francesca Giuffrida and Isabelle Tavazzi for the analyses of human milk phospholipids and Neotron Spa., Italy for the analyses of fatty acids.

Conflict of interest

TS, JH, and NS were the employees of Société des Produits Nestlé SA, Switzerland. WL was a consultant of and received travel support from Nestlé SA, Switzerland.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.919769/full#supplementary-material

References

1. Hobbs M, Jahan M, Ghorashi SA, Wang B. Current perspective of sialylated milk oligosaccharides in mammalian milk: implications for brain and gut health of newborns. *Foods.* (2021) 10:473. doi: 10.3390/foods10020473

2. Oliveros E, Martín MJ, Torres-Espínola FJ, Segura-Moreno MT, Ramírez M, Santos A, et al. Human milk levels of 2'-fucosyllactose and 6'-sialyllactose are positively associated with infant neurodevelopment and are not impacted by maternal BMI or diabetic status. *J Nutr Food Sci.* (2021) 4:100024.

3. Horta BL, Loret de Mola C, Victora CG. Breastfeeding and intelligence: a systematic review and meta-analysis. *Acta Paediatr.* (2015) 104:14–9. doi: 10.1111/apa.13139

4. Belfort MB, Rifas-Shiman SL, Kleinman KP, Guthrie LB, Bellinger DC, Taveras EM, Gillman MW, et al. Infant feeding and childhood cognition at ages 3 and 7 Years: effects of breastfeeding duration and exclusivity. *JAMA Pediatry.* (2013) 167:836–44. doi: 10.1001/jamapediatrics.2013.455

5. Rochat TJ, Houle B, Stein A, Coovadia H, Coutsoudis A, Desmond C, et al. Exclusive breastfeeding and cognition, executive function, and behavioural disorders in primary school-aged children in rural South Africa: a cohort analysis. *PLoS Med.* (2016) 13:e1002044. doi: 10.1371/journal.pmed.1002044

6. Kotelnikova Y, Mackrell SVM, Jordan PL, Hayden EP. Longitudinal associations between reactive and regulatory temperament traits and depressive symptoms in middle childhood. *J Clin Child Adolesc Psychol.* (2015) 44:775–86. doi: 10.1080/15374416.2014.893517

7. Lauzon-Guillain B, Wijndaele K, Clark M, Acerini CL, Hughes IA, Dunger DB, et al. Breastfeeding and infant temperament at age three months. *PLoS One.* (2012) 7:e29326. doi: 10.1371/journal.pone.0029326

8. Taut C, Kelly A, Zgaga L. The association between infant temperament and breastfeeding duration: a cross-sectional study. *Breastfeed Med.* (2016) 11:111–8. doi: 10.1089/bfm.2015.0184

9. Jonas W, Atkinson L, Steiner M, Meaney MJ, Wazana A, Fleming AS, et al. Breastfeeding and maternal sensitivity predict early infant temperament. *Acta Paediatr.* (2015) 104:678–86. doi: 10.1111/apa.12987

10. Uauy R, Hoffman DR, Mena P, Llanos A, Birch EE. Term infant studies of DHA and ARA supplementation on neurodevelopment: results of randomized controlled trials. *J Pediatr.* (2003) 143(Suppl. 4):S17–25. doi: 10.1067/S0022-3476(03)00398-6

11. Hadley KB, Ryan AS, Forsyth S, Gautier S, Salem N Jr. The essentiality of arachidonic acid in infant development. *Nutrients.* (2016) 8:216. doi: 10.3390/nu8040216

12. Willatts P. Effects of nutrition on the development of higher-order cognition. *Nestle Nutr Inst Workshop Ser.* (2018) 89:175–84. doi: 10.1159/000486501

13. Kotani S, Sakaguchi E, Warashina S, Matsukawa N, Ishikura Y, Kiso Y, et al. Dietary supplementation of arachidonic and docosahexaenoic acids improves cognitive dysfunction. *Neurosci Res.* (2006) 56:159–64. doi: 10.1016/j.neures.2006.06.010

14. Basak S, Mallick R, Banerjee A, Pathak S, Duttaroy AK. Maternal supply of both arachidonic and docosahexaenoic acids is required for optimal neurodevelopment. *Nutrients.* (2021) 13:2061. doi: 10.3390/nu13062061

15. Hahn KE, Dahms I, Butt CM, Salem N Jr, Grimshaw V, Bailey E, et al. Impact of arachidonic and docosahexaenoic acid supplementation on neural and immune development in the young pig. *Front Nutr.* (2020) 7:592364. doi: 10.3389/fnut.2020.592364

16. Nieto-Ruiz A, Garcia-Santos JA, Verdejo-Roman J, Dieguez E, Sepulveda-Valbuena N, Herrmann F, et al. Infant formula supplemented with milk fat globule membrane, long-chain polyunsaturated fatty acids, and synbiotics is associated with neurocognitive function and brain structure of healthy children aged 6 years: the COGNIS study. *Front Nutr.* (2022) 9:820224. doi: 10.3389/fnut.2022.820224

17. Schneider N, Bruchhage MMK, O'Neill BV, Hartweg M, Tanguy J, Steiner P, et al. A nutrient formulation affects developmental myelination in term infants: a randomized clinical trial. *Front Nutr.* (2022) 9:823893. doi: 10.3389/fnut.2022.823893

18. Henriksen C, Haugholt K, Lindgren M, Aurvåg AK, Rønnestad A, Grønn M, et al. Improved cognitive development among preterm infants attributable to early supplementation of human milk with docosahexaenoic acid and arachidonic acid. *Pediatr Am Acad Pediatr.* (2008) 121:1137–45. doi: 10.1542/peds.2007-1511

19. Carlson SE, Werkman SH, Peeples JM, Cooke RJ, Tolley EA. Arachidonic acid status correlates with first year growth in preterm infants. *Proc Natl Acad Sci USA.* (1993) 90:1073–7. doi: 10.1073/pnas.90.3.1073

20. Khan NA, Raine LB, Drollette ES, Scudder MR, Hillman CH. The relation of saturated fats and dietary cholesterol to childhood cognitive flexibility. *Appetite.* (2015) 93:51–6. doi: 10.1016/j.appet.2015.04.012

21. Schverer M, O'Mahony SM, O'Riordan KJ, Donoso F, Roy BL, Stanton C, et al. Dietary phospholipids: role in cognitive processes across the lifespan. *Neurosci Biobehav Rev.* (2020) 111:183–93. doi: 10.1016/j.neubiorev.2020.01.012

22. Bozek K, Wei Y, Yan Z, Liu X, Xiong J, Sugimoto M, et al. Organization and evolution of brain lipidome revealed by large-scale analysis of human, chimpanzee, macaque, and mouse tissues. *Neuron.* (2015) 85:695–702. doi: 10.1016/j.neuron.2015.01.003

23. Norris GH, Jiang C, Ryan J, Porter CM, Blesso CN. Milk sphingomyelin improves lipid metabolism and alters gut microbiota in high fat diet-fed mice. *J Nutr Biochem.* (2016) 30:93–101. doi: 10.1016/j.jnutbio.2015.12.003

24. Caudill MA, Strupp BJ, Muscalu L, Nevins JEH, Canfield RL. Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: a randomized, double-blind, controlled feeding study. *FASEB J.* (2018) 32:2172–80. doi: 10.1096/fj.201700692RR

25. Hahn-Holbrook J, Fish A, Glynn LM. Human milk Omega-3 fatty acid composition is associated with infant temperament. *Nutrients.* (2019) 11:2964. doi: 10.3390/nu11122964

26. Tallima H, El Ridi R. Arachidonic acid: physiological roles and potential health benefits - a review. *J Adv Res.* (2018) 11:33–41. doi: 10.1016/j.jare.2017.11.004

27. Christian P, Smith ER, Lee SE, Vargas AJ, Bremer AA, Raiten DJ. The need to study human milk as a biological system. *Am J Clin Nutr.* (2021) 113:1063–72. doi: 10.1093/ajcn/nqab075

28. Lynch SR. Interaction of iron with other nutrients. *Nutr Rev.* (1997) 55:102–10. doi: 10.1111/j.1753-4887.1997.tb06461.x

29. Cho S, Zhu Z, Li T, Baluyot K, Howell BR, Hazlett HC, et al. Human milk 3'-Sialyllactose is positively associated with language development during infancy. *Am J Clin Nutr.* (2021) 114:588–97. doi: 10.1093/ajcn/nqab103

30. Cruz-Hernandez C, Goeuriot S, Giuffrida F, Thakkar SK, Destaillats F. Direct quantification of fatty acids in human milk by gas chromatography. *J Chromatogr A.* (2013) 1284:174–9. doi: 10.1016/j.chroma.2013.01.094

31. Tavazzi I, Fontannaz P, Lee LY, Giuffrida F. Quantification of glycerophospholipids and sphingomyelin in human milk and infant formula by high performance liquid chromatography coupled with mass spectrometer detector. *J Chromatogr B Analyt Technol Biomed Life Sci.* (2018) 1072:235–43. doi: 10.1016/j.jchromb.2017.10.067

32. Gartstein MA, Rothbart MK. Studying infant temperament via the revised infant behavior questionnaire. *Infant Behav Dev.* (2003) 26:64–86. doi: 10.1016/S0163-6383(02)00169-8

33. Gower JC, Ross GJS. Minimum spanning trees and single linkage cluster analysis. *J R Stat Soc Ser C.* (1969) 18:54–64. doi: 10.2307/2346439

34. Dunn JCA. Fuzzy relative of the ISODATA process and its use in detecting compact well-separated clusters. *J Cybern.* (1973) 3:32–57. doi: 10.1080/01969727308546046

35. Song Q, Ni J, Wang G. A fast clustering-based feature subset selection algorithm for high-dimensional data. *IEEE Trans Knowl Data Eng.* (2013) 25:1–14. doi: 10.1109/TKDE.2011.181

36. Miller AJ. *Subset Selection in Regression*. 2nd ed. Boca Raton, FL: Chapman & Hall/CRC (2002).

37. Zhang Z. Variable selection with stepwise and best subset approaches. *Ann Transl Med.* (2016) 4:136. doi: 10.21037/atm.2016.03.35

38. Thomas L. *Package 'leaps'. Regression subset selection. Thomas Lumley Based on Fortran Code by Alan Miller.* (2013). Available online at: http://CRAN.R-project.org/package=leaps

39. Andreas NJ, Kampmann B, Mehring Le-Doare K. Human breast milk: a review on its composition and bioactivity. *Early Hum Dev.* (2015) 91:629–35. doi: 10.1016/j.earlhumdev.2015.08.013

40. Innis SM. Impact of maternal diet on human milk composition and neurological development of infants. *Am J Clin Nutr.* (2014) 99:734S–41S. doi: 10.3945/ajcn.113.072595

41. Berger PK, Plows JF, Jones RB, Alderete TL, Yonemitsu C, Poulsen M, et al. Human milk oligosaccharide 2'-fucosyllactose links feedings at 1 month to cognitive development at 24 months in infants of normal and overweight mothers. *PLoS One.* (2020) 15:e0228323. doi: 10.1371/journal.pone.0228323

42. Thakkar SK, Giuffrida F, Cristina CH, De Castro CA, Mukherjee R, Tran LA, et al. Dynamics of human milk nutrient composition of women from Singapore with a special focus on lipids. *Am J Hum Biol.* (2013) 25:770–9. doi: 10.1002/ajhb.22446

43. Ma L, Liu X, MacGibbon AK, Rowan A, McJarrow P, Fong BY. Lactational changes in concentration and distribution of ganglioside molecular species in human breast milk from Chinese mothers. *Lipids.* (2015) 50:1145–54. doi: 10.1007/s11745-015-4073-1

44. Ruan C, Liu X, Man H, Ma X, Lu G, Duan G, et al. Milk composition in women from five different regions of China: the great diversity of milk fatty acids. *J Nutr.* (1995) 125:2993–8. doi: 10.1093/jn/125.12.2993

45. Fu Y, Liu X, Zhou B, Jiang AC, Chai L. An updated review of worldwide levels of docosahexaenoic and arachidonic acid in human breast milk by region. *Public Health Nutr.* (2016) 19:2675–87. doi: 10.1017/S1368980016000707

46. Makrides M, Neumann MA, Gibson RA. Effect of maternal docosahexaenoic acid (DHA) supplementation on breast milk composition. *Eur J Clin Nutr.* (1996) 50:352–7.

47. Delplanque B, Gibson R, Koletzko B, Lapillonne A, Strandvik B. Lipid quality in infant nutrition: current knowledge and future opportunities. *J Pediatr Gastroenterol Nutr.* (2015) 61:8–17. doi: 10.1097/MPG.0000000000000818

48. Khor GL, Tan SS, Stoutjesdijk E, Ng KWT, Khouw I, Bragt M, et al. Temporal changes in breast milk fatty acids contents: a case study of malay breastfeeding women. *Nutrients.* (2020) 13:101. doi: 10.3390/nu13010101

49. Martin MA, Lassek WD, Gaulin SJ, Evans RW, Woo JG, Geraghty SR, et al. Fatty acid composition in the mature milk of Bolivian forager-horticulturalists:

controlled comparisons with a US sample. *Matern Child Nutr.* (2012) 8:404–18. doi: 10.1111/j.1740-8709.2012.00412.x

50. Wiedeman AM, Whitfield KC, March KM, Chen NN, Kroeun H, Sokhoing L, et al. Concentrations of water-soluble forms of choline in human milk from lactating women in Canada and Cambodia. *Nutrients.* (2018) 10:381. doi: 10.3390/nu10030381

51. Prado EL, Dewey KG. Nutrition and brain development in early life. *Nutr Rev.* (2014) 72:267–84. doi: 10.1111/nure.12102

52. Silbereis JC, Pochareddy S, Zhu Y, Li M, Sestan N. The cellular and molecular landscapes of the developing human central nervous system. *Neuron.* (2016) 89:248–68. doi: 10.1016/j.neuron.2015.12.008

53. Park HJ, Friston K. Structural and functional brain networks: from connections to cognition. *Science.* (2013) 342:1238411. doi: 10.1126/science.1238411

54. Li F, Wu SS, Berseth CL, Harris CL, Richards JD, Wampler JL, et al. Improved neurodevelopmental outcomes associated with bovine milk fat globule membrane and lactoferrin in infant formula: a randomized, controlled trial. *J Pediatr.* (2019) 215:24–31.e8. doi: 10.1016/j.jpeds.2019.08.030

55. Brink LR, Lonnerdal B. Milk fat globule membrane: the role of its various components in infant health and development. *J Nutr Biochem.* (2020) 85:108465. doi: 10.1016/j.jnutbio.2020.108465

56. Gawlik NR, Anderson AJ, Makrides M, Kettler L, Gould JF. The influence of DHA on language development: a review of randomized controlled trials of DHA supplementation in pregnancy, the neonatal period, and infancy. *Nutrients.* (2020) 12:3106. doi: 10.3390/nu12103106

57. Ramos T, Méio MDBB, Morsch DS, Moreira MEL, Carmo MDGTD, Sichieri R, et al. Preterm infant language development: a role for breast milk fatty acids. *J Hum Growth Dev.* (2013) 23:270–5. doi: 10.7322/jhgd.69499

58. Bernard JY, De Agostini M, Forhan A, de Lauzon-Guillain B, Charles MA. The dietary n6:n3 fatty acid ratio during pregnancy is inversely associated with child neurodevelopment in the EDEN mother-child cohort. *J Nutr.* (2013) 143:1481–8. doi: 10.3945/jn.113.178640

59. Kim H, Kim H, Lee E, Kim Y, Ha EH, Chang N. Association between maternal intake of n-6 to n-3 fatty acid ratio during pregnancy and infant neurodevelopment at 6 months of age: results of the MOCEH cohort study. *Nutr J.* (2017) 16:23. doi: 10.1186/s12937-017-0242-9

60. Kris-Etherton PM, Innis S, Ammerican Dietetic Assocition, Dietitians of Canada. Position of the American dietetic association and dietitians of Canada: dietary fatty acids. *J Am Diet Assoc.* (2007) 107:1599–611. doi: 10.1016/j.jada.2007.07.024

61. Stanley JC, Elsom RL, Calder PC, Griffin BA, Harris WS, Jebb SA, et al. UK food standards agency workshop report: the effects of the dietary n-6:n-3 fatty acid ratio on cardiovascular health. *Br J Nutr.* (2007) 98:1305–10. doi: 10.1017/S000711450784284X

62. Rump P, Hornstra G. Relation between arachidonic acid and docosahexaenoic acid in maternal and neonatal blood. *Eur J Clin Nutr.* (2001) 55:916–7. doi: 10.1038/sj.ejcn.1601226

63. Colombo J, Jill Shaddy D, Kerling EH, Gustafson KM, Carlson SE. Docosahexaenoic acid (DHA) and arachidonic acid (ARA) balance in developmental outcomes. *Prostaglandins Leukot Essent Fatty Acids.* (2017) 121:52–6. doi: 10.1016/j.plefa.2017.05.005

64. Carlson SE, Colombo J. Docosahexaenoic acid and arachidonic acid nutrition in early development. *Adv Pediatr.* (2016) 63:453–71. doi: 10.1016/j.yapd.2016.04.011

65. Shiner RL, Buss KA, McClowry SG, Putnam SP, Saudino KJ, Zentner M. What is temperament now? Assessing progress in temperament research on the twenty-fifth anniversary of Goldsmith et al. (1987). *Child Dev Perspect.* (2012) 6:436–44. doi: 10.1111/j.1750-8606.2012.00254.x

66. Koletzko B, Bergmann K, Brenna JT, Calder PC, Campoy C, Clandinin MT, et al. Should formula for infants provide arachidonic acid along with DHA? A position paper of the European academy of paediatrics and the child health foundation. *Am J Clin Nutr.* (2020) 111:10–6. doi: 10.1093/ajcn/nqz252

67. Caudill MA, Obeid R, Derbyshire E, Bernhard W, Lapid K, Walker SJ, et al. Building better babies: should choline supplementation be recommended for pregnant and lactating mothers? Literature overview and expert panel consensus. *Gynecol Obstet.* (2020) 2:149–61.

68. Ross RG, Hunter SK, Hoffman MC, McCarthy L, Chambers BM, Law AJ, et al. Perinatal phosphatidylcholine supplementation and early childhood behavior problems: evidence for CHRNA7 moderation. *Am J Psychiatry.* (2016) 173:509–16. doi: 10.1176/appi.ajp.2015.15091188

69. Freedman R, Hunter SK, Law AJ, Wagner BD, D'Alessandro A, Christians U, et al. Higher gestational choline levels in maternal infection are protective for infant brain development. *J Pediatr.* (2019) 208:198–206.e2. doi: 10.1016/j.jpeds.2018.12.010

70. Veereman-Wauters G, Staelens S, Rombaut R, Dewettinck K, Deboutte D, Brummer RJ, et al. Milk fat globule membrane (INPULSE) enriched formula milk decreases febrile episodes and may improve behavioral regulation in young children. *Nutrition.* (2012) 28:749–52. doi: 10.1016/j.nut.2011.10.011

71. Beijers RJ, Schaafsma A. Long-chain polyunsaturated fatty acid content in Dutch preterm breast milk; differences in the concentrations of docosahexaenoic acid and arachidonic acid due to length of gestation. *Early Hum Dev.* (1996) 44:215–23. doi: 10.1016/0378-3782(95)01710-0

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 08 September 2022
DOI 10.3389/fnut.2022.907526

Check for updates

OPEN ACCESS

EDITED BY
Andrew Scholey,
Swinburne University of Technology, Australia

REVIEWED BY
Flavia Antonucci,
University of Milan, Italy
Isis Trujillo-Gonzalez,
University of North Carolina at Chapel Hill, United States

*CORRESPONDENCE
Hui Ren
renhuispine@163.com
Xiaobing Jiang
spinedrjxb@sina.com
Gengyang Shen
15920198161@163.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

RECEIVED 29 March 2022
ACCEPTED 19 August 2022
PUBLISHED 08 September 2022

CITATION
Shang Q, Chen G, Zhang P, Zhao W, Chen H, Yu D, Yu F, Liu H, Zhang X, He J, Yu X, Zhang Z, Tan R, Wu Z, Tang J, Liang D, Shen G, Jiang X and Ren H (2022) Myristic acid alleviates hippocampal aging correlated with GABAergic signaling. *Front. Nutr.* 9:907526. doi: 10.3389/fnut.2022.907526

COPYRIGHT
© 2022 Shang, Chen, Zhang, Zhao, Chen, Yu, Yu, Liu, Zhang, He, Yu, Zhang, Tan, Wu, Tang, Liang, Shen, Jiang and Ren. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Myristic acid alleviates hippocampal aging correlated with GABAergic signaling

Qi Shang[1,2†], Guifeng Chen[1,2†], Peng Zhang[1,2†], Wenhua Zhao[1,2], Honglin Chen[1,2], Die Yu[2,3], Fuyong Yu[1,2], Huiwen Liu[1,2], Xuelai Zhang[2,3], Jiahui He[1,2], Xiang Yu[2,3], Zhida Zhang[2,3], Riwei Tan[1,2], Zixian Wu[1,2], Jingjing Tang[2,3], De Liang[2,3], Gengyang Shen[2,3,4*], Xiaobing Jiang[2,3*] and Hui Ren[2,3*]

[1]Guangzhou University of Chinese Medicine, Guangzhou, China, [2]Lingnan Medical Research Center of Guangzhou University of Chinese Medicine, Guangzhou, China, [3]The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China, [4]Nanshan Hospital, The First Affiliated Hospital of Guangzhou University of Chinese Medicine (Shenzhen Nanshan Hospital of Chinese Medicine), Guangzhou, China

Previous studies have shown that myristic acid (MA), a saturated fatty acid, could promote the proliferation and differentiation of neural stem cells *in vitro*. However, the effect of MA on hippocampal neurons aging has not been reported *in vivo*. Here we employed 22-month-old naturally aged C57BL/6 mice to evaluate the effect and mechanism of MA on hippocampal aging. First, we examined a decreased exploration and spatial memory ability in aging mice using the open field test and Morris water maze. Consistently, aging mice showed degenerative hippocampal histomorphology by H&E and Nissl staining. In terms of mechanism, imbalance of GABRB2 and GABRA2 expression in aging mice might be involved in hippocampus aging by mRNA high throughput sequencing (mRNA-seq) and immunohistochemistry (IHC) validation. Then, we revealed that MA alleviated the damage of exploration and spatial memory ability and ameliorated degeneration and aging of hippocampal neurons. Meanwhile, MA downregulated GABRB2 and upregulated GABRA2 expression, indicating MA might alleviate hippocampal aging correlated with GABAergic signaling. In conclusion, our findings revealed MA alleviated hippocampal aging correlated with GABAergic signaling, which might provide insight into the treatment of aging-associated diseases.

KEYWORDS

myristic acid, hippocampus, aging, GABAergic expressions, GABRB2, GABRA2

Introduction

With the rapid increase in the global aging population, aging-related diseases have brought a huge burden to patients, families, and socioeconomics. It is imminent to explore the pathogenesis and therapeutic drugs of aging. The hippocampus is an important part of the limbic system of the brain and contains various messenger receptors (1). It is not only a high-level regulatory center of neuroendocrine system but

also the most sensitive area (1, 2). Studies have confirmed that hippocampus function is closely related to aging (3, 4). The decline of hippocampal neurons is the most pronounced change with age (5). These changes in hippocampus are closely related to age-related exploration and spatial memory impairment as well as an imbalance of hippocampal messenger receptors (6, 7). Therefore, a more in-depth understanding of hippocampus aging is essential for the improvement of treatment and prevention of aging-associated diseases.

GABAergic neural circuits play an important role in regulating hippocampal neuronal synaptic plasticity, memory, and cognitive function (8). GABAergic interneurons synthesize and release the neurotransmitter γ-aminobutyric acid (GABA) and contribute substantially to the regulation of the adult neuronal network (8). Generally, GABA exerts its effects on nerves mainly through its receptors; GABRA2 (GABAA receptor subunit alpha2) and GABRB2 (GABAA receptor subunit beta2) are the two important subunits of GABAA receptor, respectively. Previous studies demonstrated that GABRA2 expression decreased with age in the hippocampus (9, 10). However, aging-related disorders on GABRB2 expression are contradictory and need further experimental validation.

Previous studies have shown that myristic acid (hereinafter referred to as MA, IUPAC name: tetradecanoic acid, molecular formula: $CH_3(CH_2)_{12}COOH$, CAS#: 544-63-8), a saturated fatty acid, mainly present in cardamom oil, coconut oil, palm kernel oil, butterfat, and bovine milk, could be used to prepare a variety of foods (11, 12). MA could promote the proliferation and differentiation of neural stem cells *in vitro* (13). Besides, aging was accompanied by a decline in MA, indicating MA played an important role in aging-related disorders (14). The isomer of MA, Myristoleic acid, could suppress osteoclastogenesis and bone resorption by inhibiting RANKL activation, indicating MA had a potential therapeutic effect on bone aging-related diseases, such as osteoporosis and osteoarthritis (15). However, the effect of MA on hippocampal neurons aging has not been reported *in vivo*. Here we employed 22-month-old naturally aged C57BL/6 mice to evaluate the effect and mechanism of MA on hippocampal aging. Our investigations indicated that MA alleviated hippocampal aging correlated with GABAergic signaling, which may provide insight into the treatment of aging-associated diseases.

Materials and methods

Mice

C57BL/6 male mice aged 3 and 22 months were obtained from the Laboratory Animal Center of Guangzhou University of Chinese Medicine (GZUCM) (Animal production certificate#(Yue)20180034). Mice had *ad libitum* access to tap water and standard rodent chow (Product performance standard: GB14924.3-2010, Laboratory Animal Center of GZUCM). All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of GZUCM (approval no. TCMF1-2021026). Firstly, the mice were divided into two groups: 3-month-old mice (3M) and 22-month-old mice (22M) group to assess the success of natural aging murine models. Secondly, another batch of 22-month-old mice was randomly divided into two groups: 22-month-old mice (22M, equal amount of solvent intraperitoneal injection) and MA (MA+22M, 2mg/kg·d MA intraperitoneal injection) group. They were randomized to each group after matching for age and body mass. Drugs were administered for 2 months by intraperitoneal injection. The concentration of MA was referred to a previous study (15). Following the behavioral assessment (open field test and Morris water maze), samples from murine brains were used for subsequent experimental assessments.

Open field test

The operation of open field test process was in accordance with a previous study which was used for assessing murine exploratory behavior (16). Generally, the exploration ability of aging mice decreased (17). Briefly, mice were transported to the laboratory room 2 h in advance to adapt to the environment before the experiment. The open field reaction box for C57BL/6 was a 50 × 50 cm open field chamber. The open-field chamber was divided into nine grids. The central area is defined as the center 1/9 area of open-field chamber bottom, and the remaining area is defined as the peripheral area. The longer time the mice spent in the center, longer distance the mice across the center and more times the mice across the center in the open-field chamber, the better the mouse's ability to explore (17). Wipe the bottom of open-field chamber with 75% alcohol. Gently remove the animals from the cage, place them quickly in the open-field chamber. Set up the parameters in SuperMaze software (Version 3.0, Shanghai XinRuan Information Technology Co., Ltd) and automatically record the animal activities in the box for 5 min. After one mouse was finished, the bottom of the box was wiped with 75% alcohol to remove the odor, and the next mouse was tested with the same method before. The parameters including total distance traveled by the mice (Total Distance), mice quiescent time (Quiescent Time), distance in the central region (Distance Center), number of central region entries (Number of Center), central region staying time (Time Center), distance in the periphery region (Distance Periphery), number of periphery region entries (Number of Periphery), Periphery region staying time (Time Periphery), the ratio of Distance Center vs. Distance Periphery and Time Center vs. Time Periphery were evaluated.

Morris water maze

Morris water maze was employed to assess the mouse spatial memory (6). After the open field test, the process of the Morris water maze was operated next day. Mice were placed into the pool from four quadrants facing the pool wall, and the time required by the mice from entering the water to finding and standing on the underwater concealed platform was recorded as the escape latency with SuperMaze software (Version 3.0, Shanghai XinRuan Information Technology Co., Ltd). If the mice could not find the platform 60 s after entering the water, the mice were guided to stay on the platform for 10 s. This was a positioning navigation test of mice, which was four quadrants a day for a total of four days. On the fifth day, a space exploration test was conducted. The platform was removed and the mice were placed from the opposite quadrant of the platform. The swimming time of the mice lasted 60 s. Each mouse was put into the pool from four water entry points for one training, and the interval between the two training was at least 30 min. The quadrants of each day's training were randomly arranged. The parameters including Escape Latency and Platform crossover number were recorded.

HE staining and Nissl staining

The murine cerebral hemispheres and organs (Heart, liver, spleen, lung, kidney and testis) were removed completely on ice, fixed with 4% paraformaldehyde for 48 h, then embedded in paraffin. The hemispheres were cut to the hippocampus zone at the midsagittal plane of 5 μm, and stained with H&E staining solution (Servicebio, G1005, Wuhan) and Nissl staining solution (Servicebio, G1036, Wuhan) respectively. The organs including heart, liver, spleen, lung, kidney and testis were cut into 5 μm slices, and stained with H&E staining solution (Servicebio, G1005, Wuhan). The H&E and Nissl staining of sections were intelligently photographed by Digital Pathology Scanning System (3D HISTEC Ltd., Pannoramic MIDI). The rate of Nissl-stained cells and neurodegeneration of hippocampus were calculated using Image J 1.51 analysis software (Wayne Rasband, National Institutes of Health, USA). The rate of Neurodegeneration was defined as the ratio of pathological neuronal cells to total cells in the entire hippocampus; Neuronal pathological changes included disarrangement of neurons, nuclear shrinkage, and dark staining of neuronal cells (18). Each group had three biological replicates; each mouse was randomly selected two non-overlapping visual fields in each partial of hippocampal CA1, CA3 and DG area under high magnification microscope (400×) of each slice, and counted the mean rate of Nissl-stained cells and neurodegeneration.

mRNA-seq of hippocampus

The total RNA was extracted from young (3M) and aging (22M) hippocampus samples in mice by using Trizol reagents (Invitrogen). The extracted total RNA samples were subjected to agarose electrophoresis and Nanodrop for quality inspection and quantification. Oligo magnetic beads were used to enrich the mRNA, and the KAPA Stranded RNA-Seq Library Prep Kit (Illumina, Aksomics, Shanghai) was used to construct the library. The constructed library was checked by Agilent 2100 Bioanalyzer (Aksomics, Shanghai), and the final quantification of the library was performed by qPCR. According to the quantitative results and the final sequencing data, the sequencing libraries of different samples were mixed into the sequencing process. The screening thresholds to determine the significant differential expression (DE) genes were $P < 0.05$ and $|\log2 \text{ fold change (FC)}| > 0.585$ (19). The normalized data of each group were analyzed by hierarchical clustering. The color green represented low expression, whereas red represented high expression, to investigate the possible mechanism of the upregulated and downregulated DE genes involved in the process of hippocampal aging. The raw data of mRNA-seq of hippocampus were uploaded to GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201029).

KEGG analysis

The differentially mRNAs expressions from mRNA-seq data were employed for KEGG analysis by using the KEGG database. The upregulated and downregulated mRNAs were uploaded into the KEGG database. The bar plot showed the top in front enrichment score value of the significant enrichment pathway in terms of the value of -log10 (*P*-value).

Immumohistochemical staining (IHC)

IHC was used to detect the GABRB2, GABRA2, P16, and P21 protein expression in the brain. The brain tissue sections were dewaxed, antigen repaired, and sealed successively. GABRB2 (Bioss Cat# BS-12065R, Rabbit, 1:200), GABRA2 (Affinity Biosciences Cat# DF6626, RRID: AB_2838588, Rabbit, 1:100), P16 (Affinity Biosciences Cat# AF0228, RRID: AB_2833403, Rabbit, 1:200) and P21 (Beyotime Cat#AP021, 1:200) antibodies were added respectively and incubated at 4°C overnight. Then Goat anti-rabbit or mouse IgG antibodies were added on brain tissue sections and incubated at room temperature for 1 h. After conventional DAB staining, the sections were stained with hematoxylin and sealed. IHC of brain sections for the whole film scanning was intelligently photographed by Digital Pathology Scanning System (3D HISTEC Ltd., Pannoramic MIDI). Image J 1.51 analysis software (Wayne Rasband, National Institutes

of Health, USA) was used to calculate the relative protein expressions. Each group had three biological replicates; each mouse was randomly selected two non-overlapping visual fields in each partial of hippocampal CA1, CA3 and DG area under high magnification microscope (400×) of each slice, and counted the mean optical density, and then normalized by the folds of experimental group to control group.

Immunofluorescence (IF)

The paraffin sections of hippocampal tissue were deparaffinized with xylene, anhydrous ethanol, 85% alcohol and 75% alcohol, respectively. The tissue sections were placed in citric acid buffer (pH 6.0) in a microwave oven for antigen retrieval. Then the primary antibodies including GABRB2 (Bioss Cat# BS-12065R, Rabbit, 1:200), GABRA2 (Affinity Biosciences Cat# DF6626, RRID: AB_2838588, Rabbit, 1:100), Sox2 (Bioss Cat#BS-0523R, 1:200), Nestin (Proteintech Cat#19483-1-AP, 1:100) were placed in a humid box and incubated overnight at 4°C. Next, the secondary antibody Alexa Fluor 488 AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson Cat#111-545-003, 1:200) was incubated at room temperature for 50 min in the dark. Nuclei were counterstained with DAPI (Solarbio, C0060, 1:100) and incubated for 10 min at room temperature in the dark. After tissue sections drying, the sections were mounted with anti-fluorescence quenching mounting medium. IF of hippocampus sections for the whole film scanning was intelligently photographed by Digital Pathology Scanning System (3D HISTEC Ltd., Pannoramic MIDI). Image J 1.51 analysis software (Wayne Rasband, National Institutes of Health, USA) was used to calculate the relative protein expressions. Each group had three biological replicates; each mouse was randomly selected two non-overlapping visual fields in the hippocampal CA3 area under high magnification microscope (400×) of each slice, and counted the average fluorescence intensity, and then normalized by the folds of experimental group to control group.

Statistical analysis

All data were checked for normality and homogeneity of variance. If two independent sample data fit a normal distribution, the results are expressed as means ± standard deviations (SDs) and two-tailed Student's *t*-test was used. If two independent sample data do not conform to a normal distribution, the Mann-Whitney U test was employed and a box plot was used. Two-way analysis of variance (ANOVA) following Bonferroni's multiple comparisons test was employed to analyze the Morris water maze data. $P < 0.05$ was considered statistically different. Statistical analysis was performed using GraphPad Prism version 8.4.3 for Windows (GraphPad Software, San Diego, California, USA).

Results

Aging mice displayed decreased exploration and spatial memory ability

To evaluate the exploration ability and spatial memory ability of aging mice, we normally raised 3-month-old mice (young) and 22-month-old mice (aged), and evaluated behavioral memory using the open field test and Morris water maze. In the open field test, aging mice displayed impaired exploration ability, including decreased Total Distance ($P<0.05$), Time Center ($P<0.05$), Distance Periphery ($P<0.05$), Number of Periphery ($P<0.05$), and Time Center vs Time Periphery ($P<0.05$), while increased Quiescent Time ($P<0.01$) and Time Periphery ($P<0.01$); and a slight downward trend in Distance Center, Number of Center and Distance Center vs. Distance Periphery but no statistical differences (Figure 1A). Morris water maze were used to evaluate the hippocampus-dependent spatial memory. In Morris Water Maze Test, aging mice witnessed extended escape latency in the navigation test (Two-way ANOVA, $P<0.01$ in 3d and 4d) and reduced platform crossover number in the space exploration test (Mann-Whitney U test, $P<0.05$), compared with young mice (Figures 1B–D). This showed that the exploratory ability and spatial memory ability of aging mice decreased with age.

Aging mice showed degeneration and aging of hippocampal neurons

To more intuitively reflect the pathological manifestations of the hippocampal neurons of aging mice, the CA1, CA3, and DG regions of hippocampus were assessed by HE staining and Nissl staining (Figure 2A). The nerve cells of the young group had intact cell structure, clear round shape, and were arranged compactly and neatly; while the nerve cells of aging mice in hippocampus were disordered and the granules of Nissl bodies were blurred (Figure 2A). Accordingly, quantitative analysis revealed that the number of Nissl bodies decreased ($P<0.001$), while the rate of neurodegeneration increased significantly ($P<0.001$) in the hippocampal neurons of aging mice than that of young mice (Figures 2B,C). The expressions of senescence markers p21 and p16 protein detected by IHC in aging murine hippocampal neurons was higher ($P<0.05$) than those of young mice, implying that aging murine hippocampus showed the characteristics of aging (Figures 2D–F).

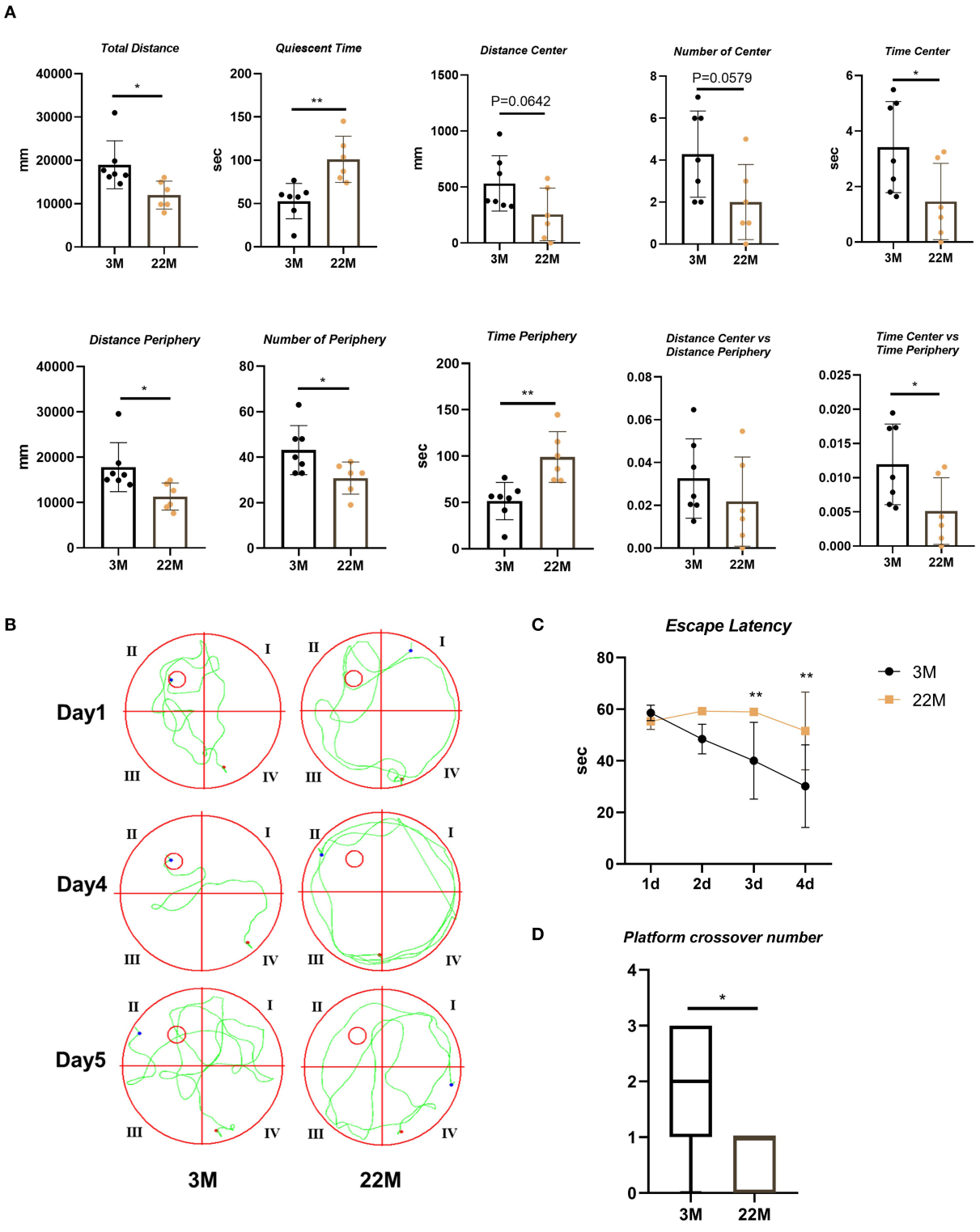


FIGURE 1
Evaluation of exploration and spatial memory ability in aging mice. **(A)** The parameters of open field test between young (3M) and aging mice (22M) group. Data were represented as mean ± SD and were analyzed by Student *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$. **(B)** The locus diagrams of Morris water maze. Day 1th to Day 4th, Navigation test; Day 5th, Space exploration test. **(C)** Escape Latency in the navigation test of Morris water maze. Data were represented as mean ± SD and were analyzed by two-way analysis of variance (ANOVA) following Bonferroni's multiple comparisons test. $^{**}P < 0.01$. **(D)** Platform crossover number in the space exploration test of Morris water maze. Mann-Whitney *U* test was employed and a box plot was used. $^{*}P < 0.05$.

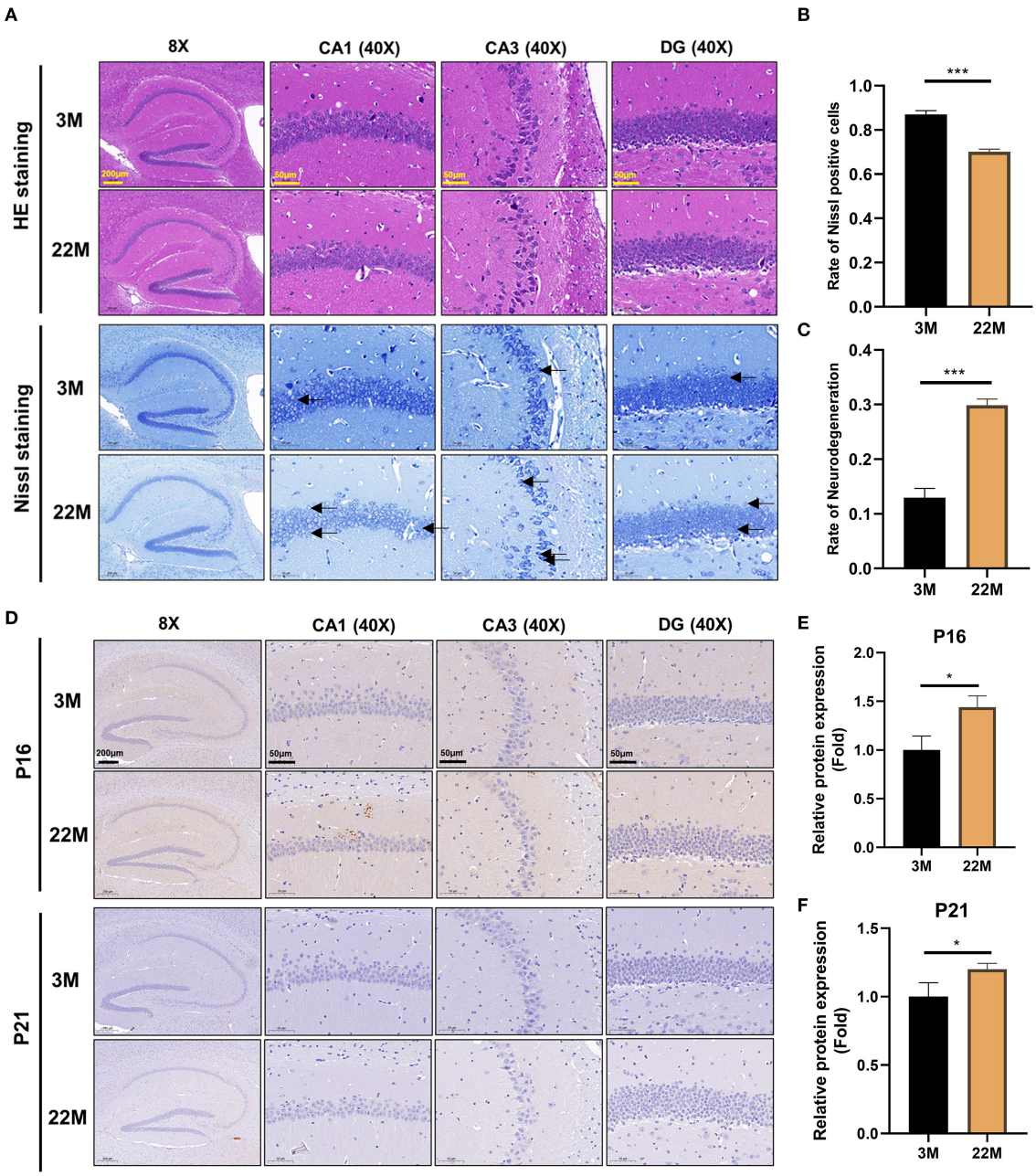


FIGURE 2
Evaluation of hippocampal histomorphology in aging mice. **(A)** H&E and Nissl staining in the hippocampus between 3M and 22M group. The magnifications and scale bars were shown in **(A)**. **(B,C)** Nissl positive cells and Neurodegeneration rate in the hippocampus were assessed by Nissl staining. Data were represented as mean ± SD and were analyzed by Student *t*-test. ***$P < 0.001$. Arrows showed degenerated neurons. **(D)** IHC of senescence markers P16 and P21 in the hippocampus. The magnifications and scale bars were shown in **(D)**. **(E,F)** Relative expressions of senescence markers P16 and P21 were calculated by IHC. Data were represented as mean ± SD and were analyzed by Student *t*-test. *$P < 0.05$.

mRNA-seq and IHC of hippocampus indicated the imbalance of GABRB2 and GABRA2 expression in aging mice

To further explore the mechanism of hippocampus aging in mice, we employed mRNA-seq to screen for possible genes and pathways in the young (3M) and aging (22M) hippocampus. The heat map displayed up-regulated and down-regulated gene sets (Figure 3A). KEGG pathway enrichment analysis found that GABAergic synapse was involved in both up-regulated and down-regulated signaling pathways (Figure 3B). We further analyzed the differential expression genes and

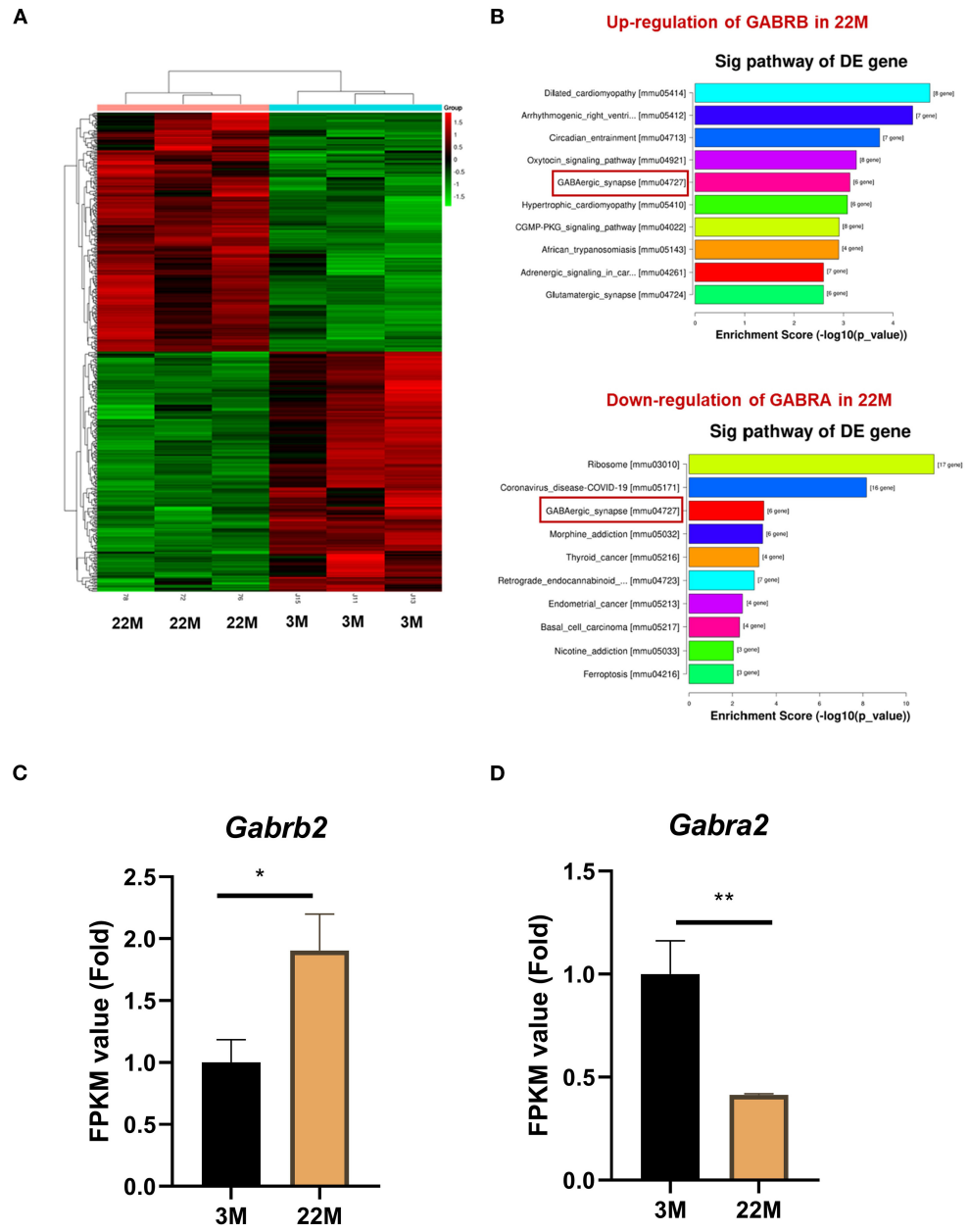


FIGURE 3
GABRB2 and GABRA2 expression were evaluated by mRNA-seq of hippocampus in aging mice. **(A)** Heat map of hippocampus between 3M and 22M group. **(B)** KEGG analysis of the hippocampus between 3M and 22M group. **(C,D)** FPKM value of GABRB2 and GABRA2 in the hippocampus by mRNA-seq. Data were represented as mean ± SD and were analyzed by Student *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$.

found that GABRB2 ($P<0.05$) was up-regulated in the aging hippocampus whereas GABRA2 ($P<0.01$) was down-regulated in the aging hippocampus, indicating an imbalance of GABRB2 and GABRA2 expressions in aging mice (Figures 3C,D). We then employed hippocampal immunohistochemistry to further validate the GABRB2 and GABRA2 protein expressions in aging mice. Consistent with the mRNA-seq results, we verified that GABRB2 protein expression ($P<0.01$) was increased in the aging hippocampus whereas GABRA2 protein expression ($P<0.01$) was decreased in the aging hippocampus (Figures 4A–C). This study laid a foundation for further study on the mechanism of MA in improving hippocampus aging.

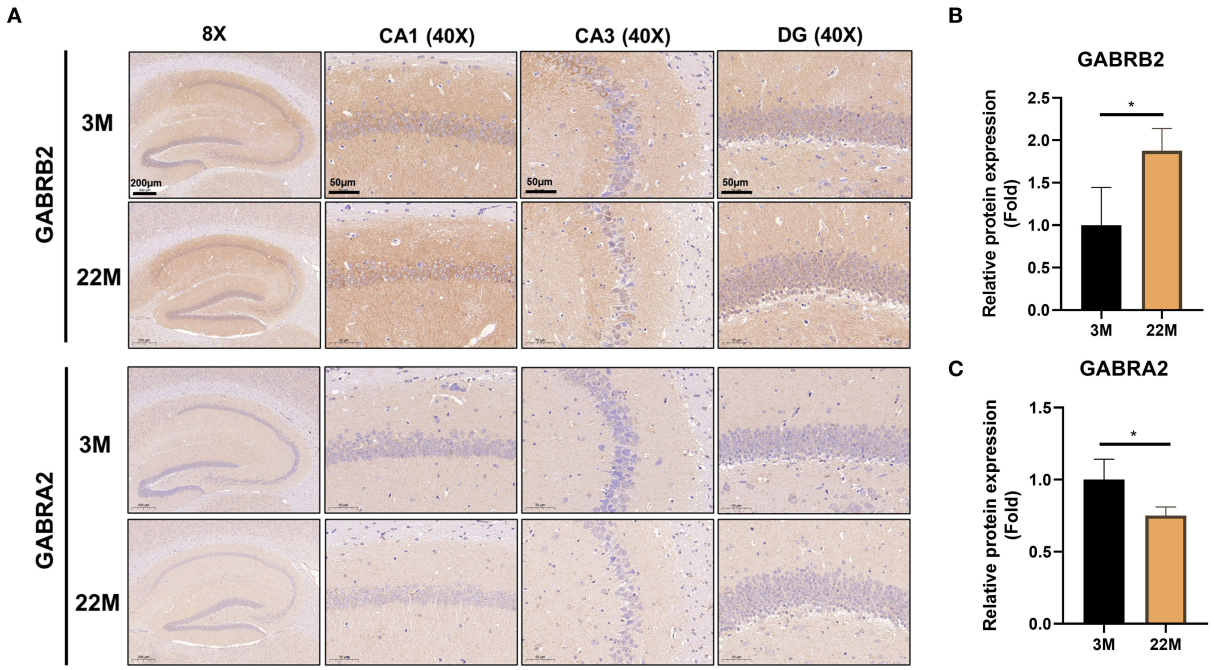


FIGURE 4
Validation of GABRB2 and GABRA2 expression by IHC of hippocampus in aging mice. **(A)** IHC of GABRB2 and GABRA2 in the hippocampus between 3M and 22M group. The magnifications and scale bars were shown in **(A)**. **(B,C)** Relative expressions of GABRB2 and GABRA2 were calculated by IHC. Data were represented as mean ± SD and were analyzed by Student *t*-test. $^*P < 0.05$.

MA alleviated the damage of exploration and spatial memory ability in aging mice

MA, a saturated fatty acid, was a promising anti-aging effective constituent in previous studies. First, we evaluated the safety of MA treatment on aging mice. Using HE staining, we found that MA could not lead to damage to organs, including heart, liver, spleen, lung, kidney, and testis (Supplementary Figure 1). Expectedly, MA alleviated hippocampal aging to some extent. In the open field test, we examined an improved exploration ability in aging mice, including increased Total Distance ($P<0.05$), Number of Center ($P<0.05$), Time Center ($P<0.05$), and Time Center vs Time Periphery ($P<0.05$); and decreased Quiescent Time ($P<0.05$); and an upward trend in Distance center and Distance Center vs Distance Periphery and a downward trend in Time Periphery (Figure 5A). In Morris water maze, we observed a reduced escape latency in the navigation test (Two-way ANOVA, $P<0.01$ in 3d and $P<0.001$ in 4d) and increased platform crossover number (Mann-Whitney U test, $P<0.05$) in the space exploration test, indicating MA improved hippocampus-dependent spatial memory (Figures 5B–D).

MA ameliorated degeneration and aging of hippocampal neurons

We further used H&E and Nissl staining to demonstrate the effect of MA on hippocampal histomorphology. We found an improvement in the disorder of hippocampal neurons and clearer Nissl bodies in MA-treated aging mice (Figure 6A). Accordingly, our quantitative results also showed that the rate of Nissl positive cells was increased ($P<0.01$) and the rate of neurodegeneration was decreased ($P<0.01$) (Figures 6B,C). Furthermore, hippocampal immunofluorescence of nerve regeneration marker Nestin demonstrated that MA increased Nestin protein expression ($P<0.05$); an increased trend of Sox2, another nerve regeneration marker, was observed in MA-treated aging hippocampus (Supplementary Figures 2A–D). IHC of Classic aging marker P16 also displayed that MA-treated aging hippocampus reduced the aging marker P16 protein expression ($P<0.01$). There was a decreasing trend of P21 protein expression in the aging hippocampus, although there was no statistical difference (Figures 6D–F). We also explored the effect of MA on cerebral cortex neurons and found that MA could alleviate neurodegeneration of cerebral cortex and reduce aging marker P16 expression (Supplementary Figures 3A–D).

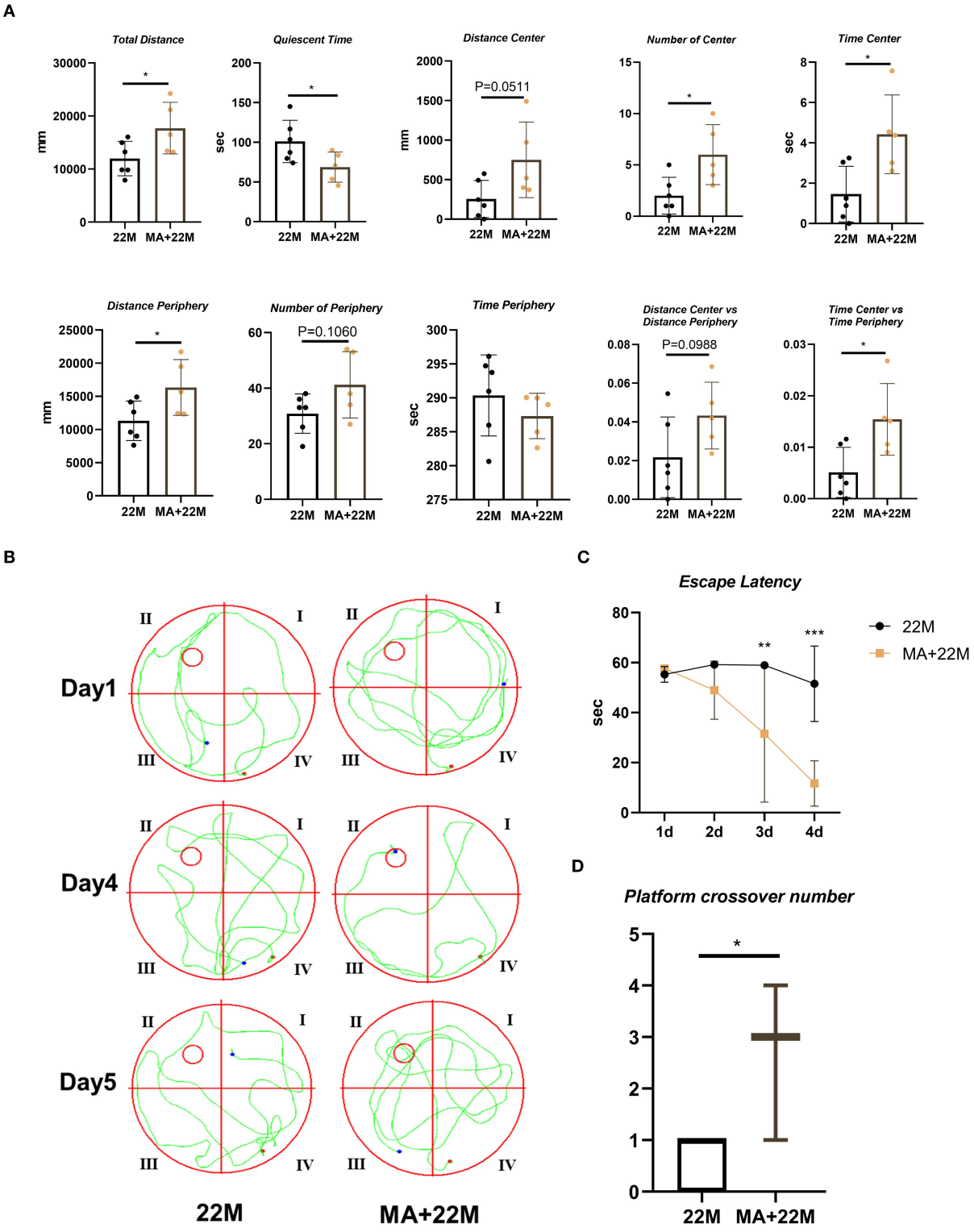


FIGURE 5
Evaluation of exploration and spatial memory ability in MA-treated aging mice. **(A)** The parameters of open field test between aging mice (22M) and MA-treated aging mice (MA+22M) group. Data were represented as mean ± SD and were analyzed by Student *t*-test. $^{*}P < 0.05$. **(B)** The locus diagrams of Morris water maze between 22M and MA+22M group. Day 1th and Day 4th, Navigation test; Day 5th, Space exploration test. **(C)** Escape Latency in the navigation test of Morris water maze between 22M and MA+22M group. Data were represented as mean ± SD and were analyzed by two-way ANOVA following Bonferroni's multiple comparisons test. $^{**}P < 0.01$ and $^{***}P < 0.001$. **(D)** Platform crossover number in the space exploration test of Morris water maze. Mann-Whitney *U* test was employed and a box plot was used. $^{*}P < 0.05$.

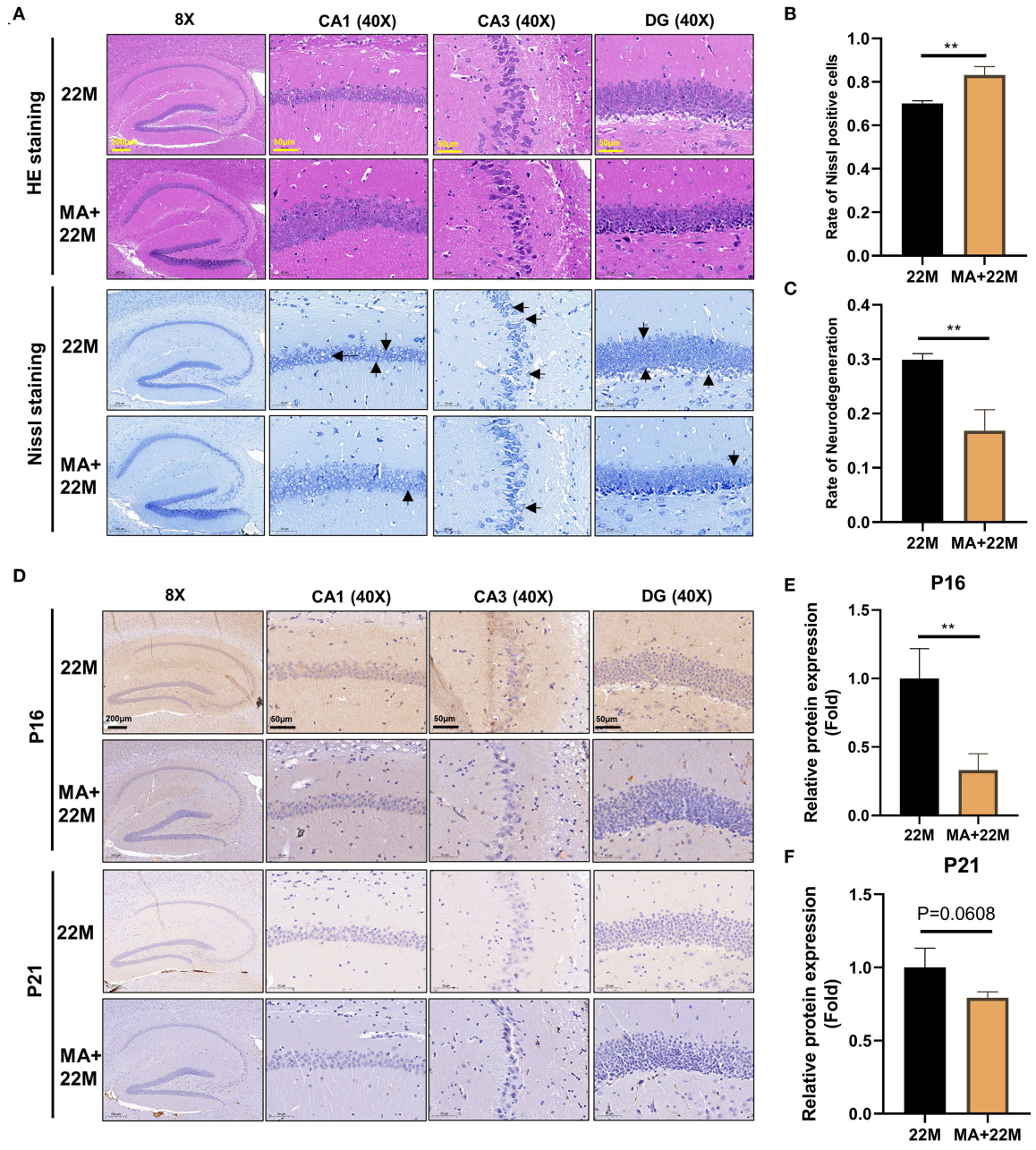


FIGURE 6
Evaluation of hippocampal histomorphology in MA-treated aging mice. **(A)** H&E and Nissl staining in hippocampus between 22M and MA+22M group. The magnifications and scale bars were shown in **(A)**. **(B,C)** Nissl positive cells and Neurodegeneration percentage in hippocampus were assessed by Nissl staining between 22M and MA+22M group. Data were represented as mean ± SD and were analyzed by Student *t*-test. $^{**}P < 0.01$. Arrows showed degenerated neurons. **(D)** IHC of senescence markers P16 and P21 in the hippocampus between 22M and MA+22M group. The magnifications and scale bars were shown in **(D)**. **(E,F)** Relative expressions of senescence markers P16 and P21 calculated by IHC. Data were represented as mean ± SD and were analyzed by Student *t*-test. $^{**}P < 0.01$.

MA alleviated hippocampal aging correlated with GABAergic signaling

To further explore the mechanism of MA-treated hippocampus aging in mice, we employed hippocampus IHC to further validate the GABRB2 and GABRA2 protein expressions in MA-treated aging mice. We verified that GABRB2 protein expression was decreased ($P<0.05$) in the aging hippocampus whereas GABRA2 protein expression was increased ($P<0.05$) in MA-treated aging hippocampus,

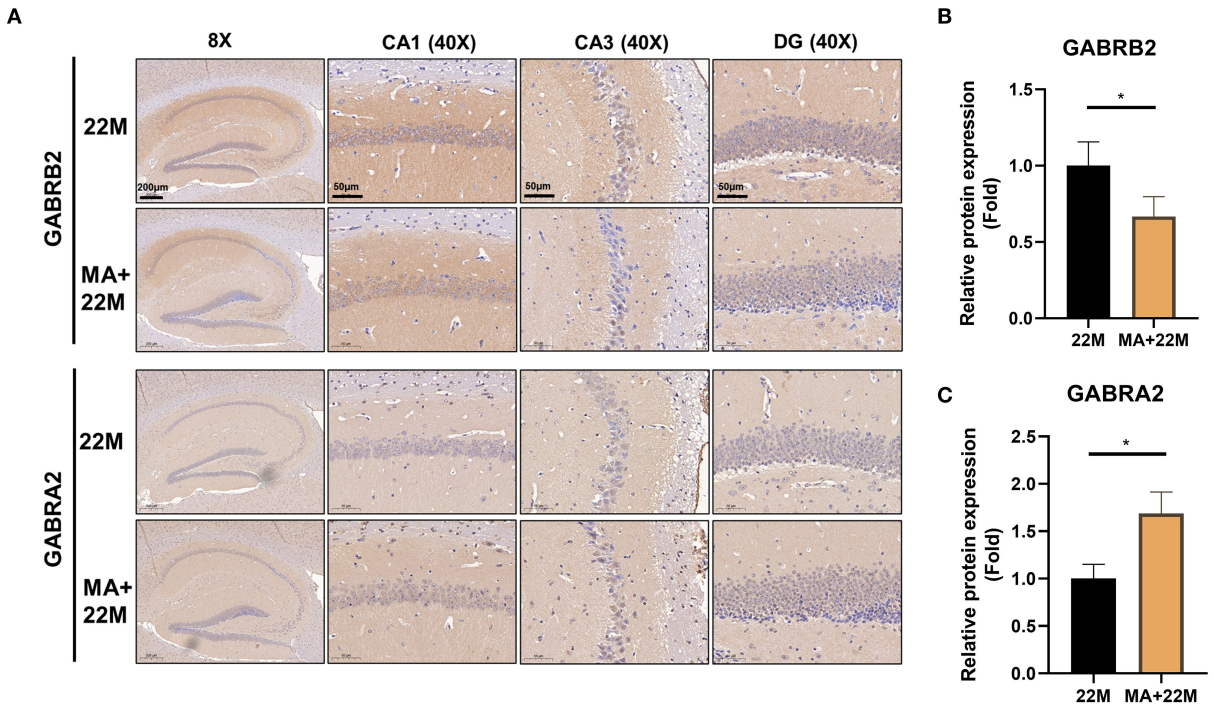


FIGURE 7
Validation of GABRB2 and GABRA2 expression by IHC of hippocampus in MA-treated aging mice. **(A)** IHC of GABRB2 and GABRA2 in the hippocampus between 22M and MA+22M group. Magnifications and scale bars were shown in **(A)**. **(B,C)** Relative expressions of GABRB2 and GABRA2 were calculated by IHC. Data were represented as mean ± SD and were analyzed by Student *t*-test. $^*P < 0.05$.

indicating that MA may improve the imbalance of GABAergic expressions (Figures 7A–C, Supplementary Figures 4A–D).

Discussion

Our study demonstrated that MA, a saturated fatty acid extracted from cardamom oil, coconut oil, palm kernel oil, etc., ameliorated the aging of hippocampus correlated with GABAergic signaling. Aging mice displayed a decline in exploration and spatial memory ability, impairment in the hippocampal histomorphology, upregulation of GABRB2 and downregulation of GABRA2 expression; these effects were improved by the administration of MA.

In aging mice of 22M, a decreased exploration and spatial memory ability were observed using open field test and Morris water maze, which were consistent with previous studies (20, 21). As we know, exploration and spatial memory ability assessed by open field test and Morris water maze respectively are hippocampus-dependent type of exploration and memory ability (22, 23). Hippocampus, as an important part of the limbic system of the brain, is a high-level regulatory center of the neuroendocrine system, whose function is closely related to aging. Aging is characterized by a decrease of hippocampal neurons (5), which was verified by H&E and Nissl staining in our study. To further explore the mechanism of hippocampus aging, we employed mRNA-seq to detect differential gene expressions in the hippocampus between young and aging mice. We found that GABAergic synapse, oxytocin and endocannabinoid signaling were involved in this process. Aging induced oxytocinergic transmission impairment in the central nervous system (24); oxytocin in people aged over 65 may be associated with aging-related changes in hippocampus (25). Furthermore, oxytocin induced GABA release onto mossy cells of the rat dentate gyrus of hippocampus (26). Endocannabinoid system activity contributes to the homeostatic defense against aging and thus may retard brain aging (27, 28). Besides, cannabinoid type 1 receptor activity on hippocampal GABAergic neurons ameliorated age-dependent cognitive decline by decreasing pyramidal cell degeneration and neuroinflammation (29). Given that both oxytocin and endocannabinoid signaling can participate in the regulation of the GABA pathway, we focused on GABAergic signaling in this study. KEGG pathway analysis was found that GABRB2 was upregulated whereas GABRA2 was downregulated, which was verified by IHC of hippocampus between young and aging mice. Previous studies also demonstrated that GABRA2 expression decreased with age in the hippocampus (9, 10). However, aging-related disorders on GABRB2 expression are contradictory. The mRNA level of GABRB2 in the nucleus

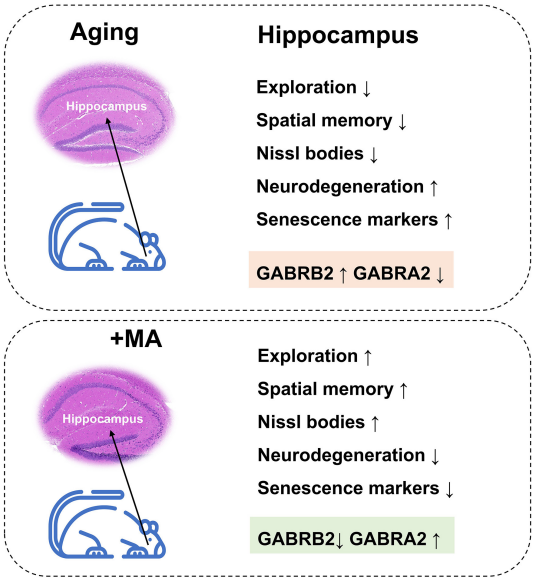


FIGURE 8
Working model. Aging mice displayed a decline in exploration and spatial memory ability, impairment in hippocampal histomorphology, upregulation of GABRB2, and downregulation of GABRA2 expression; these effects were improved by the administration of MA.

accumbens and prefrontal cortex was decreased in middle-aged female mice (30). However, kainic acid, an epileptic seizures inducer in mice, could downregulate GABRB2 expression (31). The reasons of contradictory results of GABRB2 expression may be due to the differences in brain regions and disease detections. Overall, we implied an imbalance of GABAergic signaling in the hippocampus between young and aging mice, which may provide insight into the discovery of targets in aging-related disorders.

To further explore the anti-aging effect of drugs through the GABAergic signaling pathway, MA, a saturated fatty acid, was selected and employed to verify the effect and mechanism of anti-hippocampus aging. Studies have reported that aging was accompanied by a decline in MA, indicating MA played an important role in aging-related disorders (14). However, contradicting our study, Zeng et al. pointed out that MA may promote tumor progression and drug resistance, which may be due to different diseases studied and different drug doses (32). Furthermore, previous studies have demonstrated that MA could promote the proliferation and differentiation of neural stem cells *in vitro* (13). The isomer of MA, Myristoleic acid, could suppress osteoclastogenesis and bone resorption by inhibiting RANKL activation, indicating MA had a potential therapeutic effect on aging-related diseases (15). However, the effect of MA on hippocampal neurons aging has not been reported *in vivo*. We employed 22-month-old naturally aged C57BL/6 mice to evaluate the effect and mechanism of MA on hippocampal aging. Our investigations indicated that MA alleviated hippocampal aging correlated with GABAergic signaling. To the best of our knowledge, this is the first time that we report that MA improved hippocampal aging, which was expected to provide new drugs for combating aging-related diseases.

Our current study has some limitations. We performed *in vivo* study to verify the effect of MA alleviating hippocampus aging via regulating GABAergic expressions. However, there is still a lack of *in vitro* experiments to further explore the effect and mechanism of MA in improving the aging of hippocampal neurons. Secondly, the knockout and overexpression of GABRA2 and GABRB2 in animals and hippocampal neurons are worthy of further exploration to verify the role and mechanism of hippocampal aging and MA in improving hippocampal neuronal aging. As a potential nutritional supplement for the treatment of aging-related diseases, such as Alzheimer's disease and osteoporosis, MA is worthy of further verification by clinical trials.

In conclusion, we demonstrated a decline in exploration and spatial memory ability and impairment of hippocampal histomorphology in aging mice, which may result from an imbalance of GABRB2 and GABRA2 expressions. MA, a saturated fatty acid extracted from food, alleviated the aging of hippocampus correlated with GABAergic signaling (Figure 8). Our study indicated that MA has the potential as a nutritional supplement, which may provide insight into the treatment and prevention of aging-associated diseases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI; GSE201029.

Ethics statement

The animal study was reviewed and approved by the First Affiliated Hospital of Guangzhou University of Chinese Medicine.

Author contributions

QS, XJ, HR, and GS designed the project. QS, GC, PZ, WZ, HC, DY, FY, HL, XZ, and JH performed the experiments. QS, GC, and PZ wrote the original draft of the manuscript. XJ, HR, GS, and DL reviewed and edited the manuscript, supervised the research, and all authors analyzed the data. All authors read and approved the final manuscript.

Funding

This work was supported in part by the following grants: National Natural Science Foundation of China (81904225), Guangzhou Science and Technology Project (202201011169), Guangdong Natural Science Foundation (2020A1515110322 and 2021A1515011247 and 2022A1515012062), Innovative Team Project of the Department of Education of Guangdong Province (2021KCXTD017), High-Level University Collaborative Innovation Team of GZUCM (2021xk57), Graduate Research Innovation Project of GZUCM (A1-2606-21-429-001Z22). The funding institutions had not any role in the study design, data collection, data analysis, interpretation, or writing of the report in this study.

Acknowledgments

Thanks to the Science and Technology Innovation Center of GZUCM for supporting the animal behavior platform.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.907526/full#supplementary-material

References

1. Gomez-Castro F, Zappettini S, Pressey JC, Silva CG, Russeau M, Gervasi N, et al. Convergence of adenosine and gaba signaling for synapse stabilization during development. *Science.* (2021) 374:eabk2055. doi: 10.1126/science.abk2055

2. Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA. Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature.* (2011) 476:458–61. doi: 10.1038/nature10287

3. Yousef H, Czupalla CJ, Lee D, Chen MB, Burke AN, Zera KA, et al. Aged blood impairs hippocampal neural precursor activity and activates microglia via brain endothelial cell vcam1. *Nat Med.* (2019) 25:988–1000. doi: 10.1038/s41591-019-0440-4

4. Ano Y, Ohya R, Takashima A, Uchida K, Nakayama H. Beta-lactolin reduces age-related inflammation and cognitive decline. *Front Nutr.* (2021) 8:724134. doi: 10.3389/fnut.2021.724134

5. Boldrini M, Fulmore CA, Tartt AN, Simeon LR, Pavlova I, Poposka V, et al. Human hippocampal neurogenesis persists throughout aging. *Cell Stem Cell.* (2018) 22:589-–99. doi: 10.1016/j.stem.2018.03.015

6. Bian D, Wang X, Huang J, Chen X, Li H. Maternal Neu5ac supplementation during pregnancy improves offspring learning and memory ability in rats. *Front Nutr.* (2021) 8:641027. doi: 10.3389/fnut.2021.641027

7. Kandel P, Semerci F, Mishra R, Choi W, Bajic A, Baluya D, et al. Oleic acid is an endogenous ligand of Tlx/Nr2e1 that triggers hippocampal neurogenesis. *Proc Natl Acad Sci U S A.* (2022) 119:e2023784119. doi: 10.1073/pnas.2023784119

8. Li G, Bien-Ly N, Andrews-Zwilling Y, Xu Q, Bernardo A, Ring K, et al. Gabaergic interneuron dysfunction impairs hippocampal neurogenesis in adult apolipoprotein E4 knockin mice. *Cell Stem Cell.* (2009) 5:634–45. doi: 10.1016/j.stem.2009.10.015

9. McQuail JA, Frazier CJ, Bizon JL. Molecular aspects of age-related cognitive decline: the role of gaba signaling. *Trends Mol Med.* (2015) 21:450–60. doi: 10.1016/j.molmed.2015.05.002

10. Ionescu-Tucker A, Butler CW, Berchtold NC, Matheos DP, Wood MA, Cotman CW. Exercise reduces H3k9me3 and regulates brain derived neurotrophic factor and Gabra2 in an age dependent manner. *Front Aging Neurosci.* (2021) 13:798297. doi: 10.3389/fnagi.2021.798297

11. Lee YY, Tang TK, Phuah ET, Karim NAA, Alitheen NBM, Tan CP, et al. Structural difference of palm based medium- and long-chain triacylglycerol (Mlct) further reduces body fat accumulation in Dio C57bl/6j mice when consumed in low fat diet for a mid-term period. *Food Res Int.* (2018) 103:200–7. doi: 10.1016/j.foodres.2017.10.022

12. Dos Santos NJA, Bezerra LR, Castro DPV, Marcelino PDR, Virginio Junior GF, da Silva Junior JM, et al. Effect of dietary palm kernel oil on the quality, fatty acid profile, and sensorial attributes of young bull meat. *Foods.* (2022) 11. doi: 10.3390/foods11040609

13. Mahmoudi R, Ghareghani M, Zibara K, Tajali Ardakani M, Jand Y, Azari H, et al. Alyssum homolocarpum seed oil (Ahso), containing natural alpha linolenic acid, stearic acid, myristic acid and beta-sitosterol, increases proliferation and differentiation of neural stem cells in vitro. *BMC Complement Altern Med.* (2019) 19:113. doi: 10.1186/s12906-019-2518-4

14. Ponnappan U, Holley DH, Lipschitz DA. Effect of age on the fatty acid composition of phospholipids in human lymphocytes. *Exp Gerontol.* (1996) 31:125–33. doi: 10.1016/0531-5565(95)02017-9

15. Kwon JO, Jin WJ, Kim B, Kim HH, Lee ZH. Myristoleic acid inhibits osteoclast formation and bone resorption by suppressing the rankl activation of Src and Pyk2. *Eur J Pharmacol.* (2015) 768:189–98. doi: 10.1016/j.ejphar.2015.10.053

16. van der Goot E, Vink SN, van Vliet D, van Spronsen FJ, Falcao Salles J, van der Zee EA. Gut-microbiome composition in response to phenylketonuria depends on dietary phenylalanine in Btbr Pah(Enu2) mice. *Front Nutr.* (2021) 8:735366. doi: 10.3389/fnut.2021.735366

17. Oury F, Khrimian L, Denny CA, Gardin A, Chamouni A, Goeden N, et al. Maternal and offspring pools of osteocalcin influence brain development and functions. *Cell.* (2013) 155:228–41. doi: 10.1016/j.cell.2013.08.042

18. Jian WX, Zhang Z, Zhan JH, Chu SF, Peng Y, Zhao M, et al. Donepezil attenuates vascular dementia in rats through increasing Bdnf induced by reducing Hdac6 nuclear translocation. *Acta Pharmacol Sin.* (2020) 41:588–98. doi: 10.1038/s41401-019-0334-5

19. Dai B, Sun F, Cai X, Li C, Liu H, Shang Y. Significance of Rna N6-methyladenosine regulators in the diagnosis and subtype classification of childhood asthma using the gene expression omnibus database. *Front Genet.* (2021) 12:634162. doi: 10.3389/fgene.2021.634162

20. Hendrickx JO, De Moudt S, Calus E, De Deyn PP, Van Dam D, De Meyer GRY. Age-related cognitive decline in spatial learning and memory of

C57bl/6j mice. *Behav Brain Res.* (2022) 418:113649. doi: 10.1016/j.bbr.2021.113649

21. Shen Z, Xu H, Song W, Hu C, Guo M, Li J, et al. Galectin-1 ameliorates perioperative neurocognitive disorders in aged mice. *CNS Neurosci Ther.* (2021) 27:842–56. doi: 10.1111/cns.13645

22. Kokkinou M, Irvine EE, Bonsall DR, Natesan S, Wells LA, Smith M, et al. Reproducing the dopamine pathophysiology of schizophrenia and approaches to ameliorate it: a translational imaging study with ketamine. *Mol Psychiatry.* (2021) 26:2562–76. doi: 10.1038/s41380-020-0740-6

23. Balzano T, Dadsetan S, Forteza J, Cabrera-Pastor A, Taoro-Gonzalez L, Malaguarnera M, et al. Chronic hyperammonemia induces peripheral inflammation that leads to cognitive impairment in rats: reversed by anti-Tnf-alpha treatment. *J Hepatol.* (2020) 73:582–92. doi: 10.1016/j.jhep.2019.01.008

24. Melis MR, Stancampiano R, Fratta W, Argiolas A. Oxytocin concentration changes in different rat brain areas but not in plasma during aging. *Neurobiol Aging.* (1992) 13:783–6. doi: 10.1016/0197-4580(92)90102-4

25. Orihashi R, Mizoguchi Y, Imamura Y, Yamada S, Ueno T, Monji A. Oxytocin and elderly mri-based hippocampus and amygdala volume: a 7-year follow-up study. *Brain Commun.* (2020) 2:fcaa081. doi: 10.1093/braincomms/fcaa081

26. Harden SW, Frazier CJ. Oxytocin depolarizes fast-spiking hilar interneurons and induces gaba release onto mossy cells of the rat dentate gyrus. *Hippocampus.* (2016) 26:1124–39. doi: 10.1002/hipo.22595

27. Kataoka K, Bilkei-Gorzo A, Nozaki C, Togo A, Nakamura K, Ohta K, et al. Age-dependent alteration in mitochondrial dynamics and autophagy in hippocampal neuron of cannabinoid Cb1 receptor-deficient mice. *Brain Res Bull.* (2020) 160:40–9. doi: 10.1016/j.brainresbull.2020.03.014

28. Tanveer R, Gowran A, Noonan J, Keating SE, Bowie AG, Campbell VA. The endocannabinoid, anandamide, augments Notch-1 signaling in cultured cortical neurons exposed to amyloid-beta and in the cortex of aged rats. *J Biol Chem.* (2012) 287:34709–21. doi: 10.1074/jbc.M112.350678

29. Albayram O, Alferink J, Pitsch J, Piyanova A, Neitzert K, Poppensieker K, et al. Role of Cb1 cannabinoid receptors on gabaergic neurons in brain aging. *Proc Natl Acad Sci U S A.* (2011) 108:11256–61. doi: 10.1073/pnas.1016442108

30. Cao G, Meng G, Zhu L, Zhu J, Dong N, Zhou X, et al. Susceptibility to chronic immobilization stress-induced depressive-like behaviour in middle-aged female mice and accompanying changes in dopamine D1 and gabaa receptors in related brain regions. *Behav Brain Funct.* (2021) 17:2. doi: 10.1186/s12993-021-00175-z

31. Li S, Luo Z, Lu B, Xia S, Li C, Guan X, et al. Protective effects of lycopene on kainic acid-induced seizures. *Epilepsy Res.* (2019) 151:1–6. doi: 10.1016/j.eplepsyres.2019.01.010

32. Zeng L, Wu GZ, Goh KJ, Lee YM, Ng CC, You AB, et al. Saturated fatty acids modulate cell response to DNA damage: implication for their role in tumorigenesis. *PLoS One.* (2008) 3:e2329. doi: 10.1371/journal.pone.0002329

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 09 September 2022
DOI 10.3389/fnut.2022.895803

Check for updates

OPEN ACCESS

EDITED BY
Andrew Scholey,
Swinburne University of Technology, Australia

REVIEWED BY
Chong Chen,
Yamaguchi University Graduate School of Medicine, Japan
Karolina Skonieczna-Zydecka,
Pomeranian Medical University, Poland

*CORRESPONDENCE
Yongli Gao
gylzxy1993@163.com

SPECIALTY SECTION
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

RECEIVED 14 March 2022
ACCEPTED 08 August 2022
PUBLISHED 09 September 2022

CITATION
Chen X, Li D, Liu Y, Zhu L, Jia Y and Gao Y (2022) Nutritional risk screening 2002 scale and subsequent risk of stroke-associated infection in ischemic stroke: The REMISE study. *Front. Nutr.* 9:895803. doi: 10.3389/fnut.2022.895803

COPYRIGHT
© 2022 Chen, Li, Liu, Zhu, Jia and Gao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Nutritional risk screening 2002 scale and subsequent risk of stroke-associated infection in ischemic stroke: The REMISE study

Xiaoli Chen[1], Dongze Li[1], Yi Liu[1], Ling Zhu[1], Yu Jia[2] and Yongli Gao[1]*

[1]Department of Emergency Medicine and West China School of Nursing, West China Hospital, Sichuan University, Chengdu, China, [2]Department of General Practice and National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, Chengdu, China

Background and aim: Stroke-associated infection (SAI) is a common and serious complication in patients with IS. This study aimed to evaluate the impact of nutritional status at admission assessed on SAI, explore the predictive value of the Nutritional Risk Screening 2002 (NRS-2002 for SAI.

Methods: This study included patients with IS who were admitted to five major hospitals in Chengdu from January 2017 to February 2019. The nutritional status was assessed using the NRS-2002 tool. Logistic regression analysis was performed to explore the predictive value of NRS-2002 for SAI.

Results: A total of 594 patients with IS were included in this study; among them, 215 (36.20%) patients were at risk of malnutrition, and 216 (36.36%) patients developed SAI. The area under the curve of the NRS-2002 scores was smaller than A2DS2 (0.644 vs. 0.779), and NRS-2002 improved the predictive values of the A^2DS^2 score(Age, Atrial fibrillation, Dysphagia, Sex, Stroke Severity) for SAI ($P < 0.001$). Logistic regression analysis showed that patients with NRS-2002 score ≥ 3 had significantly higher risks of SAI (NRS-2002: odds ratios (OR) = 1.450, 95% confidence interval (CI): 1.184–1.692, $P < 0.001$).

Conclusion: NRS-2002 is a useful and simple tool for identifying the risk of SAI. Malnutrition is related to the development of SAI. Malnourished patients with stroke may benefit from further nutritional supplements and management.

KEYWORDS

ischemic stroke, stroke-associated infection, NRS-2002, malnutrition, multicenter

Introduction

Ischemic stroke (IS) is the main type of stroke in clinical practice, accounting for approximately 69.6% of all strokes (1), with the characteristics of high morbidity, high disability rate, high mortality, and high recurrence rate (2). The occurrence of serious complications is the most important cause of death and disability in patients with IS. Stroke-associated infection (SAI) is one of the extremely common and serious complications, with an incidence of 23–65% (3, 4). SAI not only prolongs the length

of hospitalization and increases the economic burden but also is closely related to the increase in severe disability and mortality (5). Therefore, early evaluation and prevention of SAI is considered an important part of stroke diagnosis and treatment. The current diagnosis of SAI is mainly based on the inflammatory marker procalcitonin (PCT) and computed tomography (CT) scan, but both indicators only appear clinically after infection. Therefore, it is of great significance to find effective assessment tools for the early prevention and treatment of SAI.

Malnutrition is a common condition in patients with stroke. The prevalence of malnutrition is up to 62% in patients with stroke (6) due to reduced mobility, cognitive impairments, and dysphagia (7). Malnutrition may worsen clinical outcomes and increase mortality (8–10). The mortality rate of malnourished patients with stroke was significantly higher. Research has revealed that malnutrition leads to immune dysfunction (11), resulting in reduced resistance to bacteria and pathogens, and heightens drug resistance, which in turn leads to an increased probability of infection. It was noted that patients with stroke are prone to become immunocompromised due to malnutrition, thus increasing the probability of SAI.

The Nutritional Risk Screening 2002 (NRS-2002) tool (12, 13) is a nutritional assessment tool that is widely used in clinical applications. The effectiveness of this tool has been confirmed by a large number of clinical studies (14, 15). Kyle et al. found that the NRS-2002 tool had higher sensitivity and specificity than other nutritional assessment tools (16). Nutrition may be a reliable indicator for predicting infection. Therefore, nutritional screening tools can be used for the early risk assessment of SAI. To the best of our knowledge, no studies have shown that nutritional risk assessment tools can be used for the early identification of SAI.

This study aimed to evaluate the nutritional status of patients with IS using the NRS-2002 tool and explore its correlation with SAI occurrence to screen for high risk of SAI and early clinical intervention to improve the prognosis of patients with IS.

Materials and methods

Study design

This is a retrospective multicenter study based on recovery trajectory data to assess whether NRS-2002 can predict the incidence of SAI in patients with IS. Patients were recruited from the stroke centers of five hospitals. This study was registered at www.chictr.org.cn (Identifier: ChiCTR2100052025) and conducted in accordance with the Helsinki Declaration. The research plan was approved by the Human Ethics Committee of West China Hospital of Sichuan University. Informed consent was obtained from each participant.

Study population

Patients with acute stroke admitted to the Emergency Stroke Center of five major hospitals in Chengdu, Sichuan, from January 2017 to February 2019 were retrospectively included in this study. The inclusion criteria were as follows: (1) in line with diagnostic criteria for IS (17); (2) patients who were admitted to hospitals within 24 h after onset and diagnosed with cerebral infarction by head CT or head magnetic resonance imaging (MRI); (3) age $\geq$ 18 years. The exclusion criteria were as follows: (1) severe liver, kidney, and hematological diseases; (2) previous history of stroke and legacy sequelae; (3) cancer history or immunosuppressive therapy; (4) incomplete clinical data.

Data collection and measures

The following data were retrieved from the database of retrospective multicenter studies on IS: demographic statistics and characteristic information, vital signs, medical history, drugs, laboratory tests, hospitalization, and adverse outcomes. In this study, blood cells were counted using a hematology analysis system (LH750, Beckman Coulter, Brea, CA). Blood biochemical tests were performed using an Architect C16000 analyzer (Abbott Diagnostics, Dallas, TX, USA).

The A^2DS^2 score (Age, Atrial fibrillation, Dysphagia, Sex, Stroke Severity) was calculated (age $\geq$ 75 years = 1; male sex = 1; atrial fibrillation = 1; dysphagia = 2; stroke severity: National Institutes of Health Stroke Scale (NIHSS) score $\geq$ 5 = 3) (18), which is a validated screening tool for stroke-associated pneumonia, and the range of A^2DS^2 score was from 0 to 10, and 0–4 points for low risk and 5–10 points for high risk. The NIHSS (19) which can assess stroke severity, was administered at admission and discharge. The range of the NIHSS scores was from 0 to 42.

Malnutrition screening tools

The NRS-2002 tool (12) was used to evaluate the nutritional status of patients by trained nurses. The NRS-2002 score ranges from 0 to 7 and consists of three components: (1) disease severity (reflecting increased nutritional needs), which ranges from 0 to 3 based on the patient's comorbidities and medical history; (2) nutritional impairment status, which relates to BMI, body weight, and food, which ranges from 0 to 3; (3) age, with one point for patients $\geq$ 70 years old. According to European Society for Parental and Enteral Nutrition(ESPEN) guidelines for malnutrition diagnosis (12), patients with NRS-2002 scores $\geq$3 were identified as malnourished.

TABLE 1 Baseline patient characteristics and nutritional risk score 2002 in patients with ischemic stroke.

Characteristic	NRS-2002 <3 $N = 379$	NRS-2002 ≥3 $N = 215$	*P*
Demographic variables			
Age, years	58.79 ± 11.97	76.49 ± 9.08	<0.001
Males, *n* (%)	264 (70)	114 (53)	<0.001
Smoking, *n* (%)	179 (47)	70 (33)	<0.001
Drinking, *n* (%)	151 (40)	26 (12)	<0.001
Chronic medical conditions			
Hypertension, *n* (%)	223 (59)	137 (64)	0.242
Diabetes, *n* (%)	104 (27)	54 (25)	0.538
Hyperlipidemia, *n* (%)	49 (13)	10 (5)	0.001
Coronary heart disease, *n* (%)	31 (8)	17 (8)	0.907
History of cancer, *n* (%)	17 (4)	12 (6)	0.551
Physiological and lab variables			
BMI, kg/m^2	24.39 ± 3.02	22.94 ± 4.91	<0.001
Admission SBP, mmHg	144.09 ± 25.45	148.02 ± 26.06	0.075
Admission DBP, mmHg	88.91 ± 16.22	86.73 ± 17.91	0.132
Heart rate,/min	82.39 ± 16.22	81.28 ± 18.85	0.618
Temperature, °C	36.49 ± 0.27	36.51 ± 0.32	0.36
Hemoglobin, g/L	139.82 ± 19.67	130.00 ± 17.132	<0.001
WBC, $*10^9/L$	7.98 ± 2.99	7.78 ± 2.80	0.451
Neutrophil, $*10^9/L$	71.11 ± 11.84	76.98 ± 36.71	0.005
Platelet count, $*10^9/L$	189.64 ± 72.57	169.31 ± 61.92	0.001
Albumin, g/l	70.36 ± 5.77	70.29 ± 6.17	0.117
D-dimer, mg/L	0.41 (0.21–.23)	1.04 (0.52–2.46)	<0.001
Fibrinogen, g/L	2.91 ± 0.98	3.24 ± 1.11	0.204
Blood glucose, mmol/L	7.73 ± 3.19	8.17 ± 3.30	0.11
Creatinine, μmol/L	72.0 (63.0–84.0)	75.0 (62.0–92.0)	0.012
BUN, mmol/L	5.48 (4.30–6.50)	5.80 (4.60–7.70)	0.003
Triglycerides, mmol/L	1.79 ± 1.46	1.41 ± 0.93	0.001
Total cholesterol, mmol/L	4.88 ± 3.91	5.19 ± 5.96	0.432
HDL, mmol/L	1.19 ± 0.37	1.37 ± 0.47	<0.001
LDL, mmol/L	2.42 (1.87–3.06)	2.42 (1.88–3.06)	0.472
Risk scores			
NIHSS score	6 (2–13)	10 (4–17)	<0.001
Barthel index	55 (20–75)	25 (10–50)	<0.001
A^2DS^2 score	4 (1–4)	5 (3–6)	<0.001

Outcomes

The main outcome was SAI during hospitalization. According to the revised standard definition of the Centers for Disease Control and Prevention based on electronic medical records (20), SAI was defined as an infection in the 1st week after stroke, mainly including the following three types: pneumonia, urinary tract infection, and other infections. All infections were reviewed and verified by experienced clinical doctors.

Statistical analysis

Parametric continuous variables were represented by mean ± standard deviation, and nonparametric continuous variables were represented by the median of the quartile interval. Categorical variables were reported in the form of frequencies and percentages. The *t*-test was used for comparison between groups with parametric characteristics, and the Mann–Whitney U test was used for comparison between groups with

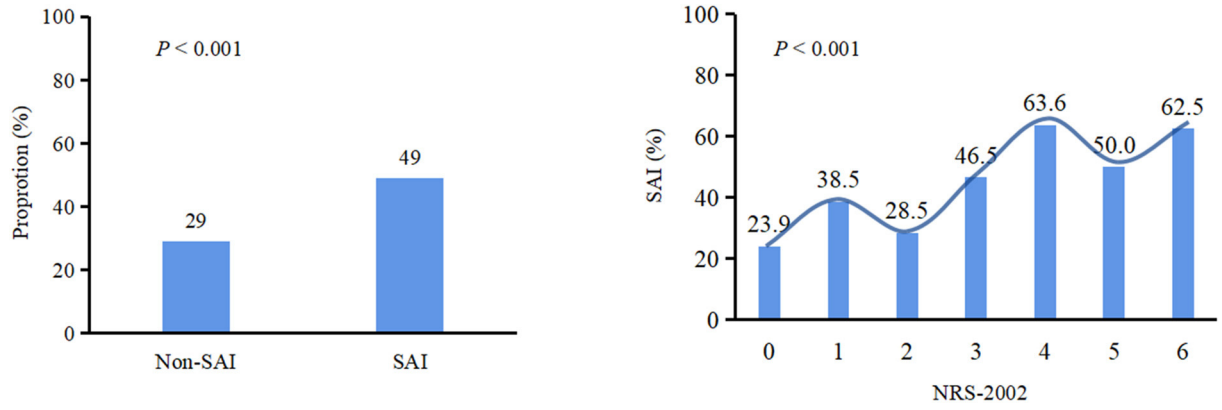


FIGURE 1
Chi square test was used to compare categorical variables. The proportion of patients with malnutrition (NRS-2002≥3) in SAI is higher than that in non-SAI ($P < 0.001$). Patients with NRS-2002 score ≥3 were more likely to develop SAI ($P < 0.001$). SAI, Stroke-associated infection;NRS-2002, Nutritional Risk Screening 2002.

TABLE 2 Adjusted ORs (95% CI) for the association of NRS-2002, NIHSS, and A^2DS^2 scores with the incidence of stroke-associated infection.

Variables	Unadjusted		Model 1		Model 2	
	OR (95% CI)	*P*-trend	OR (95% CI)	*P*-trend	OR (95% CI)	*P*-trend
NRS-2002	1.358 (1.186,1.554)	<0.001	1.371 (1.176,1.613)	<0.001	1.350 (1.144,1.592)	<0.001
NIHSS	1.145 (1.111,1.181)	<0.001	1.163 (1.120,1.208)	<0.001	1.142 (1.098,1.189)	<0.001
A^2DS^2	1.621 (1.467,1.791)	<0.001	1.590 (1.416,1.784)	<0.001	1.161 (1.117,1.207)	<0.001

Model 1: adjusted by age, sex, smoking (never, former, current), drinking (never, former, current), body mass index, systolic blood pressure, smoking, and drinking.
Model 2: adjusted by model 1 plus hemoglobin, urea nitrogen, creatinine.
OR, odds ratio; CI, confidence interval.

non-parametric characteristics. A chi-square test or Fisher's exact test was used to compare categorical variables.

Logistic regression analysis was performed to analyze the relationship between malnutrition and SAI. The logistic regression model was adjusted for demographic variables (sex, age, smoking, and drinking), physiological variables (body mass index and blood pressure), laboratory tests (hemoglobin, urea nitrogen, and creatinine), and chronic diseases (hypertension, diabetes, and hyperlipidemia) to further determine whether these relationships were independent of risk factors. In the model, the patients were divided into groups according to age (<65 years vs. ≥65 years), sex (male vs. female), hypertension (yes vs. no), diabetes (yes vs. no), treatment (conservative vs. thrombolysis or thrombectomy), and NIHSS score (≤7 vs. >7). The area under the receiver operating characteristic (ROC) curve and continuous net reclassification improvement (NRI) were established to evaluate the predictive ability of NRS-2002 and A^2DS^2 for SAI.

The significance level was set at 0.05. All statistical analyses were performed using SPSS version 26.0 (IBM Corp, Armonk, NY, USA) and R software 3.5.0 (Vienna, Austria).

Results

Baseline patient characteristics

Finally, after an overview of the inclusion and exclusion criteria, 594 patients were included in this study. The average age was 58.79 ± 11.97 years. Of these participants, 378 (63.6%) were male. In addition, 215 (36.20%) patients were at risk of malnutrition (NRS-2000≥3). A total of 216 (36.36%) patients developed SAI during hospitalization. At admission, patients with NRS-2002 score ≥3 were found to be older, have a lower Barthel index and hemoglobin, have a higher D-dimer, and high-density lipoprotein, and have a higher NIHSS score and A^2DS^2 score. The baseline characteristics of the patients are presented in Table 1. Table 1 show the baseline characteristics of the patients.

Relationship between NRS-2002 scores and SAI

Patients with NRS-2002 score ≥3 were more likely to develop SAI ($P < 0.001$) (Figure 1). In the logistic

regression model (Table 2), after adjusting for demographic variables, physical examination, laboratory testing, and chronic medical conditions, patients with malnutrition risk (assessed by NRS-2002) or neurological deficit (assessed by NIHSS) had significantly higher risks of SAI (NRS-2002: odds ratio (OR) = 1.350, 95% confidence interval (CI): 1.144–1.592, $P < 0.001$; NIHSS: OR = 1.142, 95% CI: 1.098–1.189, $P < 0.001$). Restricted cubic spline analyses demonstrated continuous relationships between NRS-2002 and odds for SAI (Figure 2).

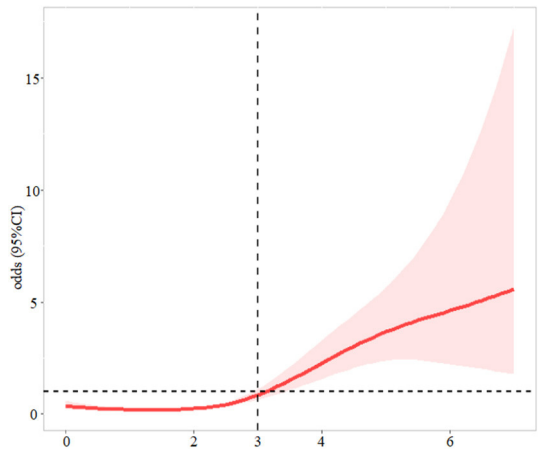


FIGURE 2
The association between NRS-2002 and odds ratio for stroke-associated infection. The solid line indicates the point estimate, and the shaded area is the 95% CI. Models were adjusted by by age, sex, smoking (never, former, current), drinking (never, former, current), body mass index, systolic blood pressure, smoking, drinking,.hemoglobin, urea nitrogen, and creatinine.

Predictive value of NRS-2002 for SAI

According to ROC curve analysis, the area under the curve (AUC) of NRS-2002 and A^2DS^2 for SAI was 0.644 (95% CI: 0.588–0.700, $P < 0.001$) and 0.779 (95% CI: 0.735–0.823, $P < 0.001$), respectively. A^2DS^2 combined with NRS-2002 achieved a higher AUC (0.822, 95% CI:0.793-0.862) than A^2DS^2 alone ($P < 0.001$). Additionally, NRS-2002 combined with A^2DS^2 achieved additional predictive values for SAI beyond A^2DS^2 according to the NRI analysis (NRI = 0.095, 95% CI: 0.048–0.139, $P < 0.001$) (Table 3, Supplementary Figure 1).

An NRS-2002 score ≥5 had optimal specificity (95.69%) to predict SAI (Table 4).

Subgroup analysis

Subgroup analyses stratified by age, sex, hypertension, diabetes, treatment, and NIHSS score were performed (Figure 3).

The results showed that patients with NRS-2002 score ≥3 combined with other factors including age ≥ 65 (OR = 2.626, 95% CI: 1.504–4.585, $P < 0.001$), female (OR =

TABLE 3 Predictive value of the NRS-2002, NIHSS and A^2DS^2 for stroke-associated infection.

Variables	AUROC (95% CI)	P for AUROC	P for AUROC comparison	NRI (95% CI)	P for NRI
NRS-2002	0.644(0.588, 0.700)	<0.001	<0.001	−0.182 (−0.121, −0.259)	<0.001
A^2DS^2	0.779(0.735, 0.823)	<0.001	Ref.	Ref.	-
NRS-2002 plus A^2DS^2	0.822(0.793, 0.862)	<0.001	<0.001	0.095 (0.048, 0.139)	<0.001

CI, confidence interval; AUROC, area under the receiver operating characteristic curve; NRI, net reclassification improvement; NIHSS, National Institutes of Health Stroke Rating Scale; NRS-2002, Nutritional Risk Screening 2002; A^2DS^2, Age, Atrial fibrillation, Dysphagia, Sex, Stroke Severity.

TABLE 4 The predictive value of nutritional risk screening 2002 for stroke associated infection in patients with ischemic stroke.

NRS-2002	Sensitivity, %	Specificity, %	Accuracy, %	PPV, %	NPV, %
≥0	100.00	0.00	36.36	36.36	—
≥1	97.66	14.74	44.61	39.21	91.80
≥2	90.27	23.37	48.82	41.98	79.63
≥3	62.96	71.16	68.18	55.51	77.08
≥4	34.01	92.44	73.06	69.07	73.84
≥5	19.92	95.69	64.31	76.56	62.83
≥6	10.33	94.75	64.48	52.38	65.40
≥7	0.93	100.00	63.97	100.00	63.85

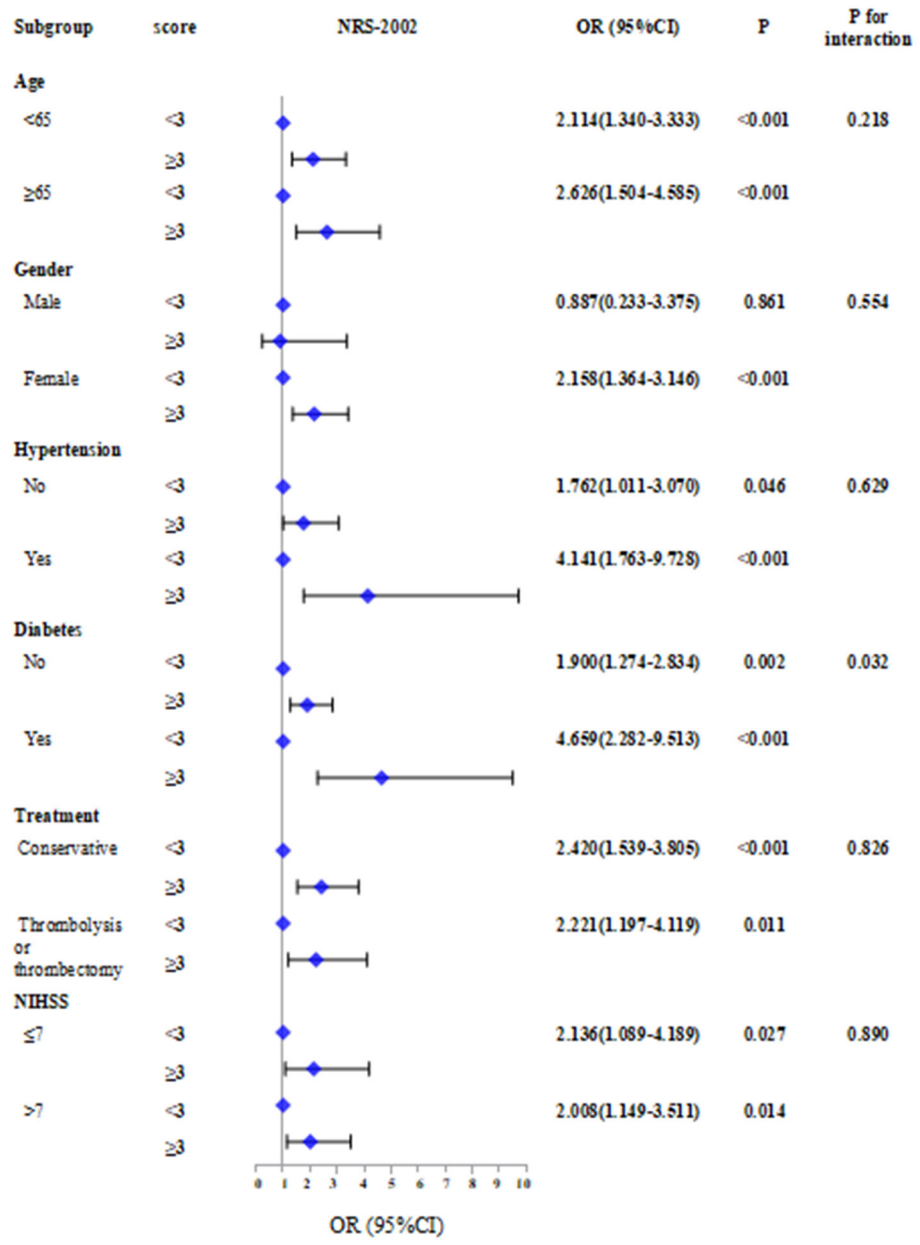


FIGURE 3
Subgroup analyses stratified by age, sex, hypertension, diabetes, treatment and NIHSS score.The absent of malnutrition group was used as reference in Logistic regression models.

2.158, 95% CI: 1.364–3.146, $P < 0.001$), hypertension (OR = 4.141, 95% CI: 1.763–9.728, $P < 0.001$), diabetes (OR = 4.659, 95% CI: 2.282–9.513, $P < 0.001$), and NIHSS score >7 (OR = 2.008, 95% CI: 1.149–3.511, $P = 0.014$) have a higher risk of SAI. In contrast, the risk of SAI associated with NRS-2002 was significantly stronger for patients with diabetes than for those without diabetes (P for interaction: 0.032).

Discussion

Stroke is the second major cause of death worldwide and the leading cause of death and disability in China (21). The clinical data of 594 patients with IS were analyzed; among them, 31.19% had malnutrition, and 36.36% had SAI. The results show that malnutrition and malnutrition risk are risk factors for SAI and using NRS-2002 to predict SAI is effective.

The comprehensive evaluation recommended early nutritional screening as an effective measure to reduce the incidence of SAI.

Recently, malnutrition has been reported to increase the incidence of infections in patients with stroke during rehabilitation and delay the recovery of motor nerve function (22, 23). Studies have shown that malnutrition can affect the size of organs, hormones, and cytokine levels and even affect the immune cells' function (24). Malnutrition is associated with immunosuppression, leading to increased susceptibility to infection. Furthermore, studies have reported that patients with IS at risk of malnutrition are 3.40 and 4.08 times more likely to develop infection complications than those with normal nutrition, respectively (25). Early nutritional support for malnourished patients with IS through nutritional assessment using NRS 2002 can help improve the prognosis. It is recommended to use NRS-2002 to guide nutritional support, which can predict short-term and long-term outcomes (26). To the best of our knowledge, this is the first study that uses the NRS-2002 tool to predict the occurrence of SAI in patients with IS. The factors that constitute the index may explain the relationship between the malnutrition index and SAI. Patients with NRS-2002 score $\geq$3 were more likely to develop SAI ($P < 0.001$). The logistic regression model showed that patients with malnutrition risk (assessed by NRS-2002) or neurological deficit (assessed by NIHSS) had significantly higher risks of SAI, which is consistent with previous studies (27, 28). The higher the NIHSS score, the more serious the neurological deficit, the higher the risk of bed rest, and the more prone to follow pneumonia and aspiration pneumonia caused by gastroesophageal reflux (29).

In this study, we validated the diagnostic value of NRS-2002 for SAI in patients with IS. Our data showed that NRS-2002 could predict the SAI. Moreover, considering sensitivity, specificity, and AUC, the results showed that NRS-2002 combined with A^2DS^2 can optimize the prediction effect of SAI. According to ROC curve analysis, the AUC of NRS-2002, A^2DS^2, and A^2DS^2 combined with NRS-2002 was 0.644, 0.779, and 0.822, respectively. Based on the NRI analysis, NRS-2002 combined with A^2DS^2 achieved additional predictive values for SAI beyond A^2DS^2 (NRI = 0.095, 95% CI: 0.048–0.139, $P < 0.001$). Because the A^2DS^2 does not include the evaluation of nutrition and malnutrition increases the SAI incidence, it is meaningful to include the evaluation of nutrition in the tool for predicting SAI. Subgroup analysis shows that NRS-2002 predicts a better value in SAI for patients with diabetes. In hyperglycemic patients, insulin is relatively insufficient due to insulin resistance, and glucose utilization is reduced. This increases the nutritional risk of the patient. The risk of malnutrition assessed using NRS-2002 is related to the development of SAI. NRS-2002 may be a useful and simple tool for identifying SAI.

This study also has some limitations. First, this is a retrospective study and needs to be confirmed by prospective studies. Second, the evaluation results of the scale were not compared with the infection biomarkers, and the evaluation results were not further verified. Third, the study failed to use NRS-2002 for continuity assessment during hospitalization.

IS is a common disease in clinical neurology. Generally, early nutritional screening should be part of the standard clinical treatment management for patients with IS. It is necessary to check the nutritional status and risk factors for patients with IS. The risk of malnutrition assessed using the NRS-2002 score is related to the development of SAI. NRS-2002 may be a useful and simple tool for identifying SAI. Patients with malnutrition and malnutrition risk should be given appropriate nutritional management as soon as possible to help reduce infection complications, improve prognosis, and reduce medical costs. However, how to provide nutritional supplements and management for malnourished patients with stroke is still a key issue faced by clinicians. Thus, further research is needed to explore an effective approach to address malnutrition in patients with IS.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by the Human Ethics Committee of West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XC, DL, and YG conceived the study design and interpreted the data and drafted the manuscript. XC, YJ, YL, and LZ collected the epidemiological and clinical data. XC, YJ, and YL summarized the data and performed the statistical analysis. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Funding

This work was supported financially by grants from Sichuan Science and Technology Program (No. 2022YFS0279, 2021YFQ0062, and 2022JDRC0148),

Sichuan University West China Nursing Discipline Development Special Fund Project (HXHL20017 and HXHL21016).

Acknowledgments

We would like to thank all the participants of this project and investigators for collecting the data.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.895803/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Receiver operating characteristic curve for NRS-2002 and A^2DS^2 of the SAP.

References

1. Feigin VL, Forouzanfar MH, Krishnamurthi R, Mensah GA, Connor M, Bennett DA, et al. Global and regional burden of stroke during 1990–2010: findings from the global burden of disease study 2010. *Lancet*. (2014) 383:245–55. doi: 10.1016/S0140-6736(13)61953-4

2. Konturek PC, Herrmann HJ, Schink K, Neurath MF, Zopf Y. Malnutrition in hospitals: it was, is now, and must not remain a problem! Medical science monitor. *Int Med J Experim Clin Res.* (2015) 21:2969. doi: 10.12659/MSM.894238

3. Wästfelt M, Cao Y, Ström JO. Predictors of post-stroke fever and infections: a systematic review and meta-analysis. *BMC Neurol*. (2018) 18:1–8. doi: 10.1186/s12883-018-1046-z

4. Vermeij FH. op Reimer WJ, De Man P, Van Oostenbrugge RJ, Franke CL, De Jong G, et al. Stroke-associated infection is an independent risk factor for poor outcome after acute ischemic stroke: data from the Netherlands. *Stroke Survey Cerebrovasc Dis.* (2009) 27:465–71. doi: 10.1159/000210093

5. Faura, J., ustamante A, Miró-Mur F, Montaner J. Stroke-induced immunosuppression: implications for the prevention and prediction of post-stroke infections. *J Neuroinflamm*. (2021) 18:1-4. doi: 10.1186/s12974-021-02177-0

6. Foley NC, Salter KL, Robertson J, Teasell RW, Woodbury MG. Which reported estimate of the prevalence of malnutrition after stroke is valid? *Stroke.* (2009) 40:e66–74. doi: 10.1161/STROKEAHA.108.518910

7. Sabbouh T, Torbey MT. Malnutrition in stroke patients: risk factors, assessment, and management. *Neurocrit Care.* (2018) 29:374–84. doi: 10.1007/s12028-017-0436-1

8. Correia MI, Waitzberg DL. The impact of malnutrition on morbidity, mortality, length of hospital stay and costs evaluated through a multivariate model analysis. *Clin Nutr*. (2003) 22:235–9. doi: 10.1016/S0261-5614(02)00215-7

9. Aliasghari F, Izadi A, Khalili M, Farhoudi M, Ahmadiyan S, Deljavan R. Impact of premorbid malnutrition and dysphagia on ischemic stroke outcome in elderly patients: a community-based study. *J Am Coll Nutr*. (2019) 38:318–26. doi: 10.1080/07315724.2018.1510348

10. Yuan K, Zhu S, Wang H, Chen J, Sun W, et al. Association between malnutrition and long-term mortality in older adults with ischemic stroke. *Clin Nutr*. (2021) 40:2535–42. doi: 10.1016/j.clnu.2021.04.018

11. Maruyama K, Nakagawa N, Koyama S, Maruyama JI, Hasebe N. Malnutrition increases the incidence of death, cardiovascular events, and infections in patients with stroke after rehabilitation. *J Stroke Cerebrovasc Dis*. (2018) 27:716–23. doi: 10.1016/j.jstrokecerebrovasdis.2017.10.002

12. Kondrup JE, Allison SP, Elia M, Vellas B, Plauth M, ESPEN. guidelines for nutrition screening 2002. *Clin Nutr.* (2003) 22:415–21. doi: 10.1016/S0261-5614(03)00098-0

13. Kondrup J, Rasmussen HH, Hamberg OL, Stanga Z, An ad hoc ESPEN Working Group. Nutritional risk screening (NRS 2002): a new method based on an analysis of controlled clinical trials. *Clin Nutr*. (2003) 22:321–36. doi: 10.1016/S0261-5614(02)00214-5

14. Guaitoli PR, Jansma EP, de Vet HC. Nutrition screening tools: does one size fit all? A systematic review of screening tools for the hospital setting. *Clin Nutr.* (2014) 33:39–58. doi: 10.1016/j.clnu.2013.04.008

15. Skipper A, Ferguson M, Thompson K, Castellanos VH, Porcari J. Nutrition screening tools: an analysis of the evidence. *JPEN J Parenter Enteral Nutr*. (2012) 36:292–8. doi: 10.1177/0148607111414023

16. Kyle ÚG, Kossovsky MP, Karsegard VL, Pichard C. Comparison of tools for nutritional assessment and screening at hospital admission: a population study. *Clin Nutr*. (2006) 25:409–17. doi: 10.1016/j.clnu.2005.11.001

17. Chinese Society of Neurology and Chinese Stroke Society, Chinese guidelines for diagnosis and treatment of acute ischemic stroke 2018. (2018) 51:666–82. doi: 10.3760/cma.j.issn1006-7876.2018.09.004

18. Hoffmann S, Malzahn U, Harms H, Koennecke HC, Berger K, Kalic M, et al. Development of a clinical score (A2DS2) to predict pneumonia in acute ischemic stroke. *Stroke*. (2012) 43:2617–23. doi: 10.1161/STROKEAHA.112.653055

19. Brott T, Adams Jr HP, Olinger CP, Marler JR, Barsan WG, Biller J, et al. Measurements of acute cerebral infarction: a clinical examination scale. *Stroke.* (1989) 20:864–70. doi: 10.1161/01.STR.20.7.864

20. Horan TC, Gaynes RP, Martone WJ, Jarvis WR, Emori TG. CDC definitions of nosocomial surgical site infections, 1992: a modification of CDC definitions of surgical wound infections. *Infect Control Hosp Epidemiol*. (1992) 13:606–8. doi: 10.2307/30148464

21. Wang W, Jiang B, Sun H, Ru X, Sun D, Wang L, et al. Prevalence, incidence, and mortality of stroke in China: results from a nationwide population-based survey of 480 687 adults. *Circulation*. (2017) 135:759–71. doi: 10.1161/CIRCULATIONAHA.116.025250

22. Scrutinio D, Lanzillo B, Guida P, Passantino A, Spaccavento S, Battista, P. Association between malnutrition and outcomes in patients with severe ischemic stroke undergoing rehabilitation. *Arch Phys Med Rehabil.* (2020) 101:852–60. doi: 10.1016/j.apmr.2019.11.012

23. Gomes F, Emery PW, Weekes CE. Risk of malnutrition is an independent predictor of mortality, length of hospital stay,

and hospitalization costs in stroke patients. *J Stroke Cerebrovasc Dis*. (2016) 25:799–806. doi: 10.1016/j.jstrokecerebrovasdis.2015.12.017

24. Alwarawrah Y, Kiernan K, MacIver NJ. Changes in Nutritional Status impact immune Cell Metabolism and Function. *Front Immunol*. (2018) 9:1055. doi: 10.3389/fimmu.2018.01055

25. Gao FJ, Zhou W, Wang J. Correlation between nutritional status screened by MNA-SF and acute stroke-associated infections in the elderly. *Pract Geriatr*. (2021) 35:1166–9. doi: 10.3969/j.issn1003-9198.2021.11.015

26. Xu JY, Zhang XN, Jiang ZM, Jie B, Wang Y, Li W, et al. Nutritional support therapy after GLIM criteria may neglect the benefit of reducing infection complications compared with NRS2002–a reanalysis of a cohort study. *Nutrition*. (2020) 79:110802. doi: 10.1016/j.nut.2020.110802

27. Suda S, Aoki J, Shimoyama T, Suzuki K, Sakamoto Y, Katano T, et al. Stroke-associated infection independently predicts 3-month poor functional outcome and mortality. *J Neurol*. (2018) 265(2):370-5. doi: 10.1007/s00415-017-8714-6

28. Ji R, Shen H, Pan Y, Wang P, Liu G, Wang Y, et al. Novel risk score to predict pneumonia after acute ischemic stroke. *Stroke*. (2013) 44:1303–9. doi: 10.1161/STROKEAHA.111.000598

29. Takizawa C, Gemmell E, Kenworthy J, Speyer R. A systematic review of the prevalence of oropharyngeal dysphagia in stroke, Parkinson's disease, Alzheimer's disease, head injury, and pneumonia. *Dysphagia*. (2016) 31:434–41. doi: 10.1007/s00455-016-9695-9

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 03 November 2022
DOI 10.3389/fnut.2022.957600

Check for updates

OPEN ACCESS

EDITED BY
Andrew Scholey,
Swinburne University of Technology, Australia

REVIEWED BY
Feng Zhu,
Xi'an Jiaotong University, China
Karolina Skonieczna-żydecka,
Pomeranian Medical University, Poland

*CORRESPONDENCE
Rui Li
rli@nwpu.edu.cn
Xingzhi Guo
guo.heinz@hotmail.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Nutrition, Psychology and Brain Health, a section of the journal Frontiers in Nutrition

RECEIVED 31 May 2022
ACCEPTED 18 October 2022
PUBLISHED 03 November 2022

CITATION
Chen L, Guo X, Hou C, Tang P, Zhang X, Chong L and Li R (2022) The causal association between iron status and the risk of autism: A Mendelian randomization study. *Front. Nutr.* 9:957600. doi: 10.3389/fnut.2022.957600

COPYRIGHT
© 2022 Chen, Guo, Hou, Tang, Zhang, Chong and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

The causal association between iron status and the risk of autism: A Mendelian randomization study

Li Chen[1,2†], Xingzhi Guo[1,2,3*†], Chen Hou[1,2], Peng Tang[1,2], Xin Zhang[1,2], Li Chong[1,2] and Rui Li[1,2,3*]

[1]Department of Geriatric Neurology, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi, China, [2]Shaanxi Provincial Clinical Research Center for Geriatric Medicine, Xi'an, Shaanxi, China, [3]Institute of Medical Research, Northwestern Polytechnical University, Xi'an, Shaanxi, China

Emerging evidence indicates a connection between serum iron levels and autism, but the underlying causal association is yet unclear. Thus, we performed two-sample Mendelian randomization (MR) analysis to evaluate the causal link between iron status on autism, using genetic instruments ($p < 5E{-}08$) strongly associated with iron status (N = 48,972), including serum iron, ferritin, transferrin levels, and transferrin saturation. Summary statistics of autism was obtained from two independent studies conducted by Psychiatric Genomics Consortium (PGC, *N*cases = 5,305, *N*controls = 5,305) and FinnGen Consortium (FC, Round six, *N*cases = 344, *N*controls = 258,095), respectively. Using the inverse-variance weighted (IVW) method, the combined results of PGC and FC demonstrated that genetically determined serum transferrin level was significantly associated with an increased risk of autism [odds ratio (OR) = 1.16, 95% CI: 1.03–1.30, p = 0.013]. There was no significant causal effect of serum iron (OR = 0.99, 95% CI: 0.72–1.37, p = 0.951), ferritin (OR = 0.88, 95% CI: 0.47–1.64, p = 0.676), and transferrin saturation (OR = 0.89, 95% CI: 0.72–1.09, p = 0.252) on autism. No obvious pleiotropy was found in this MR study. Taken together, our findings highlight that elevation of serum transferrin level might be associated with a high risk of autism, suggesting a potential role of iron deficiency in autism development. Future studies are warranted to clarify the underlying mechanism, which will pave a new path for the prevention and treatment of autism.

KEYWORDS

iron, transferrin, ferritin, autism, Mendelian randomization

Introduction

Autism, also called autism spectrum disorder (ASD), is a mental disorder characterized by challenges with communication, social skills, speech, and repetitive behaviors. Epidemic data from the Center for Disease Control and Prevention (CDC) show that autism affects about one in 44 children in the United States (1), and the signs

of autism often appear as early as 24–36 months. Although currently, the etiology of autism remains largely unknown, it is suggested that genetic background, environmental factors, and their interaction play a vital role in the development of autism (2). The imbalance of trace elements, such as iron, copper, zinc, selenium, nickel, manganese, aluminum, etc., has been reported to be tightly associated with autism risk (3).

Iron is the most important trace element in maintaining the body's metabolic process and intracellular oxygen transport. There are about 1.2 billion people who suffer from iron deficiency, especially in children and women (4). In the central nervous system, iron exerts a crucial role in regulating neurotransmitter synthesis, myelination, and neuro-inflammatory response (5). Previous studies indicated that, in autistic children, serum iron and ferritin levels were significantly decreased and increased, respectively. However, a recent meta-analysis with a large sample size demonstrated that there was no significant association between serum ferritin as well as hair iron and autism. Although iron supplementation is a common intervention for the prevention and treatment of various diseases caused by iron deficiency (6), it is also worthy to note that excess iron could increase the levels of reactive oxygen species, contributing to the oxidative damage of DNA, proteins, and lipids, which eventually lead to cell death, also known as ferroptosis (7, 8). Iron supplementation for healthy individuals may pose unexpected health risks owing to the toxicity of iron deficiency and iron overload. Thus, it invokes the need to confirm the causal effect of iron deficiency on autism, which is crucial for the prevention of autism *via* iron supplementation.

The Mendelian randomization (MR) analysis, treating germline genetic variants as instrumental variables (IVs), is now widely used in determining the potential causal link between exposures and corresponding outcomes. MR can effectively minimize the impact of confounding factors that might influence both exposure and outcome phenotype. Therefore, we here performed a two-sample MR analysis to examine whether genetically determined iron status was causally associated with autism development.

Materials and methods

Study design and instrumental variants selection

Single nucleotide polymorphisms (SNPs) strongly associated with iron status ($p < 5E{-}08$) were selected as IVs, which were obtained from genome-wide association studies (GWAS) summary statistics of European ancestry performed by Benyamin et al. (9). The iron status ($N = 48{,}927$) was indicated in four stages, including serum iron, ferritin, transferrin levels, and transferrin saturation (**Supplementary Table 1**). Instrument variables were clumped based on the 1,000 Genomes Project linkage disequilibrium (LD) structure ($r^2 < 0.001$ within 10,000 kb, European). The detailed information on study design has been well-described in the original publication (9).

Autism genome-wide association studies datasets

Two independent GWAS datasets on autism were obtained from studies performed by the Psychiatric Genomics Consortium (PGC) and the FinnGen Consortium (Round 6),[1] respectively. For summary statistics from PGC, a total of 5,305 autism cases and 5,305 controls of European descent were included (OpenGWAS: ieu-a-1184) (10). For summarized association data from FinnGen Consortium (Round 6), there were 344 cases and 258,095 normal controls of European descent. To further validate the results, we also adopted the latest autism GWAS dataset of Integrative Psychiatric Research and PGC (iPSYCH-PGC) as a replication (Ncases = 18,382, Ncontrols = 27,969, OpenGWAS: ieu-a-1185) (11), which contained GWAS summary statistics from population-based samples of iPSYCH and family-based samples of PGC (10). Autism was diagnosed according to Autism Diagnostic Interview-Revised (ADI-R) and/or Autism Diagnostic Observation Schedule (ADOS) (12).

Mendelian randomization analysis

Mendelian randomization analysis was conducted using the TwoSampleMR package (v0.5.6) (13). Moreover, if SNPs selected as IVs were absent in the summary statistics of autism, overlapping proxy SNPs in LD ($r^2 = 0.8$) were used instead. To strengthen the reliability of MR analysis, SNPs with minor allele frequency (MAF) less than 0.3 were also removed. The inverse-variance weighted (IVW), providing a robust causal evaluation under a lack of directional pleiotropy, was employed as the primary method to calculate causal estimates between iron status and autism. Meanwhile, both weighted median and MR–Egger regression methods were also applied to assess the causal estimates. After MR analysis, a meta-analysis was performed to combine the overall causal estimates from PGC or iPSYCH-PGC and FinnGen Consortium, respectively. A p-value less than 0.05 was considered statistically significant. Three corresponding principal assumptions and the flowchart in this study was described in **Figure 1**.

1 https://finngen.gitbook.io/documentation

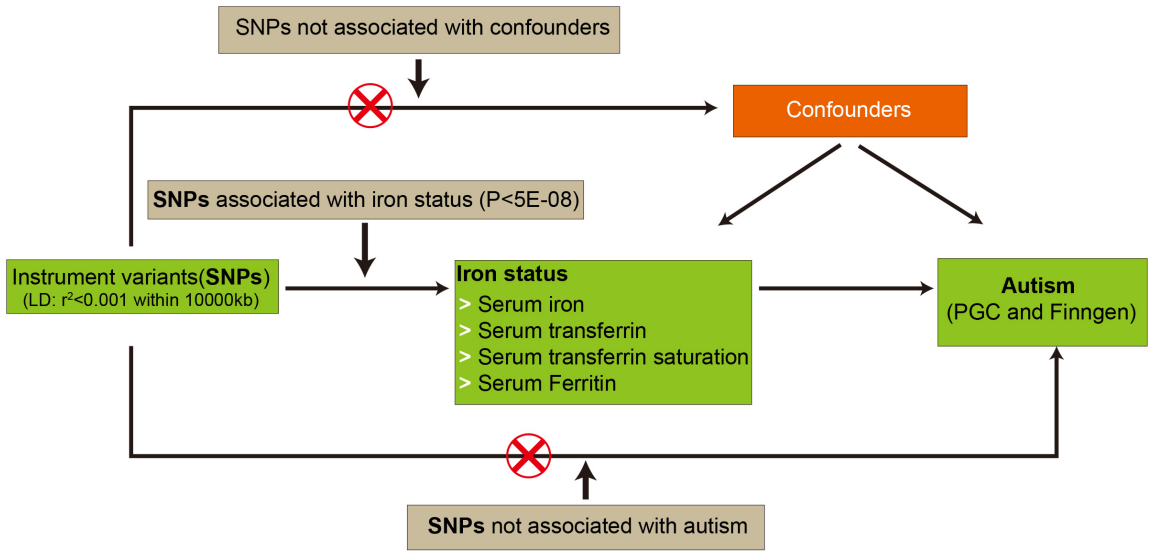


FIGURE 1
The flowchart in this two-sample Mendelian randomization study. The red cross means that genetic variants are not associated with confounding factors and outcomes. PGC, psychiatric genomics consortium; SNPs, single nucleotide polymorphisms.

Pleiotropy, heterogeneity, and power analysis

Mendelian randomization-Pleiotropy RESidual Sum and Outlier (MR-PRESSO) test with default parameters was performed to identify horizontal pleiotropic outliers (14), and the MR-Egger intercept test was conducted to assess whether there was potential horizontal pleiotropy driving the MR results. Meanwhile, the IVW method and MR-Egger regression were also used to estimate heterogeneity, which was quantified by Cochran Q statistics. To validate the reliability of the causal association between iron status and autism, we performed a power analysis using the online tool mRnd[2] (15). F values of more than 10 indicated good stability.

Results

Summary of the Mendelian randomization analysis

A detailed list of all harmonized instrumental variables, namely SNPs, for each exposure-outcome group was archived in the Supplementary Data Sheet 1. The causal estimates (OR values and 95% CI) of the IVW with *p*-values, number of SNPs, and combined estimates of the meta-analysis were presented in forest plots. The results of MR-PRESSO global with *p*-values, MR-Egger intercept with *p*-values, heterogeneity in IVW, and MR Egger test for each exposure-outcome pair were shown in **Table 1** and **Supplementary Table 2**. Causal estimates using weighted median and MR–Egger method were also calculated.

Causal effects of iron status on autism

Using the IVW method, genetically predicted serum transferrin was significantly associated with an increased risk of autism in PGC (OR = 1.16, 95% CI: 1.03–1.31, $p = 0.018$), but not FinnGen Consortium (OR = 1.15, 95% CI: 0.80–1.65, $p = 0.451$). A further meta-analysis based on the above two independent data showed that serum transferrin remained associated with an increased risk of autism (OR = 1.16, 95% CI: 1.03–1.30, $p = 0.013$). Similar results were found using both weighted median (OR = 1.179, 95% CI: 1.043–1.333) and MR Egger (OR = 1.207, 95% CI: 0.939–1.553). No obviously directional horizontal pleiotropy and heterogeneities were found in MR-PRESSO global (RSSobs = 7.94, $p = 0.659$) and MR-Egger Intercept test ($p = 0.109$). Additionally, there was no evidence showing significant heterogeneity in MR Egger (Cochran Q = 0.72, $p = 0.993$) and IVW (Cochran Q = 4.24, $p = 0.751$) regression test.

Using the IVW method, no significantly causal link between serum iron and ferritin levels and transferrin saturation on autism was observed in both PGC and FinnGen Consortium datasets (**Figure 2**). Further meta-analysis combining the causal estimates of PGC and FinnGen Consortium also showed that

2 http://cnsgenomics.com/shiny/mRnd/

TABLE 1 Heterogeneity and power analysis of iron status on autism.

Exposure\Outcome	Method	PGC (autism)				FinnGen consortium (autism)			
		MR-egger intercept (*P*)	Cochran-Q (*P*)	F-statistic	MR_PRESSO (*P*)	MR-egger intercept (*P*)	Cochran-Q (*P*)	F-statistic	MR_PRESSO (*P*)
Iron (μmol/l)	MR-Egger	0.044 (0.495)	6.64 (0.035)	368.72	11.96 (0.189)	−0.007 (0.939)	1.84 (0.397)	8957.97	3.06 (0.713)
	IVW		8.91 (0.030)				1.85 (0.603)		
Transferrin (g/l)	MR-Egger	−0.028 (0.109)	0.72 (0.993)	1191.06	7.94 (0.659)	0.012 (0.780)	1.67 (0.946)	28988.48	3.12 (0.952)
	IVW		4.24 (0.751)				1.76 (0.971)		
Transferrin saturation (%)	MR-Egger	−0.033 (0.446)	4.38 (0.111)	741.80	8.41 (0.240)	−0.091 (0.325)	0.08 (0.956)	18045.50	3.72 (0.602)
	IVW		6.31 (0.097)				1.75 (0.625)		
Ferritin (log10, μg/l)	MR-Egger	0.001 (0.978)	14.39 (0.002)	112.84	18.87 (0.053)	−0.052 (0.600)	0.44 (0.930)	2725.23	1.57 (0.915)
	IVW		14.40 (0.006)				0.78 (0.940)		

MR, Mendelian randomization; PGC, psychiatric genomics consortium; IVW, inverse-variance weighted; MR_PRESSO, Mendelian randomization pleiotropy RESidual sum and outlier; *p*, *p*-value.

there was no association between serum iron (OR = 0.99, 95% CI: 0.72–1.37, p = 0.951), ferritin (OR = 0.88, 95% CI: 0.47–1.64, p = 0.676) as well as transferrin saturation (OR = 0.89, 95% CI: 0.72–1.09, p = 0.252) and autism. Similar results were found using weighted median (**Figure 3A**) and MR Egger methods (**Figure 3B**). No obvious horizontal pleiotropy and heterogeneity were found in the MR analysis between serum iron, transferrin, and transferrin saturation and autism. To further validate the MR results above, we replicated the MR analysis using the latest GWAS dataset of iPSYCH-PGC, which showed consistent results (**Supplementary Figure 1**).

Power analysis

The overall F statistics for genetic variants selected as IVs of serum iron, ferritin, and transferrin levels and transferrin saturation in PGC were 368.72, 112.84, 1191.06, and 741.80 individually. For FinnGen Consortium, the F statistics of serum iron, ferritin, and transferrin levels and transferrin saturation in were 8957.97, 2725.23, 28988.48, and 18045.50. The F value from the iPSYCH-PGC dataset also showed that there was no obvious weak instrumental bias (**Supplementary Table 2**).

Discussion

Although various studies have been performed to explore the association between iron status and autism, this study here is the first MR analysis conducted to evaluate the naturally causal link between iron status on autism susceptibility. Using the IVW method, our results demonstrated that genetically predicted serum transferrin was causally associated with an increased risk of autism, indicating that transferrin may be a potential marker for autism development.

Iron deficiency could cause numerous detrimental effects in people, such as anemia, fatigue, atrophic glossitis, hair loss, etc. (16), and is also associated with the risk of many diseases. For example, previous studies showed that iron deficiency was associated with an increased risk of type 2 diabetes, Parkinson's disease, and cancers (17–20). Maternity with a lower mean daily iron intake led to an increased risk of autism in offspring (21). Numerous studies have suggested that iron deficiency was a common phenomenon in autism (22), and iron supplementary has been widely advised for pregnant women and children with finicky habitation. However, it is also hypothesized that excess iron intake might contribute to a higher susceptibility to autism *via* over-activating the immune system (23). Thus, it is urgent for clinicians to find an optimistic marker to guide iron supplementation, especially for those who do not need iron supplementation. There are four classical iron biological states, including serum iron, ferritin, transferrin levels, and transferrin saturation, commonly used in reflecting iron status

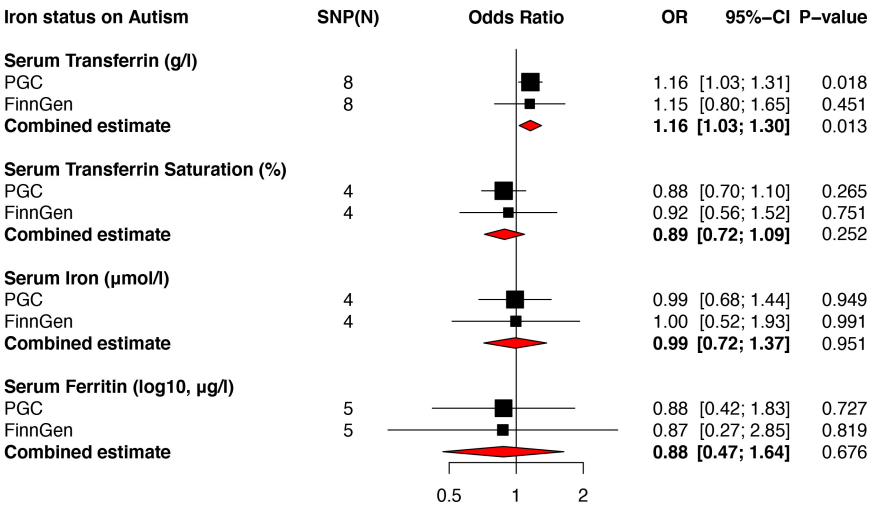


FIGURE 2
Forest plots of MR analyses showing the causal effects of iron status on autism using the IVW method. Two independent autism genome-wide association studies (GWAS) datasets from PGC and FinnGen Consortium were used to evaluate the causal effect. The combined estimates were presented as red diamonds. PGC, psychiatric genomics consortium; IVW, inverse-variance weighted; MR, Mendelian randomization.

in humans. In this study, we found that only serum transferrin was associated with an increased risk of autism, but not serum iron, ferritin, and transferrin saturation. Indeed, a recent meta-analysis also showed that hair iron and serum ferritin were not significantly different between controls and autism patients (24). Meanwhile, serum transferrin levels were significantly decreased in autistic children as compared to their non-autistic siblings (25). These data suggested that transferrin, but not iron levels might be a more sensitive and efficient marker for autism development.

The main function of transferrin, an iron-binding glycoprotein, is to mediate the transport of free iron to suitable cells with corresponding surface receptors around the body (26). Transferrin plays a vital role in transporting iron to erythroid precursors in the bone marrow, maturity dysfunction of which would cause anemia (27). Increased serum transferrin level is often observed in people with iron deficiency anemia, which is tightly associated with autism and other neurological diseases (28). It is suggested that transferrin saturation could also serve as an effective diagnostic criterion for iron deficiency (29). In addition, transferrin also was reported to have an anti-oxidative function, which could further reduce the risk of autism (30). Due to the elevation of transferrin often happening earlier than the reduction of serum iron, it might contribute to a positive association between autism and serum transferrin, but not iron.

Previous studies suggest that immune dysfunction also plays a vital role in the development of autism (31). The over-activated neuro-inflammatory response was found in the brain specimen of both children and adults with autism (32). In addition, the plasma and cerebral-spinal fluid pro-inflammatory factors were also significantly increased in autistic patients (33). Yet interestingly, it is reported that transferrin also exerts an important role in regulating the immune response in the brain. Treatment of transferrin-derived synthetic peptide could induce a pro-inflammatory phenotype of macrophage, which might contribute to an elevated cytokines level (34). A study in fish also suggested that transferrin could activate macrophages in response to pathogens (35). Taken together, these data suggested that iron deficiency might not directly increase the pathological changes in autism, and might contribute to a higher risk of autism indirectly *via* elevating the expression of transferrin. Similarly, our results showed that only transferrin was significantly associated with autism risk, but not serum iron levels. Thus, a current challenge for researchers is to determine the underlying mechanisms contributing to the association between an elevation of transferrin and autism risk, which is important for establishing prevention strategies in patients with autism susceptibility.

There are several obvious strengths in our study. No pleiotropy were found in the MRPRESSO test and MR-Egger Intercept test, suggesting the good reliability of our results. Moreover, the causal estimates obtained from three different methods (IVW, MR Egger, and weighted median) were consistent. Finally, although the MR results of serum transferrin using the latest autism GWAS dataset of iPSYCH-PGC were not statistically significant, the causal estimates showed a similar trend and were significant in the meta-analysis. Meanwhile, the results of the meta-analysis showed that genetically determined serum transferrin was still significantly associated with an increased risk of autism, even if using the negative result from FinnGen Consortium, indicating our results were robust.

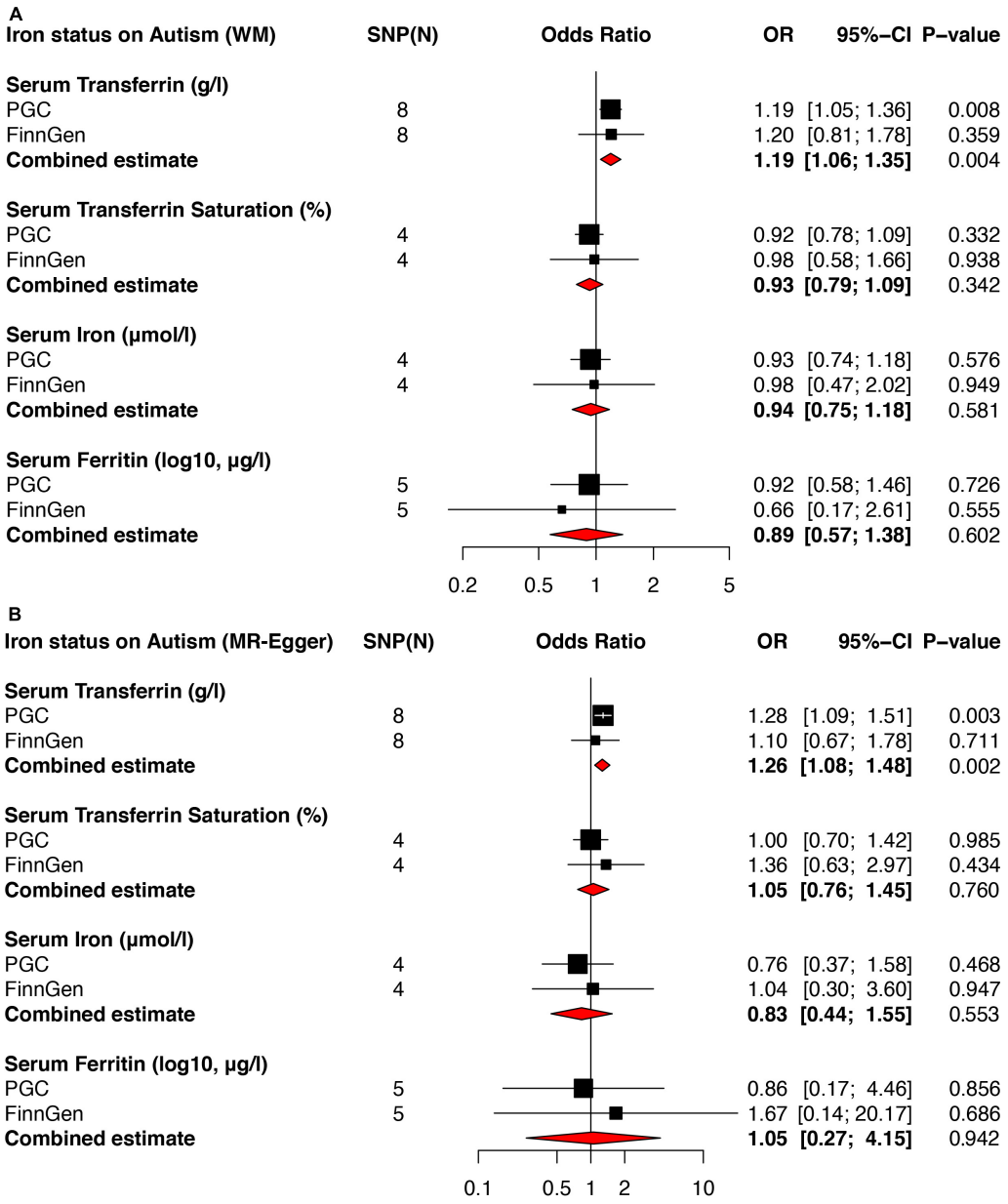


FIGURE 3
Forest plots of MR analyses showing the causal effects of iron status on autism using weighted median and MR Egger method. Two independent autism GWAS datasets from PGC and FinnGen Consortium were used to evaluate the causal effect. The combined estimates were presented as red diamonds. **(A)** Showed the causal effect of iron status on autism using the weighted median method. **(B)** Showed the causal effect of iron status on autism using the MR Egger method. PGC, psychiatric genomics consortium; WM, weighted median; MR, Mendelian randomization.

Owing to the intrinsic principles and assumptions behind MR, the results of the current study should be interpreted cautiously and some limitations should also be addressed here. First, less than ten SNPs were selected as IVs for all four traits of iron status, which might weaken the association between iron status and autism. Second, although the combined estimates of the meta-analysis showed serum transferrin was associated with an increased risk of autism, the causal effect obtained from FinnGen Consortium was not statistically significant. Third, there were only 344 autism cases from the GWAS of the FinnGen Consortium. Further released data with larger case samples from FinnGen Consortium are required to verify the associations above. Forth, all genetic data used in this MR study are from the European population. Whether the causal link between iron status and autism observed in this study is common in other ethnic groups needs to be further verified.

Conclusion

In summary, the results of this MR study demonstrate that genetically predicted serum transferrin levels might be causally associated with an increased risk of autism. Our findings reminded clinicians that more attention and concern should be paid to iron status in children with autism family history. To clarify the underlying mechanism of iron deficiency contributing to autism susceptibility is vital for guiding autism prevention and therapy.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors. All R scripts applied in MR analysis are available from the authors upon request.

Author contributions

LChe, XG, and RL conceived and designed the project. LChe, XG, CH, and XZ collected and analyzed the data and drafted the manuscript. PT, LCho, and RL revised the manuscript. All authors have read and approved the final manuscript.

Funding

This work was supported by the Project for Sanqin Academic Innovation Team in Shaanxi Province (SQ0157) and the Social Development Program of Shaanxi (Program No. 2021SF-095).

Acknowledgments

We acknowledge the participants and investigators of the FinnGen project and PGC study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.957600/full#supplementary-material

SUPPLEMENTARY DATA SHEET 1
Characteristics of harmonized instrumental variables used in Mendelian randomization (MR) analysis between iron status and autism.

References

1. Buranova N, Dampf M, Stevenson B, Sohl K. ECHO autism: early intervention connecting community professionals to increase access to best practice autism intervention. *Clin Pediatr (Phila).* (2022) 61:518–22. doi: 10.1177/00099228221090710

2. Lyall K, Croen L, Daniels J, Fallin MD, Ladd-Acosta C, Lee BK, et al. The changing epidemiology of autism spectrum disorders. *Annu Rev Public Health.* (2017) 38:81–102. doi: 10.1146/annurev-publhealth-031816-044318

3. Baj J, Flieger W, Flieger M, Forma A, Sitarz E, Skórzyńska-Dziduszko K, et al. Autism spectrum disorder: trace elements imbalances and the pathogenesis and severity of autistic symptoms. *Neurosci Biobehav Rev.* (2021) 129:117–32. doi: 10.1016/j.neubiorev.2021.07.029

4. Vos T, Abajobir AA, Abate KH, Abbafati C, Abbas KM, Abd-Allah F, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the global burden of disease study 2016. *Lancet.* (2017) 390:1211–59. doi: 10.1016/S0140-6736(17)32154-2

5. Beard JL. Effectiveness and strategies of iron supplementation during pregnancy. *Am J Clin Nutr.* (2000) 71:1288S–94S. doi: 10.1093/ajcn/71.5.1288s

6. Stoffel NU, von Siebenthal HK, Moretti D, Zimmermann MB. Oral iron supplementation in iron-deficient women: how much and how often? *Mol Asp Med.* (2020) 75:100865. doi: 10.1016/j.mam.2020.100865

7. Sousa L, Oliveira MM, Pessôa MTC, Barbosa LA. Iron overload: effects on cellular biochemistry. *Clin Chim Acta.* (2020) 504:180–9. doi: 10.1016/j.cca.2019.11.029

8. Fang X, Wang H, Han D, Xie E, Yang X, Wei J, et al. Ferroptosis as a target for protection against cardiomyopathy. *Proc Natl Acad Sci USA.* (2019) 116:2672–80. doi: 10.1073/pnas.1821022116

9. Benyamin B, Esko T, Ried JS, Radhakrishnan A, Vermeulen SH, Traglia M, et al. Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis. *Nat Commun.* (2014) 5:1–11. doi: 10.1038/ncomms5926

10. Devlin B, Kelsoe JR, Sklar P, Daly MJ, O'Donovan MC, Craddock N, et al. Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. *Nat Genet.* (2013) 45:984–94. doi: 10.1038/ng.2711

11. Grove J, Ripke S, Als TD, Mattheisen M, Walters RK, Won H, et al. Identification of common genetic risk variants for autism spectrum disorder. *Nat Genet.* (2019) 51:431–44.

12. Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium. Meta-analysis of GWAS of over 16,000 individuals with autism spectrum disorder highlights a novel locus at 10q24. 32 and a significant overlap with schizophrenia. *Mol Autism.* (2017) 8:1–17. doi: 10.1186/s13229-017-0137-9

13. Hemani G, Zheng J, Elsworth B, Wade KH, Haberland V, Baird D, et al. The MR-Base platform supports systematic causal inference across the human phenome. *Elife.* (2018) 7:e34408. doi: 10.7554/eLife.34408

14. Verbanck M, Chen C-Y, Neale B, Do R. Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat Genet.* (2018) 50:693–8. doi: 10.1038/s41588-018-0099-7

15. Brion M-JA, Shakhbazov K, Visscher PM. Calculating statistical power in Mendelian randomization studies. *Int J Epidemiol.* (2013) 42:1497–501. doi: 10.1093/ije/dyt179

16. Abbasi U, Abbina S, Gill A, Takuechi LE, Kizhakkedathu JN. Role of iron in the molecular pathogenesis of diseases and therapeutic opportunities. *ACS Chem Biol.* (2021) 16:945–72. doi: 10.1021/acschembio.1c00122

17. Hou C, Hou Q, Xie X, Wang H, Chen Y, Lu T, et al. Serum iron status and the risk of breast cancer in the European population: a two-sample Mendelian randomisation study. *Genes Nutr.* (2021) 16:1–10.

18. Yuan S, Carter P, Vithayathil M, Kar S, Giovannucci E, Mason AM, et al. Iron status and cancer risk in UK Biobank: a two-sample Mendelian randomization study. *Nutrients.* (2020) 12:526. doi: 10.3390/nu12020526

19. Wang X, Fang X, Zheng W, Zhou J, Song Z, Xu M, et al. Genetic support of a causal relationship between iron status and type 2 diabetes: a Mendelian randomization study. *J Clin Endocrinol Metab.* (2021) 106:e4641–51. doi: 10.1210/clinem/dgab454

20. Pichler I, Del Greco MF, Gögele M, Lill CM, Bertram L, Do CB, et al. Serum iron levels and the risk of Parkinson disease: a Mendelian randomization study. *PLoS Med.* (2013) 10:e1001462. doi: 10.1371/journal.pmed.1001462

21. Schmidt RJ, Tancredi DJ, Krakowiak P, Hansen RL, Ozonoff S. Maternal intake of supplemental iron and risk of autism spectrum disorder. *Am J Epidemiol.* (2014) 180:890–900.

22. Bener A, Khattab AO, Bhugra D, Hoffmann GF. Iron and vitamin D levels among autism spectrum disorders children. *Ann Afr Med.* (2017) 16:186. doi: 10.4103/aam.aam_17_17

23. Padhye U. Excess dietary iron is the root cause for increase in childhood autism and allergies. *Med Hypotheses.* (2003) 61:220–2. doi: 10.1016/s0306-9877(03)00126-9

24. Tseng P-T, Cheng Y-S, Chen Y-W, Stubbs B, Whiteley P, Carvalho AF, et al. Peripheral iron levels in children with autism spectrum disorders vs controls: a systematic review and meta-analysis. *Nutr Res.* (2018) 50:44–52. doi: 10.1016/j.nutres.2017.11.004

25. Chauhan A, Chauhan V, Brown WT, Cohen I. Oxidative stress in autism: increased lipid peroxidation and reduced serum levels of ceruloplasmin and transferrin-the antioxidant proteins. *Life Sci.* (2004) 75:2539–49. doi: 10.1016/j.lfs.2004.04.038

26. Crichton R, Charloteauxwauters M. Iron transport and storage. *Eur J Biochem.* (1987) 164:485.

27. Punnonen K, Irjala K, Rajamäki A. Iron-deficiency anemia is associated with high concentrations of transferrin receptor in serum. *Clin Chem.* (1994) 40:774–6.

28. Latif A, Heinz P, Cook R. Iron deficiency in autism and asperger syndrome. *Autism.* (2002) 6:103–14. doi: 10.1177/1362361302006001008

29. Cacoub P, Vandewalle C, Peoc'h K. Using transferrin saturation as a diagnostic criterion for iron deficiency: a systematic review. *Crit Rev Clin Lab Sci.* (2019) 56:526–32. doi: 10.1080/10408363.2019.1653820

30. Luck AN, Bobst CE, Kaltashov IA, Mason AB. Human serum transferrin: is there a link among autism, high oxalate levels, and iron deficiency anemia? *Biochemistry.* (2013) 52:8333–41. doi: 10.1021/bi401190m

31. Gładysz D, Krzywdzińska A, Hozyasz KK. Immune abnormalities in autism spectrum disorder—could they hold promise for causative treatment? *Mol Neurobiol.* (2018) 55:6387–435. doi: 10.1007/s12035-017-0822-x

32. Siniscalco D, Schultz S, Brigida AL, Antonucci N. Inflammation and neuro-immune dysregulations in autism spectrum disorders. *Pharmaceuticals.* (2018) 11:56.

33. Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, Li X-M, et al. Elevated immune response in the brain of autistic patients. *J Neuroimmunol.* (2009) 207:111–6.

34. Haddad G, Belosevic M. Transferrin-derived synthetic peptide induces highly conserved pro-inflammatory responses of macrophages. *Mol Immunol.* (2009) 46:576–86. doi: 10.1016/j.molimm.2008.07.030

35. Stafford JL, Belosevic M. Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. *Dev Comp Immunol.* (2003) 27:539–54. doi: 10.1016/s0145-305x(02)00138-6

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 04 January 2023
DOI 10.3389/fnut.2022.953326

Check for updates

OPEN ACCESS

EDITED BY
Andrew Scholey,
Swinburne University of Technology, Australia

REVIEWED BY
Giovanna Mobbili,
Marche Polytechnic University, Italy
Dongxu Wang,
Jiangsu University of Science and Technology, China

*CORRESPONDENCE
Ying Chen
✉ 2015019@htu.edu.cn
Liyong Huang
✉ liyongh0816@163.com

SPECIALTY SECTION
This article was submitted to Nutrition, Psychology and Brain Health,
a section of the journal Frontiers in Nutrition

RECEIVED 26 May 2022
ACCEPTED 25 November 2022
PUBLISHED 04 January 2023

CITATION
Yang X, Han M, Wang X, Wang J, Sun X, Zhang C, Yan S, Huang L and Chen Y (2023) Evaluation of the synergistic effects of epigallocatechin-3-gallate-loaded PEGylated-PLGA nanoparticles with nimodipine against neuronal injury after subarachnoid hemorrhage. *Front. Nutr.* 9:953326. doi: 10.3389/fnut.2022.953326

COPYRIGHT
© 2023 Yang, Han, Wang, Wang, Sun, Zhang, Yan, Huang and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Evaluation of the synergistic effects of epigallocatechin-3-gallate-loaded PEGylated-PLGA nanoparticles with nimodipine against neuronal injury after subarachnoid hemorrhage

Xianguang Yang[1], Mengguo Han[1], Xue Wang[1], Jian Wang[1], Xiaoxue Sun[1], Chunyan Zhang[1], Shuaiguo Yan[1], Liyong Huang[2,3]* and Ying Chen[1]*

[1]College of Life Sciences, Henan Normal University, Xinxiang, Henan, China, [2]Department of Neurosurgery, The First Affiliated Hospital of Xinxiang Medical University, Henan, China, [3]Henan Key Laboratory of Neurorestoratology, The First Affiliated Hospital of Xinxiang Medical University, Henan, China

Subarachnoid hemorrhage (SAH) is a devastating subtype of stroke with high mortality and morbidity. Although serious side effects might occur, nimodipine, a second-generation 1,4-dihydropyridine calcium channel blocker, is clinically used to improve neurological outcomes after SAH. Recently, (-)-epigallocatechin-3-gallate (EGCG) has been reported to inhibit Ca^{2+} overloading-induced mitochondrial dysfunction, oxidative stress, and neuronal cell death after SAH; however, low bioavailability, instability, and cytotoxicity at a high dose limited the clinical application of EGCG. To overcome these limitations, PEGylated-PLGA EGCG nanoparticles (EGCG-NPs) were constructed to enhance the bioavailability by using the double-emulsion method. Antioxidative activity, cytotoxicity, behavioral, and immunohistochemistry studies were carried out to determine the neuroprotective effectiveness after cotreatment with EGCG-NPs (75 mg/kg/d preconditioning for 7 days before SAH) and nimodipine (10 mg/kg/d after 30 min of SAH) by using *in vivo* SAH models. The optimized EGCG-NPs with a Box–Behnken design showed a small particle size of 167 nm, a zeta potential value of −22.6 mV, an encapsulation efficiency of 86%, and a sustained-release profile up to 8 days *in vitro*. Furthermore, EGCG-NPs (75 mg/kg/d) had superior antioxidative activity to free EGCG (100 mg/kg/d). EGCG-NPs combined with nimodipine exhibited significant synergistic effects against neuronal cell death by suppressing oxidative stress, Ca^{2+} overloading, mitochondrial dysfunction, and autophagy after SAH. These results suggest that cotreatment with EGCG-NPs and nimodipine may serve as a promising novel strategy for the treatment of SAH.

KEYWORDS

EGCG, subarachnoid hemorrhage, PLGA-PEG, nanoparticles, nimodipine, autophagy

Introduction

Green tea, the second most popular beverage in the world, is made from fresh leaves of *Camellia sinensis*, which contains abundant polyphenols. Polyphenols have been associated with providing beneficial health effects in the prevention of cancers, diabetes, and neurodegenerative and cardiovascular diseases (1–4). (-)-Epigallocatechin-3-gallate (EGCG) is one of the most abundant compounds, having excellent bioactivities even at low concentrations (plasma levels of $\leq$10 μM), such as antioxidant and anti-inflammatory properties (5, 6). However, the limitations of EGCG such as its low bioavailability, instability, short half-time, and cytotoxicity at a large dose have hampered its application in clinical settings. Encapsulated EGCG might be effective in overcoming the aforementioned disadvantages, which enhances its stability and increases its concentration in the plasma *via* slow and sustainable release (7). Therefore, several different nanoparticles (NPs) have been formulated, including liposomes, magnetic NPs, and polymeric NPs (8–12). Among these nanocarriers, PLGA is a safety European Medicines Agency (EMA)- and U.S. Food and Drug Administration (FDA)-approved drug carrier with higher biocompatibility and biodegradability. More importantly, PLGA microspheres provide sustained drug release over a period of a few weeks to months. Moreover, PLGA-encapsulated EGCG-NPs exhibited a more therapeutic effect against several cancers, such as non-small-cell lung cancer, breast cancer, and prostate cancer (13, 14). Most recently, loading of EGCG in PEGylated-PLGA NPs (EGCG-NPs) has been found to have neuroprotective effects with no major side effects against epilepsy and Alzheimer's disease, indicating that EGCG-NP administration might be a potential therapeutic strategy in clinical settings (15, 16).

Even though survivors of subarachnoid hemorrhage (SAH) have increased by 17% in the past few decades due to the advanced early diagnosis and interventional therapy, SAH, an uncommon subtype of stroke, remains a leading cause of death and severe disability worldwide (17). It has been reported that multiple factors, including inflammatory responses, oxidative stress, calcium overloading, and mitochondrial dysfunction, are associated with SAH progression and poor outcomes (18). Among them, calcium homeostasis is critical for the physiological maintenance of intra- and extracellular signaling cascades because overloaded calcium triggers secondary ischemia, which results in poor outcomes by inducing the narrowing of intracranial arteries and activating Ca^{2+} signaling pathway-induced cell life-and-death decisions (19). Therefore, blocking calcium channels is a promising strategy to counteract the elevation of intracellular calcium either from the endo- and/or exogenous pool after SAH. Indeed, several calcium antagonists exhibited neuroprotective properties, including nimodipine and nicardipine (20). Nimodipine, an L-type dihydropyridine calcium channel antagonist, is the only FDA-approved drug to improve neurological outcomes and to decrease mortality in patients with SAH (19, 21). The recommended dose of orally administered nimodipine is 30–120 mg every 4 h for 21 days. However, one of the serious side effects of nimodipine is hypoperfusion, which worsens neurological outcomes.

A mutual interplay between calcium signaling pathways and ROS signaling systems might contribute to the pathogenesis of various disorders. In our previous studies, EGCG has been found to act as a voltage-gated calcium channel (VGCC) antagonist by blocking the calcium influx from the extracellular space and as an antioxidative agent by reducing ROS generation (22–24). Indeed, the imbalance between ROS generation and reduction/scavenging inevitably resulted in oxidative stress, which was strongly associated with the diverse pathogenesis of early brain injury (EBI) after SAH. Kang et al. reported that EGCG decreased cell death processes and neurotoxic effects by inhibiting oxidative stress in the hippocampal tissue (25). However, some *in vivo* and *in vitro* pharmacological studies have demonstrated that EGCG at high concentrations exhibited acute and subacute toxicities (26). For example, 50 μM or 50 mg/kg/day of EGCG dramatically increased reactive oxygen species (ROS) generation and exacerbated mitochondria abnormalities (27–29). Several studies have demonstrated the neuroprotective efficacy of EGCG-NPs; however, none of them has been used in *in vivo* SAH therapy. Therefore, the aim of this study was to prepare PEGylated-PLGA EGCG-NPs and investigate its efficacy and safety against SAH. In this study, EGCG-NPs were characterized by particle size and morphology, polydispersity index, drug encapsulation efficiency, and drug loading capacity. The synergetic neuroprotective effects of EGCG-NPs (75 mg/kg/d) with nimodipine (10 mg/kg/d) were evaluated using *in vivo* and *in vitro* SAH models.

Materials and methods

Materials

mPEG-poly lactic-co-glycolic acid (PLGA) (75:25, 45,000) was purchased from Daigang Biomaterial Co., Ltd (Jinan, Shandong, China). EGCG (purity > 98%; Yuanye Biotechnology Co., Ltd, Shanghai, China) was dissolved in water (pH 3.0) and stored at −20°C. Nimodipine was obtained from Ruiyang Pharmaceutical Co., Ltd (Yi Yuan, Shandong, China). Lactate dehydrogenase (LDH), glutathione (GSH), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) kits were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). JC-1, Fluo-3 AM, DCFH-DA, Nissl staining kits, and enhanced chemiluminescence kits were purchased from Beyotime (Shanghai, China). CaMKII, Atg5, Beclin-1, and Mn-SOD antibodies were obtained from Proteintech Group, Inc

10.3389/fnut.2022.953326

TABLE 1 Box-Behnken design used for optimization of EGCG-loaded formulae.

Independent variables	Coded levels		
	Low level (−1)	Medium level (0)	High level (1)
X_1: EGCG (mg/ml)	1	5.5	10
X_2: PEG-PLGA (mg)	12	16	20
X_3: Tween-80 (%)	1.5	2.25	3
Dependent variables	**Goal**		
Y_1: Average particle size (nm)	Minimize		
Y_2: Zeta potential (mV)	Maximize in terms of absolute value		
Y_3: Encapsulation efficiency (%)	Maximize		
Y_4: Polydispersity index (PDI)	Minimize		

(Rosemont, IL, USA). SP-9002 SPlink detection and DAB kits were purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China).

Preparation of EGCG-NPs

EGCG-NPs were prepared by using the double-emulsion method in three steps: (1) EGCG was dissolved in 1 ml of the aqueous phase (W1) at pH 3.0 and emulsified with 1.5 ml of an oil phase (O) composed of ethyl acetate, which contained the dissolved polymeric matrix. Simple emulsion (W1/O) was prepared by using an ultrasound probe at 20% amplitude for 20 s; (2) 2 ml of 2.5% Tween-80 (W2) was added to W1/O, and the mixture was subjected to ultrasound at 20% amplitude for 2 min to yield a double emulsion (W1/O/W2); (3) 2 ml of 0.02% Tween-80 was added dropwise to stabilize the emulsion. Finally, the organic solvent was evaporated by stirring for 24 h.

Optimization

To obtain the optimal formulation, a 2^3 Box–Behnken design was used (Table 1). The effect of the independent variables [EGCG (X1), PEG-PLGA (X2), and Tween-80 (X3)] on the dependent variables [average particle size (Zav) (Y1), zeta potential (ZP) (Y2), polydispersity index (PDI) (Y3), and encapsulation efficiency (EE) (Y4)] was evaluated. A total of 15 experiments were required. The response Y was modeled using the following full second-order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where Y is the established response, β_0 is the intercept, and β_1-β_{33} are the measured coefficients computed from the observed experimental values of Y.

Physicochemical characterization

The Zav, PDI, and ZP were measured using dynamic light scattering and laser Doppler electrophoresis using an M3 PALS system in Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at a detection angle of 90°, 25°C with a 5-mW He–Ne laser operating at a 633-nm wavelength. Samples were diluted in water (1:10). The results were the averages of triplicate experiments with an average of 15 runs per measurement.

Morphology of EGCG-NPs

Transmission electron microscopy (TEM) (JEM 1010, JEOL, Japan) was used to investigate the morphology of the EGCG-NPs. A single drop of the diluted samples (1:10) was mounted on a carbon-coated copper grid, and then negative staining with uranyl acetate (2%) took place to visualize the particles.

Determination of the encapsulation efficiency

Free EGCG was separated from EGCG-NPs by filtration/centrifugation using an Amicon® Ultra-0.5 centrifugal filter device (Millipore® Co, Massachusetts, USA) for centrifugation at 12,000 × g for 30 min at 4°C. The concentration of EGCG was detected. An Agilent 1100 HPLC system with a UV detector was used for the HPLC analysis by using a C18 analytical column at 40°C. The optimized mobile phase consisted of acetonitrile and buffer. The flow rate was 0.8 ml/min, and the injection volume was set at 15 µl. The UV wavelength was set at 280 nm. Standards with a concentration range of 0.03–0.5 mg/ml were prepared. All experiments were carried out in triplicate. Then, encapsulation efficiency was calculated using the following equation:

$$EE = (\text{total amount of EGCG} - \text{free amount of EGCG}) / \text{total amount of EGCG} \times 100\%.$$

In vitro drug release

The release profile of EGCG from the polymeric matrix was determined by bulk equilibrium direct dialysis, as described by Albuquerque et al., with some modifications (30). For this test,

two different release mediums were chosen: one medium was composed of an acid buffer solution (pH 3.0) with ascorbic acid 0.25%, and the other medium was PBS 0.1 M buffer solution (pH 7.4) with ascorbic acid 0.25%. The dialysis sacs were equilibrated with the dissolution medium 1 h before the start of the experiment. After that, 5 ml of EGCG-NPs and free EGCG samples were enclosed in dialysis bags (Viskase Inc. MWCO 12–14,000, IL, USA) and then placed in 70 ml of buffers at 37°C with stirring at 50 rpm in a shaking incubator. At predetermined time intervals, 1 ml of the sample was withdrawn from the release medium to be analyzed with HPLC using the conditions described earlier. Then, the same amount of fresh medium was added to a dialysis container. Different kinetic models were used to adjust the data. The assay was carried out in triplicate.

Animals

The animal use and care protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Henan Normal University. A total of 60 adult male Kunming mice weighing 18–20 g were purchased from Zhengzhou University. All animals underwent institutional quarantine for 7 days before use. The animals were housed in a temperature-controlled (22 ± 3°C) and humidity-controlled (40–70%) environment with a 12-h light/dark cycle.

Experiment 1

To evaluate the antioxidant effect of EGCG-NPs, 24 mice were randomly divided into four groups ($n = 6$): Sham, SAH, SAH + free EGCG (150 mg/kg/d), and SAH + EGCG-NPs (75 mg/kg/d). The samples were collected from serum and ipsilateral hippocampus and cortex, and antioxidative enzymes activities, ROS level and the expression of Mn-SOD were detected. EGCG was intragastrically administered with water for 7 days before OxyHb injection, respectively.

Experiment 2

To explore the neuroprotective effects of cotreatment with EGCG-NPs and nimodipine, 30 mice were randomly assigned to five groups ($n = 6$): Sham, SAH, SAH + nimodipine (30 mg/kg), SAH + EGCG-NPs, and SAH + EGCG-NPs+nimodipine (10 mg/kg). Ipsilateral hippocampus and cortex were chosen for immunofluorescence and Nissl staining. Nimodipine was given after 30 min of SAH.

Mouse SAH model

SAH was induced in mice using the model reported by Huang et al. (31). The animals were placed in a prone position under 1% pentobarbital general anesthesia (50 mg/kg, intraperitoneally). The posterior cervical muscles were dissected through a suboccipital midline skin incision and retracted laterally. The exposed transparent atlantooccipital membrane was penetrated with a 30-gauge needle. Under spontaneous breathing, a 23-gauge needle without a point was percutaneously inserted into the skull at a controlled depth of 1.5 mm in the cross-position at 2 mm from the sagittal suture and 1 mm from the coronal suture. Subsequently, 50 μl (150 μmol/l) of OxyHb was injected through this hole into the subarachnoid space. Then, the animals were euthanized 48 h after SAH. Some were isolated the ipsilateral hippocanpus and cortex and stored at −80°C for enzyme activity assay. The others were perfused with 4% paraformaldehyde in phosphate buffer 0.1 M and then embedded in paraffin. Coronal sections of the brain with the cortex and hippocampus were chosen for the following studies.

LDH, GSH, SOD, T-AOC, and MDA assay

The LDH, GSH, SOD, T-AOC, and MDA contents in serum and ipsilateral hippocampus and cortex were determined by using specific commercial assay kits according to the manufacturers' instructions.

Measurement of intracellular reactive oxygen species

To detect the ROS levels of brain tissues, the brain sections were incubated with 10 μM of DCFH-DA at 37°C for 30 min. Excitation and emission wavelengths were set at 485 and 530 nm, respectively. The sections were then observed by fluorescence microscopy (AX10, Zeiss). Fluorescence intensities were quantified by using ImageJ software.

Cell culture and treatments

PC12 cells were grown in the RPMI 1640 medium with 5% fetal bovine serum (FBS), 10% horse serum, and 1% penicillin/streptomycin at 37°C under 5% CO_2. At 24 h after seeding the cells, OxyHb was added to a final concentration of 10 μM to establish an *in vitro* SAH model. Then, 25 μm of EGCG-NPs and 10 μm of nimodipine were used.

Measuring cytosolic Ca^{2+} concentration levels

To investigate the changes in the intracellular calcium level after SAH, the Ca^{2+} concentration was detected using Fluo-3 AM. In brief, 1×10^5 cells were incubated with 2 μM

of Fluo-3 AM for 1 h at 37°C in the dark. After gently washing the cells three times with Hanks' balanced salt solution (HBSS), an additional incubation was performed to equilibrate the intracellular dye concentration at room temperature. The fluorescence was continuously monitored at 10-s intervals for up to 8 min using a microplate fluorometer, with excitation wavelength of 506 nm and emission wavelength of 526 nm. The average fluorescence value was presented as relative fluorescence units (RFUs).

Immunohistochemistry assay

Deparaffinized sections were treated with 0.3% hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase activity. The sections were incubated in 0.01 M, pH 9.0 EDTA buffer for 20 min at 121°C and cooled to room temperature. After being blocked with 10% normal goat serum for 1 h at room temperature, the sections were subsequently incubated overnight with anti-Mn-SOD, CaMKII, anti-Atg5, and anti-Beclin-1 antibodies. After being extensively washed, the sections were then stained by using the SP-9002 SPlink detection kit. ImageJ software was used to evaluate the expression level.

Nissl staining

Nissl staining was performed to measure hippocampus injury. After being deparaffinized and hydrated with distilled water, the brain sections were incubated with 0.5% toluidine blue at 37°C for 30 min, then dehydrated with graded alcohols, and cleared in xylene. Finally, these sections were coverslipped using neutral resins and observed under a light microscope by a researcher who was entirely blind to experimental conditions. The neuronal density loss was estimated.

Neurological score evaluation

The modified Garcia scoring system was blindly assessed for neurological function at 48 h after SAH. The total score ranged from 3 to 18, and the scoring was used based on (1) spontaneous activity, (2) symmetry of limb movement, (3) climbing, (4) body proprioception, (5) the movement of forelimbs, and 6) response to vibrissal touch. Higher scores for the modified Garcia scoring system indicated a better neurological function.

Statistical analysis

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) program. All data were reported as means ± SD of three independent experiments. One-way ANOVA, followed by Tukey's *post-hoc* test, was performed for group comparison. The neurological scores were compared using a Kruskal–Wallis non-parametric test, followed by multiple-comparison procedures using Duncan's method. A statistical significance was set at a $P < 0.05$.

Results

Optimization study

Table 2 shows 15 experimental results of the optimization of EGCG-NPs formulation, with the obtained responses of dependent variables. The particle size of the EGCG-NPs varied from 94.97 to 256 nm and was directly proportional to the concentration of polymer and EGCG (Figure 1A). ZP was found to be in the range of −23 to −15.8 mV, which was inversely proportional to the concentration of polymer and EGCG, indicating that a low dose of EGCG and polymer led to an increase in ZP (Figure 1B). The observed PDI values varied from 0.077 to 0.243, revealing a narrow size distribution and good homogeneity of distribution (Table 2). The most influential parameters of PDI were the concentration of EGCG and polymer, suggesting that the high concentrations of EGCG and polymer induced very broad size distribution of nanoparticles (Figure 1C). The observed EE values varied from 78.62 to 85.97% (Table 2). In contrast to the lower encapsulation efficiency caused by an intermediate concentration of polymer and EGCG-NPs, either a high or a low concentration of polymer and EGCG will increase the encapsulation efficiency, indicating that larger amounts of EGCG can be loaded in the developed NPs (Figure 1D). The multiple regression analysis was performed using the following polynomial equations:

$$\begin{aligned}
Y_1 &= 24.36746 - 20.46965X_1 + 31.52943X_2 \\
&\quad +182.20278X_3 - 1.84431X_1X_2 - 2.29083X_1X_3 \\
&\quad -5.39111X_2X_3 + 1.02495X_1^2 + 1.43811X_2^2 - 31.56148X_3^2 \\
Y_2 &= -58.99547 + 3.68125X_1 + 0.80474X_2 + 4.70611X_3 \\
&\quad -0.074583X_1X_2 - 0.63083X_1X_3 + 0.48889X_2X_3 \\
&\quad -0.054427X_1^2 - 0.05177X_2^2 + 0.86519X_3^2 \\
Y_3 &= -0.12365 - 1.77083X_1 + 0.02815X_2 + 0.19430X_3 \\
&\quad -1.500002X_1X_2 - 8.83333X_1X_3 + 3.7037X_2X_3 \\
&\quad +7.91667X_1^2 - 1.25103X_2^2 - 0.01125X_3^2 \\
Y_4 &= 110.24262 - 1.72972X_1 + 0.097531X_2 - 11.60481X_3 \\
&\quad -0.041528X_1X_2 + 0.56417X_1X_3 - 0.25852X_2X_3 \\
&\quad +0.014609X_1^2 + 0.087346X_2^2 + 1.11778X_3^2.
\end{aligned}$$

TABLE 2 Box-Behnken design matrix with values and the measured response.

Run	X_1	X_2	X_3	Y_1	Y_2	PDI	Y_3
1	0	0	0	111.07 ± 1.69	−20.47 ± 1.17	0.142 ± 0.02	79.66 ± 1.23
2	1	1	0	135.87 ± 1.95	−20.37 ± 0.78	0.077 ± 0.01	81.89 ± 2.01
3	0	−1	−1	121.07 ± 4.40	−20.87 ± 0.35	0.132 ± 0.04	84.7 ± 0.87
4	−1	−1	0	118.47 ± 2.41	−23 ± 0.43	0.131 ± 0.04	85.6 ± 1.34
5	0	0	0	119.97 ± 1.95	−20.6 ± 1.30	0.118 ± 0.04	82.17 ± 0.79
6	−1	0	−1	113.83 ± 5.02	−22.8 ± 1.31	0.103 ± 0.07	84.42 ± 0.96
7	0	1	−1	189.1 ± 2.55	−21.7 ± 0.78	0.111 ± 0.04	85.65 ± 1.52
8	−1	1	0	256 ± 4.80	−20.23 ± 0.31	0.158 ± 0.02	84.61 ± 1.68
9	1	−1	0	131.13 ± 4.33	−19.77 ± 1.00	0.158 ± 0.05	85.97 ± 1.07
10	1	0	−1	133.8 ± 5.05	−17.8 ± 0.46	0.11 ± 0.03	78.62 ± 0.82
11	−1	0	1	106.93 ± 3.47	−18.03 ± 0.58	0.243 ± 0.07	84.7 ± 2.54
12	0	1	1	94.97 ± 0.28	−15.8 ± 0.95	0.117 ± 0.05	83.33 ± 2.17
13	1	0	1	99.41 ± 1.31	−20.6 ± 0.79	0.144 ± 0.04	85.67 ± 1.62
14	0	0	0	113.5 ± 0.56	−17.2 ± 0.17	0.171 ± 0.01	85.64 ± 0.77
15	0	−1	1	99.72 ± 0.86	−21.57 ± 1.41	0.088 ± 0.04	85.87 ± 1.46

X1, X2, X3, Y1, Y2, Y3, Y4. Y_1, Average particle size (nm); Y_2, Zeta potential (mV); Y_3, Encapsulation efficiency (%); Y_4, Polydispersity index (PDI).

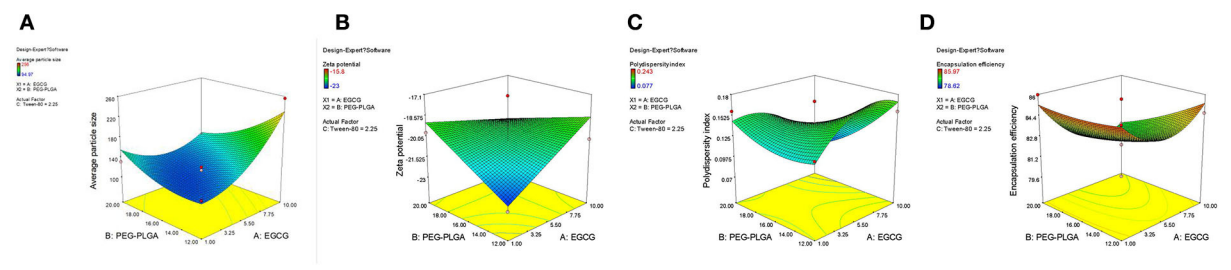


FIGURE 1
3D surface of the effect of EGCG-NPs. **(A)** Zav surface response at a fixed EGCG concentration of 1.5 mg/ml. **(B)** ZP response at a fixed Tween-80 concentration of 2.25%. **(C)** PDI response. **(D)** EE response at a fixed Tween-80 concentration of 2.25%.

Validation of optimized EGCG-NPs formulation

To promote drug release from the polymeric matrix, a high drug-loading formulation with low-surfactant level was used to develop nanoparticles. Based on the Box–Behnken design, the optimal formulation with 7.0 mg/ml of EGCG, 12 mg/ml of PLGA-PEG, and Tween-80 1.2% was obtained. The optimized formulation was composed of 167 nm Zav, −22.6 mV ZP, 86% EE, and 0.136 PDI (Figures 2A, B). The morphology of the optimized EGCG-NPs formulation was observed using TEM, with the microspheres showing no aggregation (Figure 2C). The diameter of the EGCG-NPs was consistent with the results obtained from an electrophoretic light-scattering spectrophotometer experiment. PEG located toward the aqueous phase creates a smooth surface and a hydrophilic layer surrounding the particles, which increases the stability of the sample.

In vitro release of EGCG-NPs

The diffusion/degradation process of the polymer matrix is the limited step to control the encapsulated drugs released from the polymeric delivery systems (32). *In vitro* release tests were performed to investigate the EGCG-NPs release profile on pH 3.0 and pH 7.0 medium (Figure 3). EGCG showed an initial burst (in acid buffer and in PBS) owing to the superficially entrapped drug. Free EGCG showed faster kinetics than EGCG-NPs. After 6 h, more than 90% of free drugs were released,

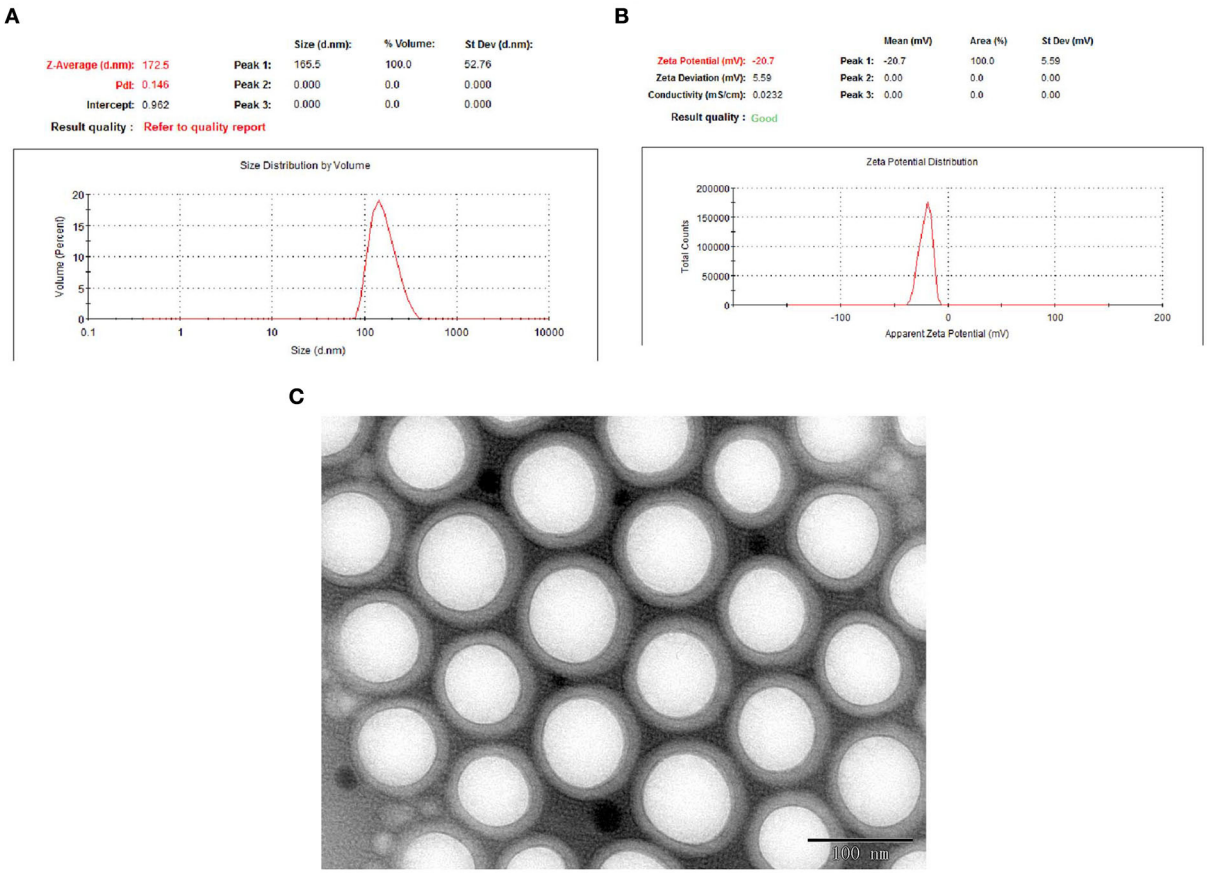


FIGURE 2
Optimization of EGCG-NPs. Particle size distribution of EGCG-NPs measured with electrophoretic light-scattering (ELS) analyzer **(A)**. Zeta potential measurement of EGCG-NPs **(B)**. TEM image of EGCG-NPs **(C)**. Scale bar = 100 nm.

whereas the released EGCG-NPs were ∼10% of the initial amount. The best fit with the highest correlation was found for the hyperbola model for free EGCG, EGCG-NPs in acid buffer, and EGCG-NPs in PBS, indicating a diffusion-governed release.

EGCG-NPs inhibition OxyHb-induced oxidative stress

To determine whether EGCG-NPs have stronger antioxidative activity than free EGCG, we examined the T-AOC, GSH, SOD, and MDA after SAH. As shown in Figures 4A–D, the lowest serum level of T-AOC, GSH, and SOD in the SAH group ($P < 0.05$ vs. Sham) was restored by both EGCG and EGCG-NPs groups ($P < 0.05$ vs. SAH). However, T-AOC activity reached its highest peak after EGCG-NPs treatment, but there was no significant difference between EGCG and EGCG-NPs groups. In contrast to the EGCG-induced upregulation of GSH and SOD, EGCG-NPs enhanced the GSH and SOD ($P < 0.01$ vs. Sham; $P < 0.01$ vs. SAH) activities and showed a significant difference compared with the EGCG group ($P < 0.01$ vs. EGCG; $P < 0.05$ vs. EGCG). As a result, the serum MDA level was significantly downregulated by EGCG-NPs ($P < 0.01$ vs. EGCG). Similar to the serum GSH, T-AOC, SOD, and MDA levels, the activities of antioxidative enzymes in ipsilateral hippocampus and cortex were higher in the EGCG-NPs group than in the EGCG group (Figures 4E–H).

EGCG-NPs reduced ROS generation by increasing Mn-SOD activity after SAH

In order to investigate the mitochondrial function after EGCG-NPs treatment, we measured the changes in intracellular ROS and Mn-SOD levels. OxyHb exposure significantly increased the intracellular ROS level ($P < 0.01$ vs. Sham), whereas preconditioning EGCG and EGCG-NPs decreased the ROS level ($P < 0.01$ vs. SAH) (Figures 5A, B). Notably, there is a significant difference between these two groups ($P < 0.01$ vs. EGCG). Considering that Mn-SOD is a major player in

10.3389/fnut.2022.953326

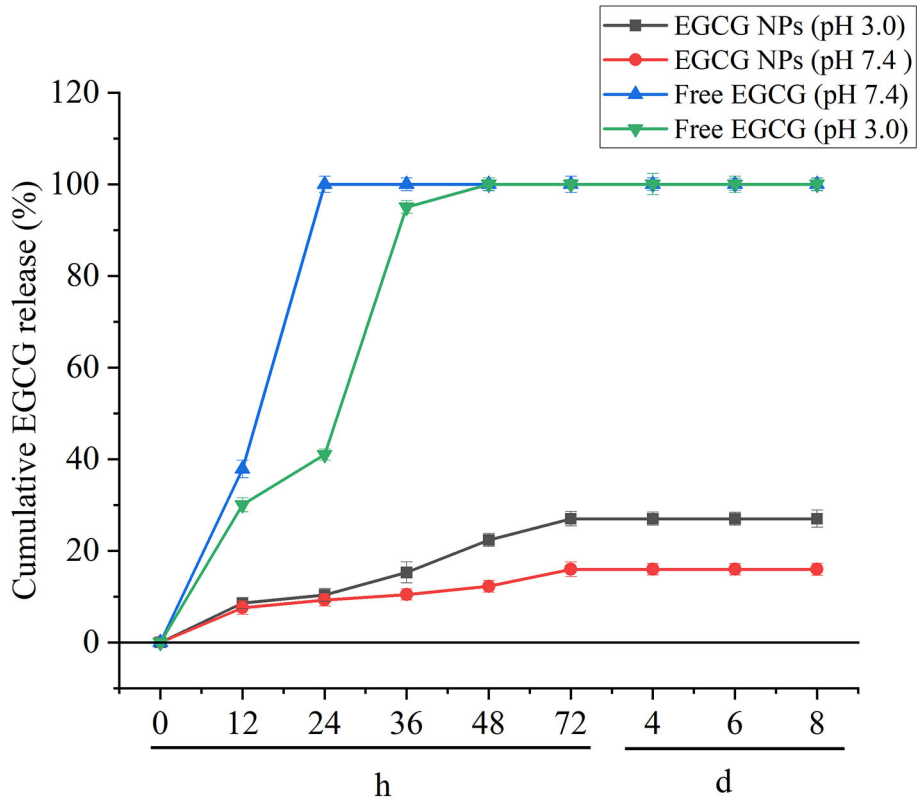


FIGURE 3
In vitro EGCG release profile of free EGCG and EGCG-NPs in pH 3.0 and pH 7.4 buffers. Data are expressed as mean ± SD ($n = 3$).

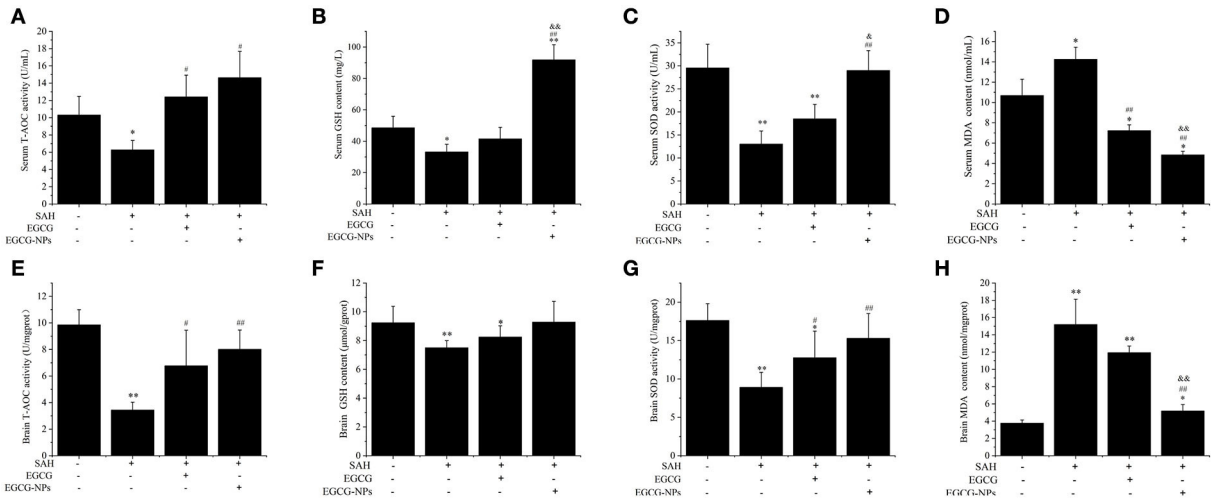


FIGURE 4
Effects of EGCG-NPs on the regulation of antioxidative enzymes activities after SAH. Serum T-AOC, GSH, SOD activities, and MDA level, respectively **(A–D)**. T-AOC, GSH, SOD activities, and MDA levels in the brain, respectively **(E–H)**. Values represent three independent experiments. $^{*}P < 0.05$ vs. Sham, $^{**}P < 0.01$ vs. Sham; $^{\#}P < 0.05$ vs. SAH, $^{\#\#}P < 0.01$ vs. SAH; $^{\&}P < 0.05$ vs. EGCG, $^{\&\&}P < 0.01$ vs. EGCG.

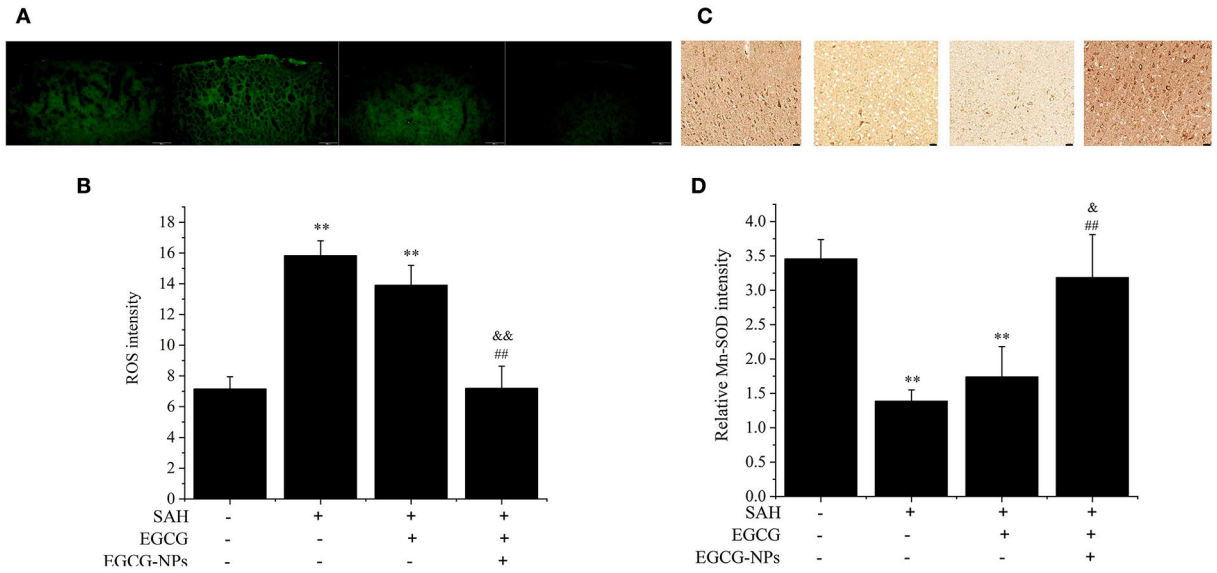


FIGURE 5
Effects of EGCG-NPs on the mitochondrial oxidative stress after SAH. ROS fluorescence distribution in the brain detected by fluorescence microscopy **(A)**. Quantification of ROS fluorescence intensity after SAH (scale bar = 100 μm) **(B)**. Identification of the expression of Mn-SOD by the immunohistochemical assay (scale bar = 20 μm) **(C)**. Quantification of the Mn-SOD level by densitometry **(D)**. Quantification represents the means and standard deviations of the results from three independent experiments. $^{**}P < 0.01$ vs. Sham; $^{\#\#}P < 0.01$ vs. SAH; $^{\&}P < 0.05$ vs. EGCG; $^{\&\&}P < 0.01$ vs. EGCG.

scavenging ROS in mitochondria, we next detected Mn-SOD expression (Figures 5C, D). Consistent with the ROS level, the low expression of Mn-SOD in SAH and EGCG groups was significantly increased in the EGCG-NPs group ($P <$ 0.01 vs. SAH, $P < 0.01$ vs. EGCG), suggesting that EGCG-NPs downregulated ROS generation after SAH mainly by maintaining the mitochondrial function.

Cotreatment with EGCG-NPs and nimodipine inhibition Ca^{2+} overloading and CaMKII activation after SAH

Previous studies have demonstrated that Ca^{2+} overloading is a major player inducing secondary brain injury. To determine the effects of EGCG-NPs and nimodipine on Ca^{2+} concentration after SAH, the cytosolic Ca^{2+} level was measured using Fluo-3 AM. A high Ca^{2+} level in the SAH group can be downregulated by preconditioning EGCG-NPs and nimodipine, but there is no significant difference between these two groups ($P < 0.05$ vs. Sham). However, EGCG-NPs + nimodipine showed stronger effects on the regulation of calcium homeostasis than EGCG-NPs or nimodipine, and a significant difference was observed ($P < 0.01$ vs. SAH; $P < 0.05$ vs. EGCG; $P < 0.01$ vs. nimodipine) (Figure 6A).

Calcium overloading is an important pathological event in SAH development, which activates the CaMKII-mediated signaling pathway. In this study, the expression of CaMKII was markedly activated after SAH, but EGCG-NPs, nimodipine, and cotreatment with EGCG-NPs and nimodipine significantly inhibited the expression of CaMKII ($P < 0.01$ vs. SAH) (Figures 6B, C). Compared with EGCG-NPs, both nimodipine and EGCG-NPs + nimodipine dramatically inhibited the expression of CaMKII ($P < 0.05$ vs. EGCG, $P < 0.01$ vs. EGCG, respectively), but there was no statistically significant difference between these two groups.

EGCG-NPs synergistic nimodipine reduction autophagy after SAH

Activated CaMKII can directly phosphorylate Beclin-1, activating autophagy, which is the main pathway for eliminating abnormal mitochondria. As shown in Figure 7A, the expression of Beclin-1 was similar to that of CaMKII. In normal brain tissue, faint Beclin-1 staining signals were observed, whereas strong Beclin-1 staining signals were found in the SAH group ($P <$ 0.01 vs. Sham). The moderate Beclin-1 was only observed in the EGCG-NPs group, but not in the nimodipine and EGCG-NPs + nimodipine group, indicating that the expression of Beclin-1 largely relied on the CaMKII activation (Figure 7B). As for Atg5, the strongest staining signals were detected in the SAH group ($P < 0.01$ vs. Sham). The expression of Atg5 can be inhibited in the other three groups ($P < 0.01$ vs. SAH), but faint signals

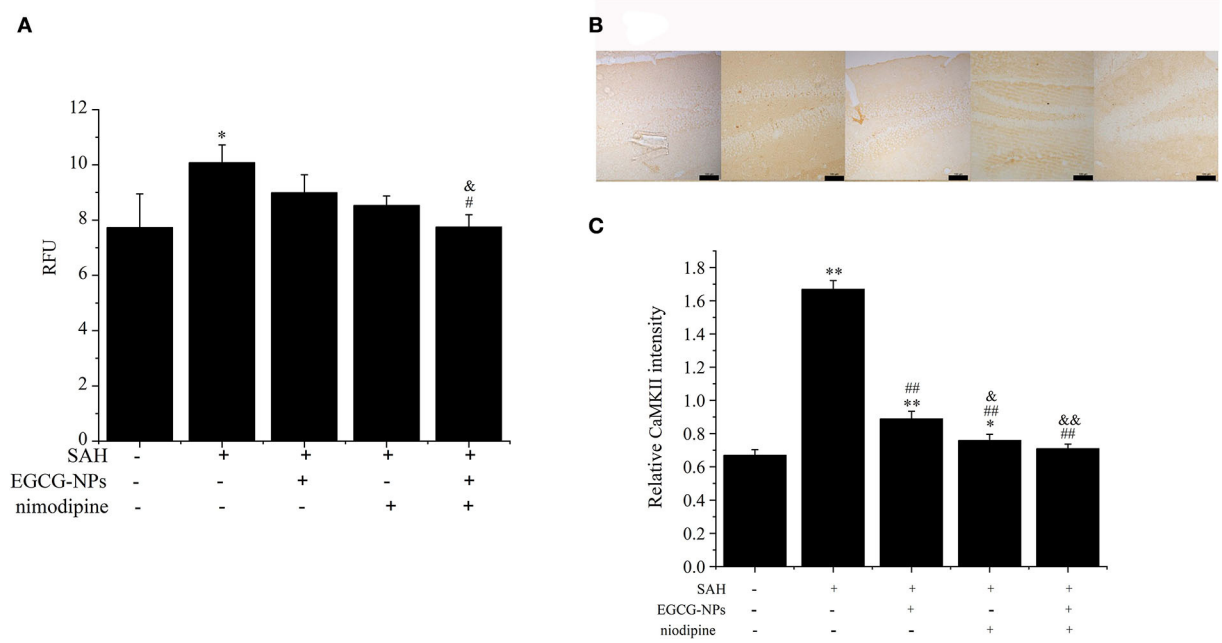


FIGURE 6
Cotreatment of EGCG-NPs and nimodipine attenuated the intracellular Ca^{2+} levels and the expression of CaMKII after SAH. EGCG-NPs + nimodipine significantly attenuated the Ca^{2+} overloading after SAH **(A)**. Fluorescence was monitored at a measurement frequency of 10 s using a fluorescence plate reader. Cotreatment of EGCG-NPs and nimodipine inhibited the expression of CaMKII **(B)**, scale bar = 100 μm. Quantification of the CaMKII level by densitometry **(C)**. Scale bar = 100 μm. $^{*}P < 0.05$ vs. Sham, $^{**}P < 0.01$ vs. Sham; $^{\#}P < 0.05$ vs. SAH, $^{\#\#}P < 0.01$ vs. SAH; $^{\&}P < 0.05$ vs. EGCG, $^{\&\&}P < 0.01$ vs. EGCG.

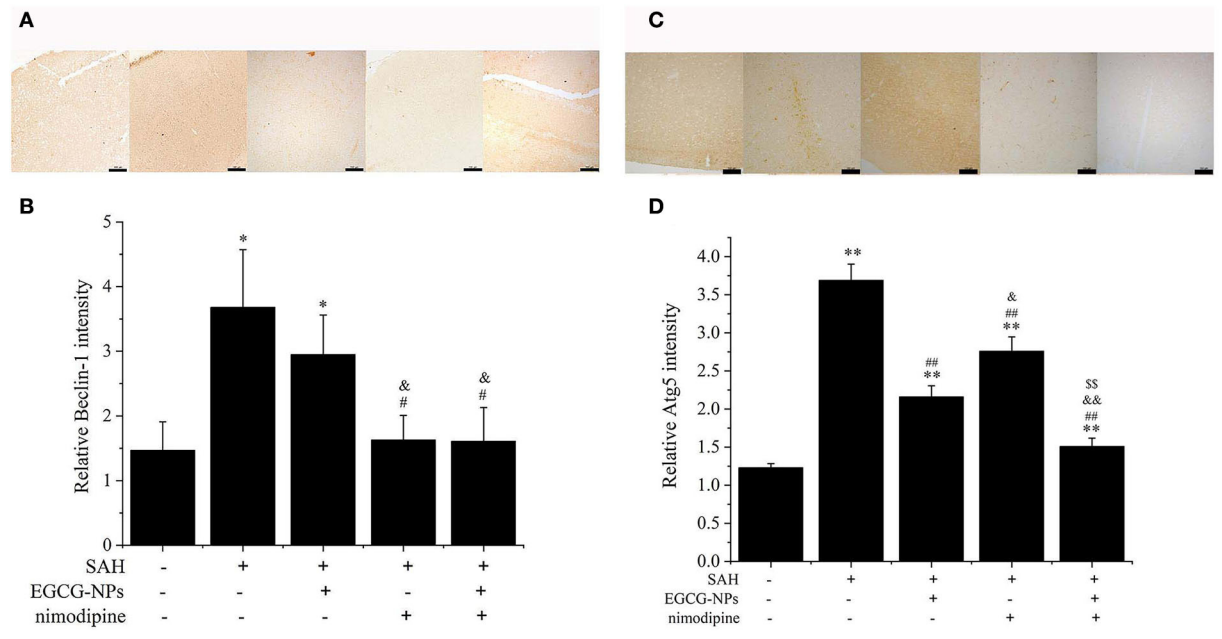


FIGURE 7
Cotreatment of EGCG-NPs and nimodipine inhibited autophagy induced by OxyHb. Identification of the expression of Beclin-1, scale bar = 100 μm **(A)**. Quantification of the expression of Beclin-1 **(B)**. Immunohistochemical microscope image of Atg5, scale bar = 100 μm **(C)**. Quantification of the Atg5 level by densitometry **(D)**. $^{*}P < 0.05$ vs. Sham, $^{**}P < 0.01$ vs. Sham; $^{\#}P < 0.05$ vs. SAH, $^{\#\#}P < 0.01$ vs. SAH; $^{\&}P < 0.05$ vs. EGCG, $^{\&\&}P < 0.01$ vs. EGCG; $^{\$\$}P < 0.01$ vs. nimodipine.

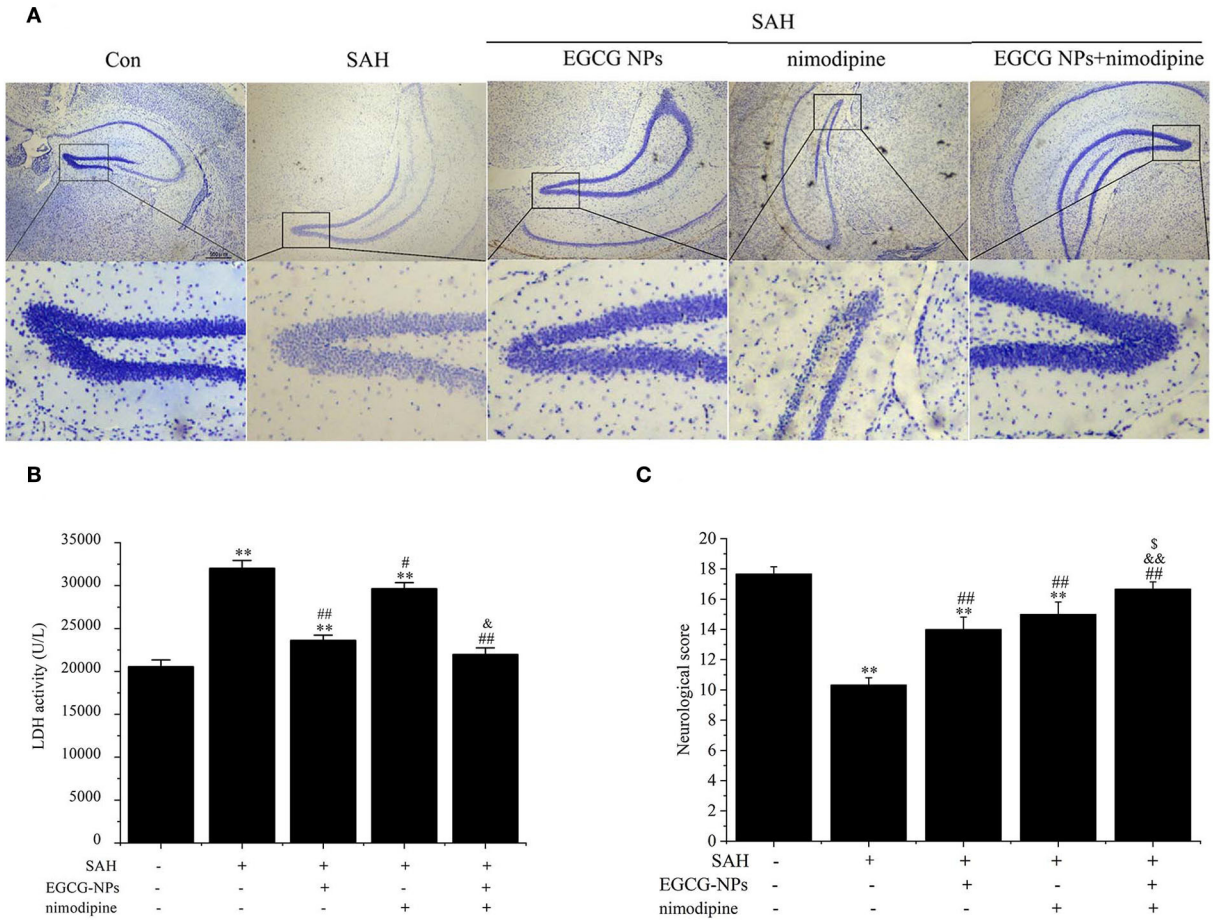


FIGURE 8
Cotreatment of EGCG-NPs and nimodipine significantly improves the pathological changes after SAH. Representative micrographs of Nissl staining in the hippocampal DG region, scale bar = 500 μm **(A)**. LDH levels after SAH **(B)**. Neurological deficits after SAH **(C)**. Results are presented as the means and standard deviations of the results of three independent experiments. $^{**}P < 0.01$ vs. Sham; $^{\#}P < 0.05$ vs. SAH, $^{\#\#}P < 0.01$ vs. SAH; $^{\&}P < 0.05$ vs. EGCG, $^{\&\&}P < 0.01$ vs. EGCG; $^{\$}P < 0.05$ vs. nimodipine.

were observed in EGCG-NPs+ nimodipine group ($P < 0.01$ vs. EGCG; $P < 0.01$ vs. nimodipine) (Figures 7C, D).

Synergistic effects of EGCG-NPs and nimodipine improve the pathological changes after SAH

Nissl staining also showed that both EGCG-NPs and nimodipine treatment groups showed an obvious reverse to the severe damage in the dentate gyrus areas of the hippocampus after SAH (Figure 8A). However, there was significantly less neuron loss and shrinkage morphology of neurons in cotreatment with EGCG-NPs and nimodipine groups than those in either EGCG-NPs or nimodipine group.

The neuroprotective role of EGCG-NPs was also evaluated by detecting the LDH level, which was widely regarded as a cell injury marker after SAH (Figure 8B). The LDH level was significantly increased after SAH ($P < 0.01$ vs. Sham), whereas treatment with EGCG-NPs and nimodipine significantly downregulated LDH activity. Specifically, treatment with EGCG-NPs led to a lower LDH activity than treatment with the nimodipine group ($P < 0.01$ vs. EGCG-NPs). However, the lowest LDH level was observed in cotreatment with EGCG and nimodipine groups ($P < 0.01$ vs. Sham, $P < 0.01$ vs. EGCG-NPs, $P < 0.01$ vs. nimodipine), which almost reached the normal level.

Similar to the LDH and Nissl staining results, the neurological scores, as shown in Figure 8C, were significantly lower 48 h after SAH than those of the Sham group ($P < 0.01$ vs. Sham). However, both EGCG-NPs and nimodipine improved neurological scores, with no significant difference between these two groups ($P < 0.01$ vs. Sham; $P < 0.01$ vs. SAH). Cotreatment with EGCG-NPs and nimodipine considerably improved the

neurological performance, showing a statistically significant difference with either EGCG-NPs or nimodipine ($P < 0.01$ vs. Sham; $P < 0.01$ vs. SAH; $P < 0.01$ vs. EGCG-NPs; $P < 0.05$ vs. nimodipine), indicating that impaired behavior function caused by SAH can be restored by EGCG-NPs + nimodipine.

Discussion

A growing body of evidence supports a correlation between EGCG intake and a reduced risk of central nervous diseases (33, 34). Recently, we have reported the efficacy of EGCG in SAH treatment by inhibiting calcium-induced autophagy (22–24). This study describes EGCG-NPs as a novel therapeutic approach for the treatment of SAH, including the following novel findings: (1) PEGylated-PLGA-loaded EGCG-NPs were successfully synthesized; (2) EGCG-NPs exhibited stronger antioxidative stress activity than free EGCG; (3) cotreatment with EGCG-NPs and nimodipine inhibited Ca^{2+} overloading and CaMKII activity; (4) cotreatment with EGCG-NPs and nimodipine eliminated dysfunctional mitochondria by autophagy; and (5) EGCG-NPs + nimodipine significantly reversed histopathological alterations after SAH.

Even though free EGCG has been found to exhibit antioxidative activities and mitochondrial protective effects after SAH, its poor bioavailability, low stability, and potential toxicity restricted its usage in clinical settings. Therefore, a nanoparticle formulation based on the biodegradable PLGA polymer was performed. Previous research studies have synthesized PEGylated-PLGA-loaded EGCG-NPs, showing an increase in the penetrating capability of the blood–brain barrier (BBB). However, low drug loading restricted its use both in the laboratory and clinical studies (10). In this study, 7 mg of EGCG was used to synthesize EGCG-NPs, and highly monodisperse and uniform EGCG-NPs were spherical in shape and had an average diameter of 167 nm, a PDI value of 0.136, a zeta potential value of −22.6 mV, and an EE of 86%. Particles smaller than 200 nm and the surfactant (Tween-80) might enhance the penetration of EGCG-NPs through the BBB. In addition, a suitable release of the drug from PLGA resulted in a prolonged permanence time in blood. Polymeric surface modification with PEG has also been demonstrated to enhance the rapid spread of carried drugs within brain tissue by reducing the reticuloendothelial system uptake (35). As a result, synthesized EGCG-NPs might exhibit stronger bioavailability and stability than free EGCG. These findings are consistent with the present study showing that the low concentration of EGCG-NPs (75 mg/kg) had stronger antioxidative properties than free EGCG (150 mg/kg) in the treatment of SAH, that is, upregulation of GSH, SOD, and T-AOC activities and downregulation of MDA and ROS levels.

ROS are not only harmful oxidative stressors but also function as well-accepted second messengers by regulating target proteins in a variety of physiological and pathological conditions, as well as in SAH progression. ROS modulates plasma membrane/intracellular Ca^{2+} channels and Ca^{2+} ATPase activities to maintain intracellular calcium homeostasis (36). Calcium signals, in turn, are essential for the formation of free radicals because an elevated intracellular Ca^{2+} level regulates ROS-generating enzymes (37). Considering that EGCG-NPs inhibited ROS generation after SAH, the intracellular Ca^{2+} level was detected. The level of Ca^{2+} concentration in cotreatment with EGCG-NPs and nimodipine groups was significantly lower than that in either EGCG-NPs or nimodipine group. In recent years, substantial antagonists/agonists have been chosen to block or activate one signaling pathway but, finally, failed to be successfully used in the clinic, such as NO donors, calcium channel blockers, antioxidants, and anti-inflammatory agents. Subsequently, some researchers proposed that the combination of two or multiple treatments may improve the SAH outcome due to the cross-talk between pathological pathways. In our previous studies, EGCG has been reported to reduce Ca^{2+} overloading, reduce ROS generation, and eventually prevent mitochondrial function and neuronal cell death. Therefore, EGCG combined with nimodipine might be a novel strategy in the treatment of SAH by targeting ROS and calcium signaling pathways. Due to the pivotal roles of mitochondria in regulating ROS generation, Ca^{2+} homeostasis, and cell growth and differentiation, a growing body of studies is focusing on developing strategies to precisely maintain sustainable mitochondrial function. In line with previous studies, cotreatment with EGCG-NPs significantly reduced mitochondrial oxidative stress than free EGCG, indicating that EGCG-NPs might directly protect mitochondrial function after SAH.

The accumulation of dysfunctional mitochondria was deleterious and caused serious consequences, even led to cell death. Autophagy plays pivotal roles in cellular quality control by alleviating abnormal mitochondria; therefore, moderate activation of autophagy predominantly acts as a pro-survival pathway after SAH (38–41). On the other hand, either defective or excess autophagy can promote cell injury and death after SAH (42–44). CaMKII, the major calcium-dependent signaling protein, has been evoked either by intracellular Ca^{2+} overloading or by ROS (45–47). Recently, CaMKII has been reported to directly phosphorylate Beclin-1 at Ser90, resulting in the ubiquitination of Beclin-1 and initiation of autophagy (48). Therefore, the CaMKII signaling pathway might be a potential target in SAH therapy (49–51). Bensalem et al. have demonstrated that polyphenols upregulated the expression of CaMKII to inhibit a cognitive decline in middle-aged mice (52). Green tea polyphenols also exhibited a similar bioactivity profile by upregulation of the expression of CaMKII to attenuate cognitive deficits (53). In contrast to these findings, our study showed that the over-expression of CaMKII activated autophagy through Beclin-1 and Atg5 in SAH, whereas

cotreatment with EGCG-NPs and nimodipine downregulated autophagy by maintaining calcium homeostasis, indicating that EGCG-NPs synergistically improved the neuroprotective effects of nimodipine by regulating mitochondrial function, ROS generation, and antioxidative enzyme activation and eventually protecting neuronal cells against OxyHb insult.

Conclusion

Results of this study confirmed that the EGCG-NP formulation represents a promising drug delivery strategy to enhance its antioxidative activity and related mitochondrial protection. The findings clearly indicated that cotreatment with EGCG-NPs and nimodipine exhibited stronger neuroprotective effects by maintaining calcium homeostasis, mitochondrial function, and autophagy flux. Taken together, EGCG-NPs combined with nimodipine can be regarded as a potential novel pharmacological strategy in SAH.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Henan Normal University.

Author contributions

XY, YC, and LH designed the study. XS, MH, and JW performed the experiments. XW, CZ, and MH performed data collection. XS, CZ, SY, and JW performed statistical analyses. XY, MH, LH, and YC wrote the paper. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Henan Key Laboratory of Neurorestoratology (HNSJXF-2021-007).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Khan N, Mukhtar H. Tea polyphenols in promotion of human health. *Nutrients.* (2018) 11:39. doi: 10.3390/nu11010039

2. Suzuki T, Pervin M, Goto S, Isemura M, Nakamura Y. Beneficial effects of tea and the green tea catechin epigallocatechin-3-gallate on obesity. *Molecules.* (2016) 21:1305. doi: 10.3390/molecules21101305

3. Di Lorenzo A, Curti V, Tenore GC, Nabav SM, Dagli M. Effects of tea and coffee consumption on cardiovascular diseases and relative risk factors: an update. *Curr Pharm Des.* (2017) 23:2474–87. doi: 10.2174/1381612823666170215145855

4. Yan L, Guo MS, Zhang Y, Yu L, Wu JM, Tang Y, et al. Dietary plant polyphenols as the potential drugs in neurodegenerative diseases: current evidence, advances, and opportunities. *Oxid Med Cell Longev.* (2022) 2022:5288698. doi: 10.1155/2022/5288698

5. Higdon JV, Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr.* (2003) 43:89–143. doi: 10.1080/10408690390826464

6. Meng Q, Velalar CN, Ruan R. Regulating the age-related oxidative damage, mitochondrial integrity, and antioxidative enzyme activity in fischer 344 rats by supplementation of the antioxidant epigallocatechin-3-gallate. *Rejuvenation Res.* (2008) 11:649–60. doi: 10.1089/rej.2007.0645

7. Granja A, Pinheiro M, Reis S. Epigallocatechin gallate nanodelivery systems for cancer therapy. *Nutrients.* (2016) 8:307. doi: 10.3390/nu8050307

8. Yavarpour-Bali H, Ghasemi-Kasman M, Pirzadeh M. Curcumin-loaded nanoparticles: a novel therapeutic strategy in treatment of central nervous system disorders. *Int J Nanomedicine.* (2019) 14:4449–60. doi: 10.2147/IJN.S208332

9. Safwat MA, Kandil BA, Elblbesy MA, Soliman GM, Eleraky NE. Epigallocatechin-3-Gallate-loaded gold nanoparticles: preparation and evaluation of anticancer efficacy in ehrlich tumor-bearing mice. *Pharmaceuticals.* (2020) 13:254. doi: 10.3390/ph13090254

10. Cano A, Ettcheto M, Chang JH, Barroso E, Espina M, Kühne BA, et al. Dual-drug loaded nanoparticles of Epigallocatechin-3-gallate (EGCG)/Ascorbic acid enhance therapeutic efficacy of EGCG in a APPswe/PS1dE9 Alzheimer's disease mice model. *J Control Release.* (2019) 301:62–75. doi: 10.1016/j.jconrel.2019.03.010

11. Han MG, Wang X, Wang J, Lang DC, Xia XH, Jia YF, et al. Ameliorative effects of epigallocatechin-3-gallate nanoparticles on 2,4-dinitrochlorobenzene induced atopic dermatitis: A potential mechanism of inflammation-related necroptosis. *Front Nutr.* (2022) 9:953646. doi: 10.3389/fnut.2022.95364

12. Minnelli C, Moretti P, Laudadio E, Gerelli Y, Pigozzo A, Armeni T, et al. Tuning curvature and phase behavior of monoolein bilayers by epigallocatechin-3-gallate: structural insight and cytotoxicity. *Colloids Surf B Biointerfaces.* (2022) 209 (Pt. 1):112171. doi: 10.1016/j.colsurfb.2021.112171

13. Zhang L, Chen W, Tu G, Chen X, Lu Y, Wu L, et al. Enhanced chemotherapeutic efficacy of PLGA-encapsulated epigallocatechin gallate (EGCG) against human lung cancer. *Int J Nanomedicine.* (2020) 15:4417–29. doi: 10.2147/IJN.S243657

14. Kazi J, Sen R, Ganguly S, Jha T, Ganguly S, Chatterjee Debnath M. Folate decorated epigallocatechin-3-gallate (EGCG) loaded PLGA nanoparticles; *in-vitro* and *in-vivo* targeting efficacy against MDA-MB-231 tumor xenograft. *Int J Pharm.* (2020) 585:119449. doi: 10.1016/j.ijpharm.2020.119449

15. Cano A, Ettcheto M, Espina M, Auladell C, Calpena AC, Folch J, et al. Epigallocatechin-3-gallate loaded PEGylated-PLGA nanoparticles: a new anti-seizure strategy for temporal lobe epilepsy. *Nanomedicine.* (2018) 14:1073–85. doi: 10.1016/j.nano.2018.01.019

16. Smith A, Giunta B, Bickford PC, Fountain M, Tan J, Shytle RD. Nanolipidic particles improve the bioavailability and alpha-secretase inducing ability of epigallocatechin-3-gallate (EGCG) for the treatment of Alzheimer's disease. *Int J Pharm.* (2010) 389:207–12. doi: 10.1016/j.ijpharm.2010.01.012

17. Macdonald RL, Schweizer TA. Spontaneous subarachnoid haemorrhage. *Lancet.* (2017) 389:655–66. doi: 10.1016/S0140-6736(16)30668-7

18. Sehba FA, Pluta RM, Zhang JH. Metamorphosis of subarachnoid hemorrhage research: from delayed vasospasm to early brain injury. *Mol Neurobiol.* (2011) 43:27–40. doi: 10.1007/s12035-010-8155-z

19. Feigin VL, Rinkel GJ, Algra A, Vermeulen M, van Gijn J. Calcium antagonists in patients with aneurysmal subarachnoid hemorrhage: a systematic review. *Neurology.* (1998) 50:876–83. doi: 10.1212/WNL.50.4.876

20. Neifert SN, Chapman EK, Martini ML, Shuman WH, Schupper AJ, Oermann EK, et al. Aneurysmal subarachnoid hemorrhage: the last decade. *Transl Stroke Res.* (2021) 12:428–46. doi: 10.1007/s12975-020-00867-0

21. Allen GS, Ahn HS, Preziosi TJ, Battye R, Boone SC, Boone SC, et al. Cerebral arterial spasm–a controlled trial of nimodipine in patients with subarachnoid hemorrhage. *N Engl J Med.* (1983) 308:619–24. doi: 10.1056/NEJM198303173081103

22. Chen Y, Chen J, Sun X, Shi X, Wang L, Huang L, et al. Evaluation of the neuroprotective effect of EGCG: a potential mechanism of mitochondrial dysfunction and mitochondrial dynamics after subarachnoid hemorrhage. *Food Funct.* (2018) 9:6349–59. doi: 10.1039/C8FO01497C

23. Zhang Y, Han M, Sun X, Gao G, Yu G, Huang L, et al. EGCG promotes neurite outgrowth through the integrin β1/FAK/p38 signaling pathway after subarachnoid hemorrhage. *Evid Based Complement Alternat Med.* (2021) 2021:8810414. doi: 10.1155/2021/8810414

24. Chen Y, Huang L, Zhang H, Diao X, Zhao S, Zhou W. Reduction in autophagy by (-)-Epigallocatechin-3-Gallate (EGCG): a potential mechanism of prevention of mitochondrial dysfunction after subarachnoid hemorrhage. *Mol Neurobiol.* (2017) 54:392–405. doi: 10.1007/s12035-015-9629-9

25. Kang KS, Wen Y, Yamabe N, Fukui M, Bishop SC, Zhu BT, et al. Dual beneficial effects of (-)-epigallocatechin-3-gallate on levodopa methylation and hippocampal neurodegeneration: *in vitro* and *in vivo* studies. *PLoS ONE.* (2010) 5:e11951. doi: 10.1371/journal.pone.0011951

26. Lee MJ, Wang ZY, Li H, Chen L, Sun Y, Gobbo S, et al. Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol Biomarkers Prev.* (1995) 4:393–9.

27. Elbling L, Weiss RM, Teufelhofer O, Uhl M, Knasmueller S, Schulte-Hermann R, et al. Green tea extract and (-)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. *FASEB J.* (2005) 19:807–9. doi: 10.1096/fj.04-2915fje

28. Weng Z, Zhou P, Salminen WF, Yang X, Harrill AH, Cao Z, et al. Green tea epigallocatechin gallate binds to and inhibits respiratory complexes in swelling but not normal rat hepatic mitochondria. *Biochem Biophys Res Commun.* (2014) 443:1097–104. doi: 10.1016/j.bbrc.2013.12.110

29. Miao Y, Sun X, Gao G, Jia X, Wu H, Chen Y, et al. Evaluation of (-)-epigallocatechin-3-gallate (EGCG)-induced cytotoxicity on astrocytes: a potential mechanism of calcium overloading-induced mitochondrial dysfunction. *Toxicol In Vitro.* (2019) 61:104592. doi: 10.1016/j.tiv.2019.104592

30. Albuquerque N, Neri JR, Lemos M, Yamauti M, de Sousa F, Santiago SL. Effect of polymeric microparticles loaded with catechin on the physicochemical properties of an adhesive system. *Oper Dent.* (2019) 44:E202–11. doi: 10.2341/18-112-L

31. Huang L, Wan J, Chen Y, Wang Z, Hui L, Li Y, et al. Inhibitory effects of p38 inhibitor against mitochondrial dysfunction in the early brain injury after subarachnoid hemorrhage in mice. *Brain Res.* (2013) 1517:133–40. doi: 10.1016/j.brainres.2013.04.010

32. Puri S, Kallinteri P, Higgins S, Hutcheon GA, Garnett MC. Drug incorporation and release of water soluble drugs from novel functionalized poly (glycerol adipate) nanoparticles. *J Control Release.* (2008) 125:59–67. doi: 10.1016/j.jconrel.2007.09.009

33. Fernandes L, Cardim-Pires TR, Foguel D, Palhano FL. Green tea polyphenol epigallocatechin-gallate in amyloid aggregation and neurodegenerative diseases. *Front Neurosci.* (2021) 15:718188. doi: 10.3389/fnins.2021.718188

34. Cai J, Jing D, Shi M, Liu Y, Lin T, Xie Z, et al. Epigallocatechin gallate (EGCG) attenuates infrasound-induced neuronal impairment by inhibiting microglia-mediated inflammation. *J Nutr Biochem.* (2014) 25:716–25. doi: 10.1016/j.jnutbio.2014.02.012

35. Salem SA, Hwei NM, Bin Saim A, Ho CC, Sagap I, Singh R, et al. Polylactic-co-glycolic acid mesh coated with fibrin or collagen and biological adhesive substance as a prefabricated, degradable, biocompatible, and functional scaffold for regeneration of the urinary bladder wall. *J Biomed Mater Res A.* (2013) 101:2237–47. doi: 10.1002/jbm.a.34518

36. Görlach A, Bertram K, Hudecova S, Krizanova O. Calcium and ROS: a mutual interplay. *Redox Biol.* (2015) 6:260–71. doi: 10.1016/j.redox.2015.08.010

37. Gordeeva AV, Zvyagilskaya RA, Labas YA. Cross-talk between reactive oxygen species and calcium in living cells. *Biochemistry.* (2003) 68:1077–80. doi: 10.1023/A:1026398310003

38. Shao A, Wang Z, Wu H, Dong X, Li Y, Tu S, et al. Enhancement of autophagy by histone deacetylase inhibitor trichostatin A ameliorates neuronal apoptosis after subarachnoid hemorrhage in rats. *Mol Neurobiol.* (2016) 53:18–27. doi: 10.1007/s12035-014-8986-0

39. Liu Y, Li J, Wang Z, Yu Z, Chen G. Attenuation of early brain injury and learning deficits following experimental subarachnoid hemorrhage secondary to cystatin C: possible involvement of the autophagy pathway. *Mol Neurobiol.* (2014) 49:1043–54. doi: 10.1007/s12035-013-8579-3

40. Zhang T, Wu P, Budbazar E, Zhu Q, Sun C, Mo J, et al. Mitophagy reduces oxidative stress via Keap1 (kelch-like epichlorohydrin-associated protein 1)/Nrf2 (nuclear factor-E2-related factor 2)/PHB2 (prohibitin 2) pathway after subarachnoid hemorrhage in rats. *Stroke.* (2019) 50:978–88. doi: 10.1161/STROKEAHA.118.021590

41. Zhou K, Enkhjargal B, Mo J, Zhang T, Zhu Q, Wu P, et al. Dihydrolipoic acid enhances autophagy and alleviates neurological deficits after subarachnoid hemorrhage in rats. *Exp Neurol.* (2021) 342:113752. doi: 10.1016/j.expneurol.2021.113752

42. Carloni S, Buonocore G, Balduini W. Protective role of autophagy in neonatal hypoxia-ischemia induced brain injury. *Neurobiol Dis.* (2008) 32:329–39. doi: 10.1016/j.nbd.2008.07.022

43. Wang Z, Shi XY, Yin J, Zuo G, Zhang J, Chen G. Role of autophagy in early brain injury after experimental subarachnoid hemorrhage. *J Mol Neurosci.* (2012) 46:192–202. doi: 10.1007/s12031-011-9575-6

44. Lee JY, He Y, Sagher O, Keep R, Hua Y, Xi G. Activated autophagy pathway in experimental subarachnoid hemorrhage. *Brain Res.* (2009) 1287:126–35. doi: 10.1016/j.brainres.2009.06.028

45. He BJ, Joiner ML, Singh MV, Luczak ED, Swaminathan PD, Koval OM, et al. Oxidation of CaMKII determines the cardiotoxic effects of aldosterone. *Nat Med.* (2011) 17:1610–8. doi: 10.1038/nm.2506

46. Hart PC, Mao M, de Abreu AL, Ansenberger-Fricano K, Ekoue DN, Ganini D, et al. MnSOD upregulation sustains the Warburg effect via mitochondrial ROS and AMPK-dependent signalling in cancer. *Nat Commun.* (2015) 6:6053. doi: 10.1038/ncomms7053

47. Kowara R, Moraleja KL, Chakravarthy B. Involvement of nitric oxide synthase and ROS-mediated activation of L-type voltage-gated Ca2+ channels in NMDA-induced DPYSL3 degradation. *Brain Res.* (2006) 1119:40–9. doi: 10.1016/j.brainres.2006.08.047

48. Li X, Wu XQ, Deng R, Li DD, Tang J, Chen WD, et al. CaMKII-mediated Beclin 1 phosphorylation regulates autophagy that promotes degradation of Id and neuroblastoma cell differentiation. *Nat Commun.* (2017) 8:1159. doi: 10.1038/s41467-017-01272-2

49. Takeuchi M, Yamamoto T. Apoptosis induced by NAD depletion is inhibited by KN-93 in a CaMKII-independent manner. *Exp Cell Res.* (2015) 335:62–7. doi: 10.1016/j.yexcr.2015.05.019

50. Zhou K, Enkhjargal B, Xie Z, Sun C, Wu L, Malaguit J, et al. Dihydrolipoic acid inhibits lysosomal rupture and NLRP3 through LAMP1/CaMKII/TAK1 pathways after subarachnoid hemorrhage in rat. *Stroke.* (2018) 49:175–83. doi: 10.1161/STROKEAHA.117.018593

51. Edvinsson L, Povlsen GK, Ahnstedt H, Waldsee R. CaMKII inhibition with KN93 attenuates endothelin and serotonin receptor-mediated vasoconstriction and prevents subarachnoid hemorrhage-induced deficits in sensorimotor function. *J Neuroinflammation.* (2014) 11:207. doi: 10.1186/s12974-014-0207-2

52. Bensalem J, Servant L, Alfos S, Gaudout D, Layé S, Pallet V, et al. Dietary polyphenol supplementation prevents alterations of spatial navigation in middle-aged mice. *Front Behav Neurosci.* (2016) 10:9. doi: 10.3389/fnbeh.2016.00009

53. Song Y, Li X, Gong X, Zhao X, Ma Z, Xia T, et al. Green tea polyphenols improve isoflurane-induced cognitive impairment via modulating oxidative stress. *J Nutr Biochem.* (2019) 73:108213. doi: 10.1016/j.jnutbio.2019.07.004

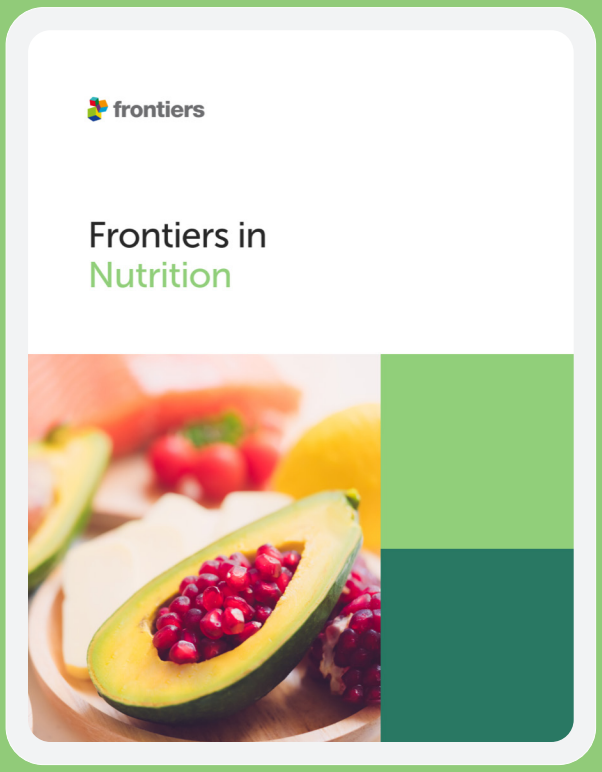
Frontiers in Nutrition

Explores what and how we eat in the context of health, sustainability and 21st century food science

A multidisciplinary journal that integrates research on dietary behavior, agronomy and 21st century food science with a focus on human health.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact