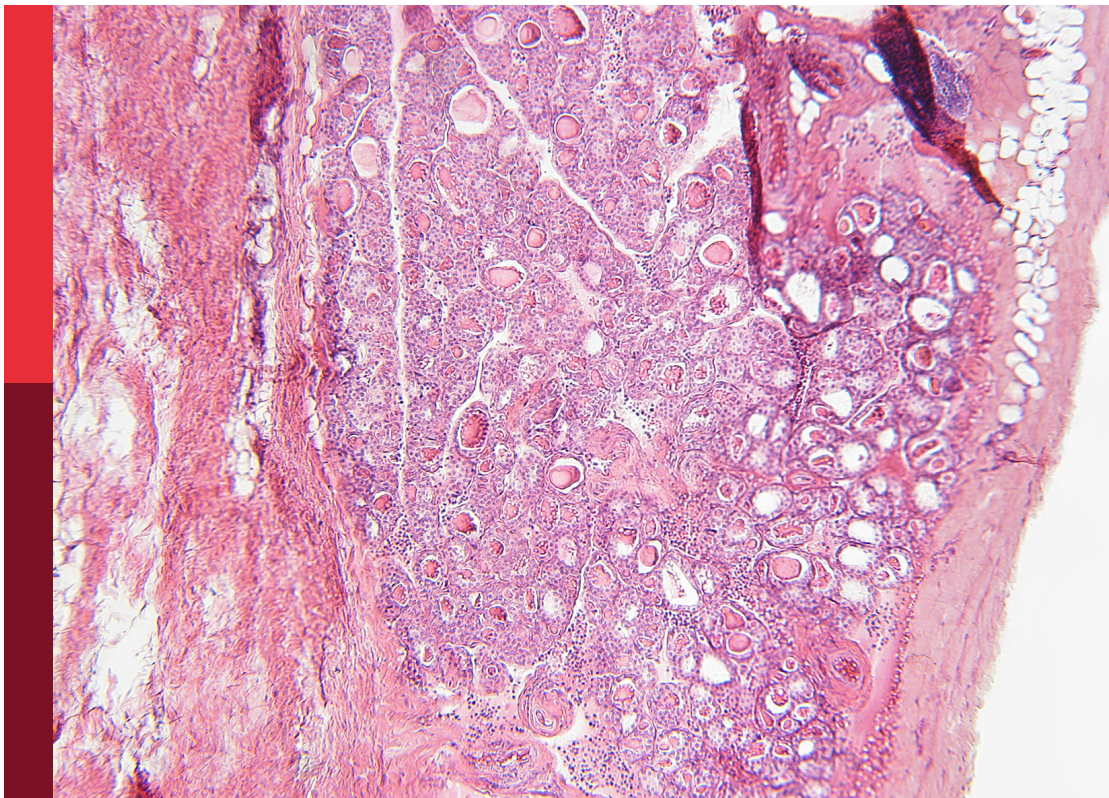
frontiers | Research Topics

Endocrine regulation of mineral ions and their relevance to metabolic diseases

Edited by
Mor-Li Hartman and Mohammed S. Razzaque

Published in
Frontiers in Endocrinology
Frontiers in Nutrition



ISSN 1664-8714
ISBN 978-2-8325-4768-7
DOI 10.3389/978-2-8325-4768-7

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

Endocrine regulation of mineral ions and their relevance to metabolic diseases

Topic editors

Mor-Li Hartman — The Forsyth Institute, United States

Mohammed S. Razzaque — Lake Erie College of Osteopathic Medicine, United States

Citation

Hartman, M.-L., Razzaque, M. S., eds. (2024). *Endocrine regulation of mineral ions and their relevance to metabolic diseases*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-4768-7

Table of contents

frontiers | Frontiers in Endocrinology

TYPE Editorial
PUBLISHED 03 April 2024
DOI 10.3389/fendo.2024.1391096

OPEN ACCESS

EDITED AND REVIEWED BY
James M. Olcese,
Florida State University, United States

*CORRESPONDENCE
Mor-Li Hartman
morlihartman2000@gmail.com
Mohammed S. Razzaque
mohammed.razzaque@utrgv.edu;
msr.nagasaki@gmail.com

RECEIVED 24 February 2024
ACCEPTED 11 March 2024
PUBLISHED 03 April 2024

CITATION
Hartman M-L and Razzaque MS (2024) Editorial: Endocrine regulation of mineral ions and their relevance to metabolic diseases. *Front. Endocrinol.* 15:1391096. doi: 10.3389/fendo.2024.1391096

Editorial: Endocrine regulation of mineral ions and their relevance to metabolic diseases

Mor-Li Hartman[1]* and Mohammed S. Razzaque[2]*

[1]Department of Inflammation and Immunology, ADA Forsyth Institute, Cambridge, MA, United States, [2]Department of Medical Education, School of Medicine, University of Texas Rio Grande Valley (UTRGV), Edinburg, TX, United States

KEYWORDS

endocrine regulation, minerals ion, metabolic diseases, metabolic homeostasis, health

Editorial on the Research Topic
Endocrine regulation of mineral ions and their relevance to metabolic diseases

One of the significant global challenges over the last couple of decades has been the emergence of the metabolic syndrome, a major global health concern (1). Extensive research is being conducted to understand and address this issue, with a particular focus on the role of minerals in metabolic diseases. Essential nutrients and minerals such as phosphate, calcium, magnesium, zinc, selenium, and iron, along with vitamins, play a vital role in maintaining the body's metabolic balance. There is a growing body of research aimed at better comprehending the significance of minerals and their levels in the body, especially concerning metabolic syndrome (2–5). Previous study by Hartman et al. have indicated that salivary phosphate levels can potentially predict the onset of obesity in children (6). Despite advancements in understanding the underlying mechanisms of metabolic diseases, there are still gaps in knowledge regarding how imbalances in various minerals and nutrients contribute to the development and progression of metabolic disorders.

It is well-documented that minerals and trace elements are essential micronutrients with well-defined biochemical and biological functions. Inadequacy in these micronutrients is associated with widespread human health issues. For instance, some diabetic patients may have deficiencies in zinc, chromium, and magnesium, highlighting the importance of these minerals in health and disease (7).

Magnesium is an essential nutrient that plays a crucial role in various physiological functions in the body (8, 9). It has been associated with a protective role in metabolic disorders, particularly in relation to the activation of vitamin D and its impact on immune function (10, 11). The paper by Eskander and Razzaque provided evidence that magnesium may help reduce the severity of asthma symptoms, reduce the intensity of COVID-19 symptoms, and inflammation. Additionally, there is evidence suggesting that magnesium is involved in improving glucose and insulin metabolism, making it relevant to conditions such as obesity, metabolic syndrome, and type 2 diabetes (12, 13). However, while the findings offer valuable insights, further research, including randomized controlled trials, is needed to validate these associations and determine causality with potential impact on vaccination and related adverse events (14). The significance of vitamin D and its impact on

healthy glucose metabolism is an ongoing area of research, particularly in relation to magnesium. Zittermann et al. described the potential role of magnesium in the synthesis of vitamin D (15). Gong et al. concluded that the level of vitamin D is independently correlated with the HOMA-β index and pancreatic β-cell dysfunction, and that magnesium intake enhances this association. However, the cross-sectional nature of the study prevents the determination of causality. Liu et al. found an independent association between the level of vitamin D and insulin resistance, which was modified by different levels of magnesium intake. The research suggests that optimal magnesium status may be important for optimizing vitamin D status (16, 17), and that the interaction between magnesium and vitamin D has significant implications for metabolic health.

Liu et al. conducted a study to examine the association between different levels of vitamin D and metabolic health indicators. The research focused on the relationship between triglycerides/high-density lipoprotein (TG/HDL) ratio with vitamin D levels and insulin resistance, impaired glucose tolerance, and diabetes mellitus. Using a cross-sectional analysis of the American National Health and Nutrition Examination Survey (NHANES) database, the authors found that both too-low and too-high levels of vitamin D3 can support the association of TG/HDL as a risk factor for Insulin resistance, impaired glucose tolerance, and diabetes mellitus. They concluded that maintaining vitamin D levels within an optimal range is essential to delay the progression of metabolic health issues such as insulin resistance, impaired glucose tolerance, and type 2 diabetes. The research has also indicated a connection between obesity and vitamin D deficiency, as well as low calcium levels (18). Yang et al. suggested that exposure to famine may be a factor contributing to changes in circulating calcium concentrations. They proposed that establishing a normal range of serum calcium and considering famine exposure history, especially in females, could help identify individuals with abnormal calcium levels and related diseases early. On the other hand, Li et al. proposed that low serum calcium could contribute to retinopathy in non-diabetic individuals. They suggested that maintaining a higher serum calcium level may be recommended to reduce the development of retinopathy.

The association between high phosphate levels and glucose impairment has been a subject of research (4, 19). This line of investigation originated from the observation of elevated phosphate levels in the saliva of obese children in Kuwait (6, 20). This led to the exploration of the potential link between obesity and phosphorus, and why high phosphate levels are found in obese children. Alhareky et al. conducted a study examining the beverage consumption of children in Kuwait, which revealed that those with high soda consumption had a significantly higher prevalence of obesity. While high obesity prevalence was also observed with high milk consumption, it was not as significant, and no such association was found with high juice consumption. The investigators discussed the possibility that the phosphate from soda drinks, in combination with sugar, may contribute to the development of obesity. The study sheds light on the potential impact of beverage choices on obesity and the need for additional studies to validate these findings. Using the same Kuwaiti cohort, a study published in 2016 found a significant association between dental decay and calorie-adjusted sugar intake. Interestingly, the study also revealed a significantly high percentage of dental decay in children who consumed a diet low in sugar but high in phosphate, compared to those who consumed a diet low in both sugar and phosphate (21). This result highlights the potential impact of both high sugar and high phosphate consumption on dental health (21, 22). Hetz et al. took a distinct approach to investigating the effects of excessive phosphate on subcellular cell signaling. They utilized quantitative proteomics and phosphoproteomics to explore the role of inorganic phosphate in protein expression and phosphorylation modification. The researchers also conducted bioinformatic analyses and literature reviews, revealing that elevated inorganic phosphate levels can rewire cell signaling through extensive cross talk. Additionally, their use of western blot analysis confirmed significant changes in the regulators responsible for pre-mRNA alternative splicing. The study sheds light on the potential impact of high phosphate intake on cellular processes and the need for further research to fully understand its consequences (23, 24).

The studies have elucidated the complex mechanisms involved in regulating phosphate homeostasis and its associated cytotoxicity. The kidney plays a critical role in maintaining the balance of mineral ions, such as calcium, phosphate, zinc, and magnesium, in the body, through processes like renal excretion, tubular reabsorption, and fine adjustments of urinary excretion to maintain the balance related to net intake. Imbalances in these ions can lead to unwanted clinical complications, and the kidney's role in regulating their balance is of great physiologic importance (25–28).

The article by Michigami et al. provides an overview of the role of osteocytes in phosphate metabolism, focusing on their function as dendritic cells in mineralized bone. The paper discusses how osteocytes control bone mass by producing sclerostin, an inhibitor of bone formation, and receptor activator of nuclear factor κ B ligand, an inducer of osteoblastic bone resorption. Additionally, it highlights the role of osteocytes in governing phosphate homeostasis through the production of fibroblast growth factor 23 (29). The article also delves into the molecular mechanisms through which osteocytes regulate phosphate metabolism, shedding light on their critical role in growth-related alterations and phosphate sensing in the body.

The study by Mohammadifard et al. provides a systematic review of randomized clinical trials, summarizing the impact of consuming whole soy, soy products, and its isolated components on metabolic syndrome features in adults. The research revealed that the inclusion of soy products in the diet of patients with metabolic syndrome effectively improved lipid profile and glycemic parameters, independent of its impact on anthropometric measures (30). In a separate study, Yan et al. aimed to determine the role of soy intake in the risk of type 2 diabetes. The findings indicated that soy intake from tofu was inversely associated with the

risk of diabetes among women, with the inverse association being more pronounced among overweight and postmenopausal women, but not among men.

These studies underscore the potential benefits of soy and its products in improving metabolic parameters and reducing the risk of type 2 diabetes, particularly among specific demographic groups. A study by Wang et al. sought to investigate the link between minerals and obesity in children, using data from the NHANES database from 2007-2014. This cross-sectional study aimed to enhance understanding of the role of minerals in preventing and managing obesity in children at high risk (31).

The existing research and reviews underscore the critical role of minerals in metabolic health and the need for comprehensive studies to further understand their impact on metabolic diseases, including obesity, metabolic syndrome, and diabetes. The findings emphasize the importance of monitoring mineral intake, addressing potential imbalances, and conducting further research to advance our understanding of mineral metabolism in the context of metabolic disorders (32, 33).

The study by Zhang et al. investigated the impact of iron overload on the development of non-alcoholic fatty liver disease (NAFLD). The research suggested that introducing iron to male Sprague Dawley rats fed a high-fat diet could synergistically exacerbate lipid metabolism disorders, liver injury, and oxidative damage. Additionally, treating the rats with deferoxamine (DFO) indicated potential support in reducing lipid metabolism dysfunction and the progression of NAFLD. The role of iron in chronic liver disease has been a subject of interest, as iron accumulation has been observed in various liver conditions, including hereditary hemochromatosis, alcoholic liver disease, nonalcoholic fatty liver disease, and hepatitis C viral infection. Higher iron levels are present not only in patients with hereditary hemochromatosis but also in those with acquired metabolic disorders and viral infections. Iron regulation may be disrupted in patients with chronic liver disease, as the liver's synthetic functions, including the production of hepcidin, are decreased (34). Iron overload can lead to hepatic inflammation and alter lipid metabolism, contributing to the pathophysiology of chronic liver disease. The accumulation of iron in the liver is a common occurrence in conditions such as hereditary hemochromatosis, alcoholic liver disease, and nonalcoholic fatty liver disease. Ding et al. conducted a systematic review and meta-analysis of observational studies to investigate the associations of dietary iron, copper, and selenium levels with metabolic syndrome. The results of the study suggested a positive association between dietary iron levels and metabolic syndrome, while a negative association was found between dietary copper and selenium levels and metabolic syndrome.

Zaborova et al. conducted a study to investigate the levels of macro- and microelements, including potassium, rubidium, magnesium, calcium, and cesium, in the bodies of young adult athletes. The researchers aimed to understand the mineral levels in athletes during physical activity. The study found that wrestlers had higher levels of several macro- and microelements, including some toxic ones. The research highlights the significance of monitoring mineral levels in athletes to support their overall health and performance.

The articles published in this Research Topic emphasize the significance of minerals, their levels, their interrelationships, and their role in maintaining metabolic homeostasis. Experimental and clinical studies have revealed a connection between mineral intake, concentration in the body, and the incidence of metabolic disorders. The involvement of minerals in muscle contraction, heart rhythm, nerve impulse conduction, oxygen transport, enzyme activation, immune functions, antioxidant activity, and bone health underscores their essential role in the human body.

The articles in this Research Topic also serve to raise awareness and enhance the knowledge of healthcare providers regarding the importance of maintaining appropriate mineral levels to support metabolic homeostasis and prevent or delay the onset of diseases. Collectively, this body of research underscores the necessity for dialogue within the healthcare community to further explore the role of minerals in the prevention and management of metabolic diseases. Additionally, it emphasizes the importance of implementing effective screening and monitoring of mineral levels during routine physical examinations to increase awareness and encourage the consumption of healthier foods that are rich in essential minerals (35).

Author contributions

M-LH: Writing – original draft, Writing – review & editing. MR: Writing – original draft, Writing – review & editing.

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

References

1. Saklayen MG. The global epidemic of the metabolic syndrome. *Curr Hypertens Rep*. (2018) 20:12. doi: 10.1007/s11906-018-0812-z

2. Peacock M. Calcium metabolism in health and disease. *Clin J Am Soc Nephrol*. (2010) 5 Suppl 1:S23–30. doi: 10.2215/CJN.05910809

3. Robberecht H, Bruyne T, Hermans N. Biomarkers of the metabolic syndrome: Influence of minerals, oligo- and trace elements. *J Trace Elem Med Biol*. (2017) 43:23–8. doi: 10.1016/j.jtemb.2016.10.005

4. Mironov N, Haque M, Atfi A, Razzaque MS. Phosphate dysregulation and metabolic syndrome. *Nutrients*. (2022) 14(21):4477. doi: 10.3390/nu14214477

5. Gulseth HL, Gjelstad IM, Birkeland KI, Drevon CA. Vitamin D and the metabolic syndrome. *Curr Vasc Pharmacol*. (2013) 11:968–84. doi: 10.2174/15701611113119990169

6. Hartman ML, Groppo F, Ohnishi M, Goodson JM, Hasturk H, Tavares M, et al. Can salivary phosphate levels be an early biomarker to monitor the evolvement of obesity? *Contrib Nephrol*. (2013) 180:138–48. doi: 10.1159/000346793

7. Lin CC, Huang YL. Chromium, zinc and magnesium status in type 1 diabetes. *Curr Opin Clin Nutr Metab Care*. (2015) 18:588–92. doi: 10.1097/MCO.0000000000000225

8. Maruotti N, Cantatore FP. Vitamin D and the immune system. *J Rheumatol*. (2010) 37:491–5. doi: 10.3899/jrheum.090797

9. Uwitonze AM, Rahman S, Ojeh N, Grant WB, Kaur H, Haq A, et al. Oral manifestations of magnesium and vitamin D inadequacy. *J Steroid Biochem Mol Biol*. (2020) 200:105636. doi: 10.1016/j.jsbmb.2020.105636

10. Ao T, Kikuta J, Ishii M. The effects of vitamin D on immune system and inflammatory diseases. *Biomolecules*. (2021) 11(11):1624. doi: 10.3390/biom11111624

11. Sung CC, Liao MT, Lu KC, Wu CC. Role of vitamin D in insulin resistance. *J BioMed Biotechnol*. (2012) 2012:634195. doi: 10.1155/2012/634195

12. Kisters S, Kisters K, Werner T, Westhoff T, Predel HG, Reuter H. Magnesium supplementation reduces interleukin-6 levels in metabolic syndrome. *Magnes Res*. (2023) 36:22. doi: 10.1684/mrh.2023.0511

13. López-Baltanás R, Rodríguez-Ortiz ME, Díaz-Tocados JM, Martinez-Moreno JM, Membrives C, Rodelo-Haad C, et al. Dietary mg supplementation decreases oxidative stress, inflammation, and vascular dysfunction in an experimental model of metabolic syndrome with renal failure. *Antioxidants (Basel)*. (2023) 12(2):283. doi: 10.3390/antiox12020283

14. Razzaque MS. Can adverse cardiac events of the COVID-19 vaccine exacerbate preexisting diseases? *Expert Rev Anti Infect Ther*. (2024). doi: 10.1080/14787210.2024.2311837

15. Zittermann A. Magnesium deficit? overlooked cause of low vitamin D status? *BMC Med*. (2013) 11:229. doi: 10.1186/1741-7015-11-229

16. Erem S, Atfi A, Razzaque MS. Anabolic effects of vitamin D and magnesium in aging bone. *J Steroid Biochem Mol Biol*. (2019) 193:105400. doi: 10.1016/j.jsbmb.2019.105400

17. Razzaque MS. Magnesium: are we consuming enough? *Nutrients*. (2018) 10 (12):1863. doi: 10.3390/nu10121863

18. Harahap IA, Landrier JF, Suliburska J. Interrelationship between vitamin D and calcium in obesity and its comorbid conditions. *Nutrients*. (2022) 14(15):3187. doi: 10.3390/nu14153187

19. Mironov N, Atfi A, Razzaque MS. Phosphate burden and organ dysfunction. *Front Aging*. (2022) 3:890985. doi: 10.3389/fragi.2022.890985

20. Razzaque MS. Salivary phosphate as a biomarker for human diseases. *FASEB Bioadv*. (2022) 4:102–8. doi: 10.1096/fba.2021-00104

21. Goodson JM, Shi P, Mumena CH, Haq A, Razzaque MS. Dietary phosphorus burden increases cariogenesis independent of vitamin D uptake. *J Steroid Biochem Mol Biol*. (2017) 167:33–8. doi: 10.1016/j.jsbmb.2016.10.006

22. Razzaque MS. Overconsumption of sugar-sweetened beverages: Why is it difficult to control? *J Popul Ther Clin Pharmacol*. (2020) 27:e51–7. doi: 10.15586/jptcp.v27i2.678

23. He P, Mann-Collura O, Fling J, Edara N, Hetz R, Razzaque MS. High phosphate actively induces cytotoxicity by rewiring pro-survival and pro-apoptotic signaling networks in HEK293 and HeLa cells. *FASEB J*. (2021) 35:e20997. doi: 10.1096/fj.202000799RR

24. Alexander R, Debiec N, Razzaque MS, He P. Inorganic phosphate-induced cytotoxicity. *IUBMB Life*. (2022) 74:117–24. doi: 10.1002/iub.2561

25. Amos A, Razzaque MS. Zinc and its role in vitamin D function. *Curr Res Physiol*. (2022) 5:203–7. doi: 10.1016/j.crphys.2022.04.001

26. Razzaque MS. Interactions between FGF23 and vitamin D. *Endocr Connect*. (2022) 11(10):e220239. doi: 10.1530/EC-22-0239

27. Razzaque MS. Phosphate metabolism: from physiology to toxicity. *Adv Exp Med Biol*. (2022) 1362:1–6. doi: 10.1007/978-3-030-91623-7

28. Razzaque MS. Bone-kidney axis in systemic phosphate turnover. *Arch Biochem Biophys*. (2014) 561:154–8. doi: 10.1016/j.abb.2014.06.031

29. Razzaque MS. The FGF23-Klotho axis: endocrine regulation of phosphate homeostasis. *Nat Rev Endocrinol*. (2009) 5:611–9. doi: 10.1038/nrendo.2009.196

30. Mohammadifard N, Sajjadi F, Haghighatdoost F. Effects of soy consumption on metabolic parameters in patients with metabolic syndrome: A systematic review and meta-analysis. *Excli J*. (2021) 20:665–85. doi: 10.17179/excli2021-3348

31. Wang L, Liu W, Bi S, Zhou L, Li L. Association between minerals intake and childhood obesity: A cross-sectional study of the NHANES database in 2007-2014. *PloS One*. (2023) 18:e0295765. doi: 10.1371/journal.pone.0295765

32. Park Y, Han J. Mineral balance and metabolic syndrome in adolescents: focus on calcium and phosphorus intake. *Healthcare (Basel)*. (2021) 9(11):1525. doi: 10.3390/healthcare9111525

33. Cui Z, Zhou H, Liu K, Wu M, Li S, Meng S, et al. Dietary copper and selenium intakes and the risk of type 2 diabetes mellitus: findings from the China health and nutrition survey. *Nutrients*. (2022) 14(10):2055. doi: 10.3390/nu14102055

34. Milic S, Mikolasevic I, Orlic L, Devcic E, Starcevic-Cizmarevic N, Stimac D, et al. The role of iron and iron overload in chronic liver disease. *Med Sci Monit*. (2016) 22:2144–51. doi: 10.12659/MSM.896494

35. Alexander R, Khaja A, Debiec N, Fazioli A, Torrance M, Razzaque MS. Health-promoting benefits of lentils: Anti-inflammatory and anti-microbial effects. *Curr Res Physiol*. (2024) 7:100124. doi: 10.1016/j.crphys.2024.100124

frontiers in Nutrition

ORIGINAL RESEARCH
published: 10 January 2022
doi: 10.3389/fnut.2021.813742

Soy Intake and Risk of Type 2 Diabetes Among Japanese Men and Women: JACC Study

*Fangyu Yan[1], Ehab S. Eshak[2,3], Kokoro Shirai[1], Jia-Yi Dong[1], Isao Muraki[1], Akiko Tamakoshi[4] and Hiroyasu Iso[1]**

[1] Department of Social Medicine, Public Health, Osaka University Graduate School of Medicine, Osaka, Japan, [2] Department of Public Health and Preventive Medicine, Faculty of Medicine, Minia University, Minia, Egypt, [3] Advanced Clinical Epidemiology, Medical Data Science, Public Health, Graduate School of Medicine, Osaka University, Osaka, Japan, [4] Department of Public Health, Hokkaido University Graduate School of Medicine, Sapporo, Japan

The evidence on the protective effects of soy foods against type 2 diabetes has been inconsistent. We thought to examine the association between the dietary intakes of soy and the risk of diabetes in a prospective study encompassing 21,925 healthy Japanese men and women aged 40–79 years. A validated self-administered food frequency questionnaire determined the intakes of soy, and their associations with risk of type 2 diabetes were evaluated by the logistic regression analysis. During the 5-year follow-up period, we observed 593 new cases of type 2 diabetes (302 in men and 291 in women). There was no association between dietary intakes of soy foods and the risk of type 2 diabetes among men. Whereas among women, higher tofu intake was inversely associated with risk of type 2 diabetes; the multivariable odds ratios (ORs) of type 2 diabetes were 0.92 (95% CI: 0.69–1.21) for 3–4 times per week and 0.67 (95% CI: 0.49–0.94) for almost daily (p-trend = 0.03) in reference to those consuming tofu less than 3 times per week. Intakes of boiled beans and miso soup were not associated with the risk in both genders. The inverse association tended to be more evident among overweight women and postmenopaused women. In conclusion, the frequency of tofu intake was inversely associated with the risk of type 2 diabetes among women.

Keywords: soy, tofu (soybean curd), type 2 diabetes, Japan, isoflavone

OPEN ACCESS

Edited by:
Mohammed S. Razzaque, Lake Erie College of Osteopathic Medicine, United States

Reviewed by:
Reema Fayez Tayyem, Qatar University, Qatar
Mohammed Srour, University of Palestine, Palestine

****Correspondence:***
Hiroyasu Iso
iso@pbhel.med.osaka-u.ac.jp

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

Received: *12 November 2021*
Accepted: *06 December 2021*
Published: *10 January 2022*

Citation:
Yan F, Eshak E, Shirai K, Dong J-Y, Muraki I, Tamakoshi A and Iso H (2022) Soy Intake and Risk of Type 2 Diabetes Among Japanese Men and Women: JACC Study. Front. Nutr. 8:813742. doi: 10.3389/fnut.2021.813742

INTRODUCTION

Type 2 diabetes is becoming a heavy burden worldwide. Diabetes mellitus is the 7th leading cause of death, which ignores its secondary complications, whereas it is the 3rd cause of mortality when secondary complications are also considered. According to the data published by the International Diabetes Federation (IDF) in the 9th atlas, 463 million diabetics existed worldwide in 2019. It is expected to rise by 51% and reach 578 million by 2030 and 700 million by 2045 (1). In Japan, the Ministry of Health, Labor, and Welfare reported in 2019 that 11 million people are suspected of having diabetes, with a prevalence of 19.7% in men and 10.8% in women (2).

Soy products have long been considered a source of high-quality protein and healthful fat (3). The low frequency of obesity and related metabolic disorders in Asian populations is thought to be, at least partially, the credit of high soy intake, a characteristic component in Asiatic diets (4). Several animal studies showed that a soy-based diet increased insulin sensitivity and lowered insulin requirement (5–7).

However, the evidence of the protective effects of soy foods against type 2 diabetes in humans has been inconsistent. A meta-analysis based on randomized clinical trials showed no overall significance of soy intake on improvements of fasting glucose or insulin concentrations (8). Meta-analyses based on observational studies reached inconsistent conclusions (9–12). Two meta-analyses suggested that the soy products were associated with a lower risk of type 2 diabetes mellitus (9, 10). A third meta-analysis showed that only tofu, but not total soy, was associated with the reduced risk (11). On the contrary, a fourth meta-analysis suggested a weak positive association between total legume (soy + pulses + peanuts) consumption and the risk of type 2 diabetes, while soy intake per se was not associated with the risk (12).

In the aforementioned meta-analyses, several heterogeneities related to the participants' gender and ethnicity and the type and the amount of soy food consumption under each study were discussed. Therefore, in this study, we aim to clarify the gender-specific association between the dietary intakes of three different soy foods with the risk of type 2 diabetes among the Japanese population, whose soy intake is almost 10 times higher than in western countries (13).

METHODS

Study Population

The Japan Collaborative Cohort Study for Evaluation of Cancer Risk (JACC Study) is a large prospective cohort study sponsored by the Japanese Ministry of Education, Sports, and Science in 1988–1990. A total of 1,10,585 residents (46,395 men and 64,190 women) aged 40–79 years from 45 areas throughout Japan were enrolled in the baseline survey after having informed individual consent from the participants in 36 study areas or group consent from community leaders in 9 study areas (14). Participants completed a self-administered questionnaire at baseline, and a 5-year follow-up survey was conducted in 31 of the 45 areas involved in the baseline survey, with 46,540 participants responding. A total of 27,427 (10,589 men and 16,838 women) individuals completed the 5-year follow-up survey of diabetes and did not have a history of diabetes at baseline survey time. Of whom, 21,925 participants (8,413 men and 13,512 women) had the baseline information on soy intake, including tofu, boiled beans, and miso soup. Finally, these 21,925 participants were included in this study (**Supplementary Figure S1**). The study design was approved by the ethics committees of Hokkaido University and Osaka University.

Baseline Survey

The baseline data were collected using a self-administered questionnaire that included queries on demographic characteristics, medical history, and lifestyle habits. For soy foods, the baseline questionnaire included a 40-item food frequency questionnaire (FFQ) that inquired about the diet over the past year. The frequency of tofu and boiled beans intake consisted of five levels (almost never, one or two times per month, one or two times per week, three or four times per week, and almost daily). The frequency of miso soup intake consisted of four levels (almost never, a few days per month, three or four days per week, and almost daily). The number of bowls of miso soup consumed per day was also given for those who responded to daily intake. The FFQ was validated by referring to 12-day weighed dietary records as a standard. The Spearman's correlation coefficients for the intake of soy foods between the FFQ and dietary records were 0.50, 0.30, and 0.69 for tofu, boiled beans, and miso soup, respectively (15).

Ascertainment of Diabetes

The incidence of type 2 diabetes was defined as self-reported physician-diagnosed diabetes at the 5-year follow-up survey for participants without such a history at the baseline survey. The validity of self-reporting physician-diagnosed diabetes was evaluated by comparing self-reported diabetes with the participants' glucose concentrations or treatment history among 1,230 men and 1,837 women. Diabetes cases were defined as $\geq$7.8 mmol/L ($\geq$140 mg/dL) fasting serum glucose concentration or $\geq$ 11.1 mmol/L ($\geq$200 mg/dL) randomly measured concentration, or treatment with oral hypoglycemic agents or insulin (16). The sensitivity and specificity of self-reporting were 70 and 95%, respectively, for men and 75 and 98%, respectively, for women.

Statistical Analysis

We categorized the frequency of tofu consumption into three groups: less than 3 times per week, 3 to 4 times per week, and almost daily. The frequency of miso soup consumption was categorized into four groups (less than one bowl per day, one bowl per day, two bowls per day, and at least three bowls per day). The frequency of boiled beans consumption was categorized into three groups (less than weekly, one to two times per week, and at least three times per week).

Statistical interaction was examined between the intake of soy foods groups and sex toward the risk of type 2 diabetes. Then, we calculated the sex-specific mean values and prevalence of type 2 diabetes risk factors and baseline participants' characteristics based on the categories of intake of soy foods. Using the lowest intake frequency category as a reference, we estimated the sex-specific odds ratios (ORs) and 95% confidence intervals (CIs) of having developed type 2 diabetes related to the intake of soy foods by the logistic regression analysis after adjusting for age and area of residence (Model 1). Model 2 was further adjusted for body mass index (BMI) ($<$18.5, 18.5–25.0, or $\geq$25 kg/m^2), history of hypertension (yes or no), family history of diabetes (yes or no), sports hours ($<$5h/ week or $\geq$5h/ week), walking hours ($<$1h/ day or $\geq$1h/ day), alcohol intake (never, ex-drinker, or current drinker of 0.1–22.9, 23.0–45.9, 46.0–68.9, or $\geq$69.0 g ethanol/day), educational status ($<$18 or $\geq$19 years), sleep duration ($<$6.0, 6.0–7.0, 7.0–8.0, 8.0–9.0, or $\geq$9.0 h/day), smoking status (never, ex-smoker, current smoker of 1–19 cigarettes/day, or $\geq$20 cigarettes/day), mental stress (high or

TABLE 1 | Sex-specific baseline participants' characteristics according to intake of soy foods.

	Tofu intake			*p*-trend	Boiled beans intake			*p*-trend	Miso soup intake				*p*-trend
	<3 times/week	3–4 times/week	Almost daily		Less than weekly	1–2 times/week	≥ 3 times /week		<1 bowl/day	1 bowl/day	2 bowls/day	≥3 bowls/day	
Men, n	3476	2727	2210		5202	2034	1177		2171	1531	2202	2509	
Age, year	55.9 ± 9.7	56.3 ± 9.5	57.7 ± 9.3	<0.0001	55.3 ± 9.5	57.9 ± 9.4	59.5 ± 9.0	<0.0001	56.9 ± 9.9	57.5 ± 9.7	55.9 ± 9.4	56.1 ± 9.3	<0.0001
History of hypertension, %	20.0	21.8	21.5	0.77	20.8	21.2	21.3	0.02	23.3	22.8	19.4	19.3	0.002
Current smoker, %	53.4	48.4	49.5	0.05	53.6	46.2	45.9	<0.0001	52.4	45.9	50.6	52.5	0.16
Sports≥5 h/week, %	7.1	8.4	9.2	0.10	7.0	9.0	11.5	0.005	8.8	7.5	7.5	8.4	0.01
Walking≥1 h/day, %	50.6	50.1	53.2	<0.0001	50.0	52.8	53.2	0.12	47.6	44.6	51.0	58.3	<0.0001
Higher education, %	16.6	18.9	18.4	0.06	17.1	18.3	20.3	0.01	20.5	21.4	19.7	11.7	<0.0001
High mental stress, %	22.3	22.2	20.3	0.74	23.5	20.2	17.0	0.02	25.3	22.1	22.7	17.5	<0.0001
BMI, kg/m²	22.5 ± 2.7	22.6 ± 2.6	22.7 ± 2.7	0.001	22.7 ± 2.7	22.4 ± 2.6	22.3 ± 2.7	0.0005	22.5 ± 2.7	22.4 ± 2.8	22.7 ± 2.6	22.7 ± 2.7	0.02
Alcohol intake, g/day	33.1 ± 22.0	33.4 ± 21.3	35.0 ± 22.1	0.0007	35.3 ± 21.9	30.9 ± 22.1	30.9 ± 20.3	<0.0001	31.9 ± 22.5	31.2 ± 20.9	33.4 ± 20.8	36.9 ± 22.4	<0.0001
Sleep duration, h	7.4 ± 1.1	7.4 ± 1.0	7.5 ± 1.1	0.0012	7.5 ± 1.1	7.4 ± 1.0	7.5 ± 1.0	0.04	7.4 ± 1.1	7.4 ± 1.0	7.4 ± 1.0	7.6 ± 1.1	<0.0001
Unemployed, %	15.8	15.8	19.1	0.61	15.4	18.1	19.6	0.002	20.9	19.8	13.4	13.7	<0.0001
≥ 1 cup of coffee/day, %	45.0	42.4	37.8	<0.0001	42.1	43.3	41.6	0.002	53.2	43.1	42.4	31.5	<0.0001
≥ 1 cup of green tea/day, %	78.2	78.3	79.4	0.64	76.7	81.2	82.1	<0.0001	79.6	79.8	79.9	75.6	0.06
≥ 3 bowls of rice/day, %	49.2	50.4	48.4	0.04	50.1	49.1	46.3	0.9	43.8	35.6	47.9	63.8	<0.0001
Women, n	4689	4436	4387		7942	3322	2248		4432	3331	3386	2363	
Age, year	56.7 ± 9.9	55.5 ± 9.4	56.4 ± 9.0	0.1309	55.0 ± 9.3	57.4 ± 9.5	58.6 ± 9.0	<0.0001	56.5 ± 9.9	56.3 ± 9.5	55.4 ± 9.1	56.6 ± 9.0	0.08
History of hypertension, %	21.0	19.6	21.2	0.3821	20.0	21.4	21.5	0.02	22.6	21.0	18.6	19.2	0.0002
Current smoker, %	5.1	3.8	2.8	<0.0001	4.6	3.3	2.4	<0.0001	6.0	3.4	2.8	2.5	<0.0001
Sports≥ 5 h/week, %	4.0	4.3	5.0	<0.0001	3.9	4.5	6.2	0.0003	4.4	3.8	3.7	6.4	<0.0001

(Continued)

TABLE 1 | Continued

	Tofu intake			*p*- trend	Boiled beans intake			*p*-trend	Miso soup intake				*p*-trend
	<3 times/week	3–4 times/week	Almost daily		Less than weekly	1–2 times/week	≥ 3 times /week		<1 bowl/day	1 bowl/day	2 bowls/day	≥3 bowls/day	
Walking≥1 h/day, %	52.7	51.2	51.8	0.019	50.8	53.2	54.0	0.0007	49.5	49.8	52.7	58.2	<0.0001
Higher education, %	8.6	10.0	10.8	0.0004	9.3	10.1	10.8	<0.0001	10.8	11.1	9.5	6.1	<0.0001
High mental stress, %	20.4	19.6	20.6	0.9123	21.1	20.0	17.4	0.24	21.9	20.6	19.3	17.5	<0.0001
BMI, kg/m²	22.7 ± 3.1	22.8 ± 2.9	23.0 ± 3.0	0.0005	22.9 ± 3.0	22.7 ± 2.9	22.6 ± 3.0	0.0004	22.7 ± 3.0	22.7 ± 2.9	22.9 ± 2.9	23.1 ± 3.1	<0.0001
Alcohol intake, g/day	10.4 ± 14.6	8.9 ± 11.1	9.2 ± 10.6	0.0555	10.3 ± 13.2	8.2 ± 9.8	7.9 ± 10.7	0.0002	10.4 ± 13.4	8.1 ± 10.1	9.5 ± 12.4	9.7 ± 12.0	0.29
Sleep duration, h	7.1 ± 1.0	7.0 ± 1.0	7.1 ± 1.0	0.0527	7.0 ± 1.0	7.1 ± 1.0	7.1 ± 1.0	0.54	7.0 ± 1.0	7.0 ± 1.0	7.1 ± 1.0	7.3 ± 1.0	<0.0001
Unemployed, %	49.1	48.4	54.4	<0.0001	47.9	53.2	56.0	0.58	51.3	51.4	48.1	51.4	0.54
≥1 cup of coffee/day, %	43.5	44.7	37.5	<0.0001	42.3	41.8	41.0	0.002	51.9	42.4	37.0	27.6	<0.0001
≥1 cup of green tea/day, %	73.9	76.2	76.8	0.0003	73.0	79.3	79.3	<0.0001	77.1	79.0	74.0	70.1	<0.0001
≥3 bowls of rice/day, %	24.3	21.3	19.4	0.09	22.3	21.8	19.6	0.08	22.2	15.7	19.3	32.9	<0.0001

TABLE 2 | Odds ratios (ORs) and 95% confidence intervals (CIs) for risk of type 2 diabetes according to intake of soy foods.

	Tofu			***p*-trend**	**Boiled beans**			***p*-trend**	**Miso soup**				***p*-trend**
	< 3 times/week	**3–4 times/week**	**Almost daily**		**Less than weekly**	**1–2 times/week**	**≥3 times /week**		**<1 bowl/day**	**1 bowl/day**	**2 bowls/day**	**≥3 bowls/day**	
Men, n	3476	2727	2210		5202	2034	1177		2171	1531	2202	2509	
No. of Cases, n	120	95	87		195	59	48		88	59	79	76	
Age– and area–adjusted OR (95% CI)	1.00	1.06(0.80–1.40)	1.22(0.91–1.63)	0.19	1.00	0.71(0.53–0.96)	0.99(0.71–1.37)	0.40	1.00	1.06(0.75 −1.50)	1.11(0.78 −1.59)	0.96(0.65 −1.43)	0.96
Multivariable adjusted OR (95% CI)*	1.00	1.09(0.82–1.45)	1.26(0.93–1.7)	0.15	1.00	0.77(0.57–1.05)	1.10(0.78–1.55)	0.90	1.00	1.02(0.72–1.46)	1.09(0.76–1.58)	0.94(0.62–1.43)	0.89
Multivariable adjusted OR (95% CI)**	1.00	1.09(0.82–1.46)	1.25(0.91–1.7)	0.17	1.00	0.77(0.56–1.04)	1.07(0.76–1.51)	0.79	1.00	0.99(0.69–1.42)	1.07(0.74–1.55)	0.92(0.6–1.41)	0.82
Women, n	4689	4436	4387		7942	3322	2248		4432	3331	3386	2363	
No. of Cases, n	123	97	71		182	66	43		113	69	57	52	
Age– and area–adjusted OR (95% CI)	1.00	0.86(0.66 −1.14)	0.64(0.48 −0.87)	0.005	1.00	0.79(0.59–1.05)	0.75(0.53–1.05)	0.05	1.00	0.88(0.64 −1.21)	0.73(0.50 −1.07)	0.85(0.55 −1.32)	0.26
Multivariable adjusted OR (95% CI)*	1.00	0.90(0.68–1.19)	0.66(0.48–0.91)	0.01	1.00	0.82(0.61–1.10)	0.82(0.57–1.17)	0.17	1.00	0.93(0.67–1.29)	0.82(0.55–1.21)	1.00(0.63–1.59)	0.73
Multivariable adjusted OR (95% CI)**	1.00	0.92(0.69–1.21)	0.67(0.49–0.94)	0.02	1.00	0.82(0.61–1.10)	0.84(0.59–1.20)	0.21	1.00	1.01(0.73–1.41)	0.87(0.59–1.29)	1.05(0.66–1.67)	0.93
P for sex interaction	0.01				0.27				0.65				

** Adjusted for age, area, energy, body mass index, history of hypertension, family history of diabetes, sports hours, walking hours, alcohol intake, educational status, sleep duration, smoking status, mental stress, work status, and nutritional factors (coffee, green tea, rice). **Adjusted further for intake of other two soy foods.*

TABLE 3 | Odds ratios (ORs) and 95% confidence intervals (CIs) for risk of type 2 diabetes according to tofu intake among different BMI groups.

	Tofu			*p*–trend
	<3 times/week	**3–4 times/week**	**Almost daily**	
Men, BMI <25kg/m^2, n	2770	2216	1765	
No. of cases, n	77	68	62	
Age– and area–adjusted OR (95% CI)	1.00	1.16(0.83–1.62)	1.32(0.94–1.88)	0.11
Multivariable adjusted OR (95% CI)*	1.00	1.17(0.83–1.65)	1.35(0.94–1.94)	0.10
Multivariable adjusted OR (95% CI)**	1.00	1.2(0.85–1.69)	1.42(0.98–2.05)	0.06
Men, BMI≥25kg/m^2, n	576	438	369	
No. of cases, n	35	26	24	
Age– and area–adjusted OR (95% CI)	1.00	1.07(0.62–1.84)	1.19(0.68–2.11)	0.55
Multivariable adjusted OR (95% CI)*	1.00	1.29(0.71–2.35)	1.63(0.84–3.16)	0.14
Multivariable adjusted OR (95% CI)**	1.00	1.25(0.69–2.29)	1.47(0.75–2.88)	0.25
P for BMI interaction	0.91			
Women, BMI <25kg/m^2, n	3548	3420	3333	
No. of cases, n	65	51	45	
Age– and area–adjusted OR (95% CI)	1.00	0.89(0.61–1.3)	0.82(0.56–1.22)	0.32
Multivariable adjusted OR (95% CI)*	1.00	0.89(0.61–1.31)	0.81(0.53–1.23)	0.32
Multivariable adjusted OR (95% CI)**	1.00	0.90(0.61–1.32)	0.80(0.53–1.23)	0.31
Women, BMI≥25kg/m^2, n	918	860	913	
No. of cases, n	53	44	26	
Age– and area–adjusted OR (95% CI)	1.00	0.87(0.57–1.33)	0.48(0.29–0.79)	0.01
Multivariable adjusted OR (95% CI)*	1.00	0.92(0.59–1.43)	0.50(0.29–0.85)	0.01
Multivariable adjusted OR (95% CI)**	1.00	0.95(0.61–1.49)	0.53(0.31–0.91)	0.03
P for BMI interaction	0.19			

**Adjusted for age, area, energy, body mass index, history of hypertension, family history of diabetes, sports hours, walking hours, alcohol intake, educational status, sleep duration, smoking status, mental stress, work status, and nutritional factors (coffee, green tea, rice). **Adjusted further for intake of other two soy foods (boiled beans and miso soup).*

TABLE 4 | Odds ratios (ORs) and 95% confidence intervals (CIs) for risk of type 2 diabetes according to tofu intake among premenopausal and postmenopausal women.

	Tofu			*p*-trend
	<3 times/week	**3–4 times/week**	**Almost daily**	
Premenopausal women, n	1553	1536	1310	
No. of cases, n	29	22	19	
Age– and area–adjusted OR (95% CI)	1.00	0.77(0.44–1.36)	0.80(0.44–1.48)	0.45
Multivariable adjusted OR (95% CI)*	1.00	0.78(0.43–1.43)	0.68(0.34–1.33)	0.24
Multivariable adjusted OR (95% CI)**	1.00	0.80(0.43–1.47)	0.70(0.36–1.40)	0.30
Postmenopausal women, n	3136	2900	3077	
No. of cases, n	94	75	52	
Age– and area–adjusted OR (95% CI)	1.00	0.88(0.64 −1.20)	0.59(0.42 −0.84)	0.004
Multivariable adjusted OR (95% CI)*	1.00	0.92(0.67–1.27)	0.63(0.44–0.92)	0.02
Multivariable adjusted OR (95% CI)**	1.00	0.94(0.68–1.30)	0.64(0.43–0.93)	0.02
P for menopausal status interaction	0.34			

**Adjusted for age, area, energy, body mass index, history of hypertension, family history of diabetes, sports hours, walking hours, alcohol intake, educational status, sleep duration, smoking status, mental stress, work status, and nutritional factors (coffee, green tea, rice). **Adjusted further for intake of other two soy foods (boiled beans and miso soup).*

not high), having a paid job (yes or no), and nutritional factors, including intakes of total energy (quartiles), coffee (never, 1–2 cups per month, 1–2 cups per week, 3–4 cups per week, 1–2 cups per day, or ≥ 3 cups day), green tea (never, less than 2 cups per week, 3–4 cups per week, 1–2 cups per day, 3–9 cups per day, or ≥ 10 cups per day), and rice intake (1–2 bowls per day, 3 bowls per day, or ≥ 3 bowls day). These variables were associated, through previous studies, with the risk of type 2 diabetes. Mutual adjustments were made for each soy food with the other two soy foods in Model 3. Additionally, stratified analyses by the BMI (< 25 and ≥ 25 kg/m^2) among both men and women and by the menopausal status (premenopausal and postmenopausal) among the women group were conducted. The statistical significance of soy food intake interactions with BMI and menopausal status

toward the risk of type 2 diabetes was also tested for crossproduct terms between each soy food intake categorical variable and dichotomous BMI and menopausal status. SAS 9.4 software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses, and two-tailed p-value < 0.05 indicated statistical significance.

RESULTS

Table 1 shows the sex-specific mean values and prevalence of diabetes risk factor at the baseline based on the intakes of different kinds of soy foods. Men and women who had more frequent soy intake were older, more educated, and more likely to practice sports but were less likely to smoke, have hypertension, and perceived mental stress than their counterparts who consumed soy less frequently.

During the 5-year follow-up period, we ascertained 593 new cases of type 2 diabetes (302 in men and 291 in women). **Table 2** presents the ORs (95% CIs) for type 2 diabetes according to the intake of soy foods. There was no association between dietary intakes of soy foods and risk of type 2 diabetes among men. Whereas among women, higher tofu intake was inversely associated with the risk of type 2 diabetes (p-trend = 0.005 in Model 1). Boiled beans and miso soup intakes were not associated with the risk in women. After controlling for type 2 diabetes traditional risk factors, the multivariable OR for type 2 diabetes among women who consuming tofu almost daily in reference to those consuming tofu <3 times per week was 0.66 (95% CI: 0.48–0.91, p-trend = 0.01 in Model 2), and the association was still significant after further adjustment for the intakes of other two soy foods; OR = 0.67 (95% CI:0.49–0.94, p-trend = 0.02 in model 3), p-value for sex-interaction = 0.01.

There was no significant interaction with BMI (< or ≥ 25 kg/m^2) for the associations between soy foods and risk of type 2 diabetes; p-interactions were > 0.05 (**Table 3**). However, of note, the multivariable OR (95%CI) for type 2 diabetes risk comparing the highest vs. lowest intake frequencies of tofu was 0.53 (0.31–0.91; p-trend = 0.03) in overweight women and 0.80 (0.53–1.23; p-trend = 0.31) in lean women and that for miso soup consumption in men was 3.21 (1.19–8.67; p-trend = 0.02) in overweight men and 0.65 (0.39–1.08; p-trend = 0.11) in lean men.

Similarly, no significant interaction by the women's menopausal status was found for the inverse association between tofu intake and risk of type 2 diabetes (p-interaction = 0.34) (**Table 4**). The multivariable OR (95% CI) that compares the almost daily tofu intake to <3 times/week was 0.70 (0.36–1.40; p-trend = 0.30) in premenopausal women and 0.64 (0.43–0.93; p-trend = 0.02) in postmenopausal women.

DISCUSSION

In this prospective population-based cohort study of Japanese men and women, we found that among the soy foods (tofu, boiled beans, and miso soup), tofu intake was associated with the reduced risk of type 2 diabetes among women but not among men. The association tended to be more pronounced among overweight and postmenopausal women than lean and premenopausal women; however, no significant effect modifications were detected.

Previous epidemiological studies reported inconsistent associations between intake of soy foods and the risk of diabetes (17–21). The discrepant findings could be attributed to differences in the study design, ethnicity of the studied population, and type and assessment of intake of soy foods. For the evidence in Japan, our findings are consistent with that of the Takayama cohort study of 13,521 (5,883 men and 7,638 women) residents aged 35–69 years and followed for a median 10-year period (17). For that study, the multivariable HR (95% CI) or type 2 diabetes risk for the highest vs. lowest tertiles of total soy intake was 0.45 (0.30–0.68; p-trend < 0.001) among women and 1.02 (0.74–1.42; p-trend = 0.94) among men, but the analyses for specific soy foods were not provided (17). On the contrary, the findings from 25,872 men and 33,919 women aged 45–75 years in the Japan Public Health Center-based Prospective (JPHC) study indicated no association between intake of total soy products and the risk of 5-year incidence of type 2 diabetes in either gender, and again, the analyses for specific soy foods were not provided (18).

The evidence outside Japan was also inconsistent. Matching our findings, a Vietnamese case-control study of 599 patients with type 2 diabetes and 599 age- and sex-matched controls showed that the multivariable OR (95 % CI) of type 2 diabetes for the highest vs. lowest quintiles of intake was 0.66 (0.42–1.02; p-trend = 0.009) for fresh tofu, 0.51 (0.35–0.75; p-trend = 0.001) for fried tofu, and 0.31 (0.21–0.46; p-trend < 0.001) for total soy foods (19). However, a pooled analysis of 3 large cohorts of US populations (63,115 women from the nurses' health study, 79,061 women from the nurses' health study II, and 21,281 men from the health professionals follow-up study) showed no association between consumption of soy foods with the risk of type 2 diabetes; the multivariable HR (95% CI) for ≥1 serving/week compared with no consumption was 0.93 (0.83–1.03; p-trend = 0.14) (20). On the contrary to our finding, the Multiethnic Cohort (MEC) study showed a positive association between soy intake and the risk of type 2 diabetes among 29,719 Caucasians, HR (95% CI) for consuming ≥ 10 g/d vs. <5 g/d was 1.22 (1.02–1.46) in men and 1.44 (1.16–1.80) in women, respectively; among 35,141 Japanese Americans, HR (95% CI) was 1.14 (1.01–1.28) in men and 1.11 (0.98–1.26) in women, respectively; and among 10,484 Native Hawaiians; HR (95% CI) was 1.29 (1.08–1.53) in men and 1.19 (1.02–1.40) in women (21).

The conclusions of the available meta-analyses also showed the same inconsistent result. According to a meta-analysis of 24 randomized trials with a total of 1,518 subjects, there was no overall impact of soy intake on improvements of fasting glucose [mean (95% CI) difference was 20.69 mg/dL (21.65, 0.27 mg/dL), $p = 0.16$] or insulin concentrations [20.18 mg/dL (20.70, 0.34 mg/dL), $p = 0.50$] (8). On the contrary, a meta-analysis of 19 independent reports from 8 observational studies (6 cohort and 2 cross-sectional) with a total of 1,74,561 participants concluded that the consumption of soy foods was associated with a lower risk of type 2 diabetes mellitus. The overall relative

risk (RR) (95% CI) was 0.77 (0.66–0.91) and the association was evident in women, RR = 0.65 (0.49–0.91) but not in men, RR = 0.82 (0.58–1.16), and in Asian population, RR = 0.73 (0.61–0.88) but not in non-Asians, RR = 1.05 (0.88–1.25) (10). In a recent meta-analysis of 15 prospective studies involving 11,232 cases among 2,71,709 individuals among the soy foods, only tofu was inversely associated with the risk of type 2 diabetes; RR (95% CI) was 0.92 (0.84, 0.99) for tofu, 0.89 (0.71–1.11) for soy milk, and 0.83 (0.68–1.01) for total soy intake (11).

Although we did not find significant interactions by BMI on the associations between soy foods and risk of type 2 diabetes, the inverse association of tofu was more evident in overweight women. The evidence on effect modifications by BMI from previous studies was inconclusive. Soy intake was associated with the reduced risk of type 2 diabetes in overweight but not lean men of the Saku study (22) and women of the JPHC study (18). However, the Takayama study reported the reduced risk with soy intake in lean but not overweight women (17). On the contrary, the weak positive association between soy intake and the risk of diabetes was confined to overweight and obese men and women of Caucasians, Hawaiians, and Japanese Americans in the MEC study (21). On the other hand, no effect modifications by BMI were found in the Vietnamese case-control study (19), the Shanghai Women's Health study (23), the Singapore Chinese Health Study (24), and the USA cohorts (20).

We do not have a clear explanation for the discrepant directions of BMI effect modification on the association between soy intake and the risk of type 2 diabetes. However, according to BMI cut point and also ethnicity, the amount of soy consumption and the type of soy foods varied between those previous studies. The MEC study (21) mentioned that the mean soy intake among Japanese American entity was 14.5 g, which was much lower than the 88 g among JPHC study (18) population and 87.1 g among men and 81.7 g among women in Takayama study (17). The authors of the MEC study assumed that these different soy intakes might affect the BMI interaction. The low levels of soy intake would not lead to the effect modification by BMI in the association between soy intake and the risk of type 2 diabetes. Alternatively, the differences may also be due to a random variation, residual confounding, or other dietary or lifestyle factors correlated with soy food consumption, BMI, and risk of type 2 diabetes. We recommend further longitudinal and clinical trials to assess the effect modification by BMI.

The significant inverse association between tofu intake and the risk of type 2 diabetes in postmenopausal women in our study matches the finding of the JPHC study where a tendency toward reduced risk was observed in the fourth quintile of total soy intake in postmenopausal women: HR (95% CI) = 0.69 (0.48–1.00; p-trend = 0.49) and premenopausal women with HR (95% CI) = 0.79 (0.57–1.09; p-trend = 0.66), p-interaction = 0.36 (18). However, the interaction by the menopausal status was not statistically significant in our study, in the JPHC study and other studies (17, 18, 20, 23, 24).

There are several potential mechanisms for soy foods' protective function against type 2 diabetes. Soy-derived protein and isoflavone contents could be the key protective factors (10). The Chinese Singaporean study (24) indicated that the isoflavone intake tended to be associated with a lower risk of diabetes; HR= 0.76 (0.58–1.00; p-trend = 0.08) for the highest vs. lowest quintiles of isoflavone intake. The previously mentioned Vietnamese case-control study showed that the multivariable ORs (95% CIs) for type 2 diabetes were 0.35 (0.24–0.49 p-trend < 0.001) for the highest (>23.2 mg/day) vs. lowest (≤12.6 mg/day) tertiles of major isoflavones intake, 0.36 (0.24–0.49; p-trend < 0.001) for daidzein, 0.35 (0.26–0.50; p-trend < 0.001) for genistein, and 0.37 (0.26–0.53; p-trend < 0.001) for glycitein (19). Total isoflavones were inversely associated with the risk of type 2 diabetes in the pooled analysis of the 3 US cohorts; the HR in the highest quintile of isoflavones vs. the lowest was 0.89 (0.83–0.96; p-trend = 0.009) (20). Isoflavones in the soy may inhibit insulin release from the pancreas and act as α-glucosidase inhibitors that restrict the intestinal brush border uptake of glucose (25). Also, isoflavone genistein could increase the beta-cell proliferation by ERK1/2 (26) and cAMP/PKA(27) pathways and enhance cell replication (28, 29). Moreover, pharmacological actions of isoflavones on glycemic control include a tyrosine kinase inhibitory action, changes in insulin receptor numbers and affinity, intracellular phosphorylation, and alterations in glucose transport (25, 30, 31). In addition to the isoflavones, soy protein may reduce insulin resistance by activating peroxisome proliferator-activated receptors (PPARs), which are nuclear transcription factors that regulate the expression of genes involved in glucose homeostasis, lipid metabolism, and fatty acid oxidation (32–34).

As for strengths, this study was based on a large sample of the community-based population, used a validated FFQ, and controlled for a wide range of potential confounding factors. As for limitations, the outcome variable was based on self-report; however, as shown above, there were high sex-specific sensitivity and specificity for the self-reported physician-diagnosed diabetes in our participants (35). Second, the data were collected from 36% of the eligible participants because the 5-year follow-up survey was limited to some but not to all the baseline study areas. However, no significant differences in participants' characteristics such as age, BMI, and other variables were found between individuals who responded or did not respond to the 5-year survey (36). Third, the dietary questionnaire did not include the data on portion size or some soy foods such as natto and soy milk; therefore, it was not possible to estimate the intake of total soy isoflavones. Finally, the residual confounding, such as by the glycemic index or load, cannot be eliminated.

In conclusion, soy intake from tofu was inversely associated with the risk of diabetes among women but not among men. The inverse association tended to be more evident among overweight and postmenopausal women.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon justified requests to the steering committee of the JACC study.

ETHICS STATEMENT

The JACC study protocol was approved by the Ethics Committees of Hokkaido University, Nagoya University, and Osaka University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FY, EE, and HI designed the study and methods of the analyses. FY drafted the manuscript. EE, KS, J-YD, IM, AT, and HI provided a critical review of the content. FY is the first author and HI is the corresponding author who both have the primary responsibility of the content. All authors contributed to the revisions and read and approved the final manuscript.

FUNDING

The JACC study was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) (Monbusho); grants-in-aid for Scientific Research on Priority Areas of Cancer; and grants-in-aid for Scientific Research on Priority Areas of Cancer Epidemiology from MEXT (MonbuKagaku-sho) (Numbers 61010076, 62010074, 63010074, 1010068, 2151065, 3151064, 4151063, 5151069, 6279102, 11181101, 17015022, 18014011, 20014026, 20390156, and 26293138); grants-in-aid from the Ministry of Health, Labor and Welfare, Health and Labor Sciences research grants, Japan (Research on Health Services: H17–Kenkou−007; Comprehensive Research on Cardiovascular Disease and Life–Related Disease: H18–Junkankitou [Seishuu]–Ippan−012; H19–Junkankitou [Seishuu]–Ippan−012; H20–Junkankitou [Seishuu]–Ippan−013; H23–Junkankitou [Seishuu]–Ippan−005; H26-Junkankitou [Seisaku]-Ippan-001; H29–Junkankitou–Ippan−003; 20FA1002); National Cancer Center Research and Development Fund (27-A-4, 30-A-15, 2021-A-16) and JSPS KAKENHI Grant Numbers JP 16H06277 and JP25330039.

ACKNOWLEDGMENTS

We express our appreciation to Drs Kunio Aoki and Yoshiyuki Ohno, Professors Emeriti of the Nagoya University School of Medicine and former chairpersons of the JACC Study. We are also greatly indebted to Dr Haruo Sugano, former Director of the Cancer Institute, Tokyo, who greatly contributed to the initiation of the JACC Study; Dr Tomoyuki Kitagawa, Director Emeritus of the Cancer Institute of the Japanese Foundation for Cancer Research and former chairman of the grant-in-aid for Scientific Research on the Priority Area Cancer; and Dr Kazao Tajima of the Aichi Cancer Center, who was the previous chairman of the grant-in-aid for Scientific Research on Priority Areas of Cancer Epidemiology, for their warm encouragement and support of this study. The whole members of JACC study were presented in https://publichealth.med.hokudai.ac.jp/jacc/member.html.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.813742/full#supplementary-material

REFERENCES

1. Saeedi P, Salpea P, Karuranga S, Petersohn I, Malanda B, Gregg EW, et al. Mortality attributable to diabetes in 20-79 years old adults, 2019 estimates: results from the International Diabetes Federation Diabetes Atlas. *Diabetes Res Clin Pract.* (2020) 162:108086. doi: 10.1016/j.diabres.2020.108086
2. Tomikue K, Saito Y. Results of "National Health and Nutrition Survey" in the first year of Reiwa. In: *Press release material.* Ed Tomikue, K. the Ministry of Health, Labor and Welfare Available online at: https://www.mhlw.go.jp/stf/newpage_14156.html. (accssed 10, 2020)
3. Messina M. Soy and health update: evaluation of the clinical and epidemiologic literature. *Nutrients.* (2016) 8:754. doi: 10.3390/nu8120754
4. Cederroth CR, Nef S. Soy, phytoestrogens and metabolism: a review. *Mol Cell Endocrinol.* (2009) 304:30–42. doi: 10.1016/j.mce.2009.02.027
5. Iritani N, Sugimoto T, Fukuda H, Komiya M, Ikeda H. Dietary soybean protein increases insulin receptor gene expression in Wistar fatty rats when dietary polyunsaturated fatty acid level is low. *J Nutr.* (1997) 127:1077–83. doi: 10.1093/jn/127.6.1077
6. Lavigne C, Marette A, Jacques H. Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. *Am J Physiol Endocrinol Metabol.* (2000) 278:E491–500. doi: 10.1152/ajpendo.2000.278.3.E491
7. Tachibana N, Iwaoka Y, Hirotsuka M, Horio F, Kohno M. Beta-conglycinin lowers very-low-density lipoprotein-triglyceride levels by increasing adiponectin and insulin sensitivity in rats. *Biosci Biotechnol Biochem.* (2010) 74:1250–5. doi: 10.1271/bbb.100088
8. Liu ZM, Chen YM, Ho SC. Effects of soy intake on glycemic control: a meta-analysis of randomized controlled trials. *Am J Clin Nutr.* (2011) 93:1092–101. doi: 10.3945/ajcn.110.007187
9. Tian S, Xu Q, Jiang RY, Han TS, Sun CH, Na LX. Dietary protein consumption and the risk of type 2 diabetes: a systematic review and meta-analysis of cohort studies. *Nutrients.* (2017) 9:982. doi: 10.3390/nu9090982
10. Li WZ, Ruan WY, Peng Y, Wang DM. Soy and the risk of type 2 diabetes mellitus: a systematic review and meta-analysis of observational studies. *Diabetes Res Clin Pract.* (2018) 137:190–9. doi: 10.1016/j.diabres.2018.01.010
11. Tang J, Wan Y, Zhao M, Zhong H, Zheng JS, Feng F. Legume and soy intake and risk of type 2 diabetes: a systematic review and meta-analysis of prospective cohort studies. *Am J Clin Nutr.* (2020) 111:677–88. doi: 10.1093/ajcn/nqz338
12. Pearce M, Fanidi A, Bishop TR, Sharp SJ, Imamura F, Dietrich S, et al. Associations of total legume, pulse, and soy consumption with incident type 2 diabetes: Federated meta-analysis of 27 studies from diverse world regions. *J Nutr.* (2021) 151:1231–40. doi: 10.1093/jn/nxaa447
13. Khankari NK, Yang JJ, Sawada N, Wen W, Yamaji T, Gao J, et al. Soy intake and colorectal cancer risk: results from a pooled analysis of prospective

cohort studies conducted in China and Japan. *J Nutr.* (2020) 150:2442–50. doi: 10.1093/jn/nxaa194
14. Tamakoshi A, Yoshimura T, Inaba Y, Ito Y, Watanabe Y, Fukuda K, et al. Profile of the JACC study. *J Epidemiol.* (2005) 15:S4–8. doi: 10.2188/jea.15.S4
15. Date C, Fukui M, Yamamoto A, Wakai K, Ozeki A, Motohashi Y, et al. Reproducibility and validity of a self-administered food frequency questionnaire used in the JACC study. *J Epidemiol.* (2005) 15:S9–S23. doi: 10.2188/jea.15.S9
16. Iso H, Date C, Wakai K, Fukui M, Tamakoshi A, Grp JS. The relationship between green tea and total caffeine intake and risk for self-reported type 2 diabetes among Japanese adults. *Ann Intern Med.* (2006) 144:554–62. doi: 10.7326/0003-4819-144-8-200604180-00005
17. Konishi K, Wada K, Yamakawa M, Goto Y, Mizuta F, Koda S, et al. Dietary soy intake is inversely associated with risk of type 2 diabetes in Japanese women but not in men. *J Nutr.* (2019) 149:1208–14. doi: 10.1093/jn/nxz047
18. Nanri A, Mizoue T, Takahashi Y, Kirii K, Inoue M, Noda M, et al. Soy product and isoflavone intakes are associated with a lower risk of type 2 diabetes in overweight Japanese women. *J Nutr.* (2010) 140:580–6. doi: 10.3945/jn.109.116020
19. Nguyen CT, Pham NM, Do W, Binns CW, Hoang VM, Dang DA, et al. Soyfood and isoflavone intake and risk of type 2 diabetes in Vietnamese adults. *Eur J Clin Nutr.* (2017) 71:1186–92. doi: 10.1038/ejcn.2017.76
20. Ding M, Pan A, Manson JE, Willett WC, Malik V, Rosner B, et al. Consumption of soy foods and isoflavones and risk of type 2 diabetes: a pooled analysis of three US cohorts. *Eur J Clin Nutr.* (2016) 70:1381–7. doi: 10.1038/ejcn.2016.117
21. Morimoto Y, Steinbrecher A, Kolonel LN, Maskarinec G. Soy consumption is not protective against diabetes in Hawaii: the Multiethnic Cohort. *Eur J Clin Nutr.* (2011) 65:279–82. doi: 10.1038/ejcn.2010.228
22. Tatsumi Y, Morimoto A, Deura K, Mizuno S, Ohno Y, Watanabe S. Effects of soybean product intake on fasting and postload hyperglycemia and type 2 diabetes in Japanese men with high body mass index: the saku study. *J Diabetes Investig.* (2013) 4:626–33. doi: 10.1111/jdi.12100
23. Villegas R, Gao YT, Yang G, Li HL, Elasy TA, Zheng W, et al. Legume and soy food intake and the incidence of type 2 diabetes in the Shanghai Women's Health Study. *Am J Clin Nutr.* (2008) 87:162–7. doi: 10.1093/ajcn/87.1.162
24. Mueller NT, Odegaard AO, Gross MD, Koh WP, Yu MC, Yuan JM, et al. Soy intake and risk of type 2 diabetes in Chinese Singaporeans [corrected]. *Eur J Nutr.* (2012) 51:1033–40. doi: 10.1007/s00394-011-0276-2
25. Vedavanam K, Srijayanta S, O'Reilly J, Raman A, Wiseman H. Antioxidant action and potential antidiabetic properties of an isoflavonoid-containing soyabean phytochemical extract (SPE). *Phytother Res.* (1999) 13:601–8. doi: 10.1002/(SICI)1099-1573(199911)13:7<601::AID-PTR550>3.0.CO;2-O
26. Gilbert ER, Liu D. Anti-diabetic functions of soy isoflavone genistein: mechanisms underlying its effects on pancreatic beta-cell function. *Food Funct.* (2013) 4:200–12. doi: 10.1039/C2FO30199G
27. Babu PVA, Si HW, Fu Z, Zhen W, Liu DM. Genistein prevents hyperglycemia-induced monocyte adhesion to human aortic endothelial cells through preservation of the cAMP signaling pathway and ameliorates vascular inflammation in obese diabetic mice. *J Nutr.* (2012) 142:724–30. doi: 10.3945/jn.111.152322
28. Park SA, Choi MS, Cho SY, Jung UJ, Kim MJ, Sung MK, et al. Genistein and daidzein modulate hepatic glucose and lipid regulating enzyme activities in C57BL/KsJ-db/db mice. *Life Sci.* (2006) 79:1207–13. doi: 10.1016/j.lfs.2006.03.022
29. Cavaghan MK, Ehrmann DA, Polonsky KS. Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. *J Clin Investig.* (2000) 106:329–33. doi: 10.1172/JCI10761
30. Sorenson RL, Brelje TC, Roth C. Effect of tyrosine kinase inhibitors on islets of langerhans - evidence for tyrosine kinases in the regulation of insulin-secretion. *Endocrinology.* (1994) 134:1975–8. doi: 10.1210/endo.134.4.8137766
31. Jonas JC, Plant TD, Gilon P, Detimary P, Nenquin M, Henquin JC. Multiple effects and stimulation of insulin-secretion by the tyrosine kinase inhibitor genistein in normal mouse islets. *Br J Pharmacol.* (1995) 114:872–80. doi: 10.1111/j.1476-5381.1995.tb13285.x
32. Velasquez MT, Bhathena SJ. Role of dietary soy protein in obesity. *Int J Med Sci.* (2007) 4:72–82. doi: 10.7150/ijms.4.72
33. Mezei O, Banz WJ, Steger RW, Peluso MR, Winters TA, Shay N. Soy isoflavones exert antidiabetic and hypolipidemic effects through the PPAR pathways in obese Zucker rats and murine RAW 264.7 cells. *J Nutr.* (2003) 133:1238–43. doi: 10.1093/jn/133.5.1238
34. Morifuji M, Sanbongi C, Sugiura K. Dietary soya protein intake and exercise training have an additive effect on skeletal muscle fatty acid oxidation enzyme activities and mRNA levels in rats. *Br J Nutr.* (2006) 96:469–75. doi: 10.1079/bjn20061855
35. Eshak ES, Iso H, Muraki I, Tamakoshi A. Fat-soluble vitamins from diet in relation to risk of type 2 diabetes mellitus in Japanese population. *Br J Nutr.* (2019) 121:647–53. doi: 10.1017/S000711451800377X
36. Eshak ES, Iso H, Muraki I, Tamakoshi A. Among the water-soluble vitamins, dietary intakes of vitamins C, B2 and folate are associated with the reduced risk of diabetes in Japanese women but not men. *Br J Nutr.* (2019) 121:1357–64. doi: 10.1017/S000711451900062X

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.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 14 January 2022
doi: 10.3389/fnut.2021.765391

Excessive Inorganic Phosphate Burden Perturbed Intracellular Signaling: Quantitative Proteomics and Phosphoproteomics Analyses

*Rebecca Hetz[1], Erik Beeler[1], Alexis Janoczkin[1], Spencer Kiers[1], Ling Li[2], Belinda B. Willard[2], Mohammed S. Razzaque[3] and Ping He[1]**

[1] Department of Biochemistry, Lake Erie College of Osteopathic Medicine, Erie, PA, United States, [2] Proteomics and Metabolomics Core, Cleveland Clinic Lerner Research Institute, Cleveland, OH, United States, [3] Department of Pathology, Lake Erie College of Osteopathic Medicine, Erie, PA, United States

Inorganic phosphate (Pi) is an essential nutrient for the human body which exerts adverse health effects in excess and deficit. High Pi-mediated cytotoxicity has been shown to induce systemic organ damage, though the underlying molecular mechanisms are poorly understood. In this study, we employed proteomics and phosphoproteomics to analyze Pi-mediated changes in protein abundance and phosphorylation. Bioinformatic analyses and literature review revealed that the altered proteins and phosphorylation were enriched in signaling pathways and diverse biological processes. Western blot analysis confirms the extensive change in protein level and phosphorylation in key effectors that modulate pre-mRNA alternative splicing. Global proteome and phospho-profiling provide a bird-eye view of excessive Pi-rewired cell signaling networks, which deepens our understanding of the molecular mechanisms of phosphate toxicity.

Keywords: inorganic phosphate, cytotoxicity, cell signaling, proteomics, phosphoproteomics, alternative splice

OPEN ACCESS

Edited by:
Omar A. Obeid, American University of Beirut, Lebanon

Reviewed by:
Tommaso Filippini, University of Modena and Reggio Emilia, Italy
Ming Chang Hu, University of Texas Southwestern Medical Center, United States

****Correspondence:***
Ping He
pinghe718@gmail.com

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

Received: *27 August 2021*
Accepted: *22 November 2021*
Published: *14 January 2022*

Citation:
Hetz R, Beeler E, Janoczkin A, Kiers S, Li L, Willard BB, Razzaque MS and He P (2022) Excessive Inorganic Phosphate Burden Perturbed Intracellular Signaling: Quantitative Proteomics and Phosphoproteomics Analyses. Front. Nutr. 8:765391. doi: 10.3389/fnut.2021.765391

INTRODUCTION

Inorganic phosphate (Pi) is an essential mineral for life due to its fundamental role in diverse cellular processes. These include nucleic acid synthesis, energy storage, and transfer, cell signal transmission, bone formation, bone growth, and skeletal mineralization (1, 2). Humans routinely intake phosphate through food, which maintains normal musculoskeletal functions. Excessive intake of dietary phosphate, especially from processed food, may result in various health issues, such as dental diseases (3, 4), cardiovascular disease (5), diabetes (6), infertility (7), kidney disease (8), and tumors (9). These disorders are mechanistically mediated by high phosphate-induced pathological calcification (10), oxidative stress (11), cell death, and abnormal signal transduction (12).

Although excessive phosphate (Pi)-dysregulated AKT, mitogen-activated protein kinase (MAPK), and fibroblast growth factor receptor (FGFR) cell signaling pathways have been reported (12–15), the global protein expression and protein phosphorylation profiles perturbed by extracellular Pi remains elusive. Comprehensive analysis of the Pi-related protein phosphorylation landscape can offer a panoramic view of Pi-associated signaling networks, expanding our understanding of how extracellular Pi shapes varied cell behaviors and may provide potential therapeutic targets to manage high phosphate-induced organ damage. Herein, we applied

quantitative proteomic and phosphoproteomic strategies to uncover high Pi-mediated alterations in protein expression and protein phosphorylation. The following bioinformatic analyses and literature searching revealed excess Pi-rewired cell signaling networks with extensive cross-talk. Western blot (WB) analysis confirmed a profound change in the regulators that govern the pre-mRNA alternative splicing.

MATERIALS AND METHODS

Materials

Most of the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). HeLa cell line was an in-kind gift from Dr. Don Newmeyer (La Jolla Institute for Immunology, San Diego, CA, USA). HEK293 cells, Dimethylsulfoxide (DMSO), XTT Cell Proliferation Assay Kit, and Universal Mycoplasma Detection Kit were purchased from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), Ultrapure water, EDTA (0.5 M), Halt™ Protease and Phosphatase Inhibitor Cocktail, Acclaim™ PepMap™ 100 C18 HPLC Column, TMT10plex™ Isobaric Label Reagent Set, High-Select™ Fe-NTA Phosphopeptide Enrichment Kit, UltraPure™ SDS Solution (10%), Pierce™ Rapid Gold BCA Protein Assay Kit, NuPAGE™ Bis-Tris 4–12% precast gels, NuPAGE™ MOPS SDS Running Buffer (20×), NuPAGE™ MES SDS Running Buffer (20×), Restore™ PLUS Western Blot Stripping Buffer, PageRuler™ Plus Prestained 10–250kDa Protein Ladder, Instant Non-fat Dry Milk, Trypsin-EDTA (0.25%), Fetal bovine serum (FBS) and L-Glutamine (200 mM), anti-Phospho-epitope SR proteins antibody (Clone 1H4), and anti-SR protein family protein antibody (Clone 16H3) were obtained from Thermo Fisher Scientific (Carlsbad, CA, USA). Nitrocellulose Membrane, Precision Plus Protein™ Dual Color Standards, and Trans-Blot® Turbo™ RTA Midi Nitrocellulose Transfer Kit were purchased from Bio-Rad (Hercules, CA, USA). Antibodies against AKT1, GAPDH, Phospho-C-JUN (S63), Phospho-C-JUN (S73), C-JUN, Cleaved Caspase-3 (Asp175), and SRSF10 were obtained from Cell Signaling Technology (Danvers, MA, USA). SRPK (D-7) and Caspase-2 (F-2) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-SRPK1 and SRPK2 antibodies were purchased from BD Biosciences (San Jose, CA, USA). SRSF1, SRSF9, and SRSF11 antibodies were purchased from MyBioSource (San Diego, CA, USA). Direct-Blot™ HRP anti-β-actin, HRP Goat anti-mouse IgG, HRP donkey anti-rabbit IgG, HRP mouse anti-rat IgG antibodies, Western-Ready™ ECL Substrate Kit, and antibodies against MYC, Bcl-XS/L, and DYKDDDDK (Flag) Tag were purchased from BioLegend (San Diego, CA). PP1 Catalytic Subunit (PP1C) and Caspase-9 antibodies were purchased from R&D Systems (Minneapolis, MN, USA). The pIRES-EGFP plasmid was obtained from Addgene (Watertown, MA, USA). ORF cDNAs of Wild type (WT) human TRA2A, TRA2B, SRSF1, SRSF2, SRSF3, SRSF4, SRSF5, SRSF6, SRSF7, SRSF11, and SRSF12 cloned into pcDNA3.1+/C-(K)DYK vectors were purchased from Genscript (Piscataway, NJ, USA). FuGENE® HD Transfection Reagent was purchased from Promega (Madison, WI, USA).

Cell Culture and Treatment

The protocol was followed as detailed by He et al. in an earlier publication (12). Briefly, HEK293 and HeLa were maintained in DMEM (with 1 mM Pi) supplemented with 10% FBS. All cells were maintained at 37°C and 5% CO_2. Monosodium phosphate (NaH_2PO4, 2M, pH 6.8) was used as the source of Pi. Cells were treated with an increased amount of Pi for 24 h. For PFA treatment, the cells were pre-treated with PFA at indicated concentrations for 0.5 h followed by Pi treatment at different concentrations in the presence of PFA for 24 h.

Total Protein Extraction

The protocol was followed as detailed by He et al. in an earlier publication (12). Briefly, the cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% Sodium deoxycholate, 1% NP-40, 0.1% SDS) supplemented with 1× Protease and Phosphatase Inhibitor Cocktail.

Electrophoresis and Western Blot (WB)

The protocol was followed as detailed by He et al. in an earlier publication (12). Protein concentration was measured by Rapid Gold BCA Protein Assay Kit according to the manufacturer's protocol. A total of 30 μg extracted protein was resolved by electrophoresis and transferred to the nitrocellulose membrane. After blocking with 5% non-fat milk, the membrane was probed with diluted primary antibodies used at the manufacturer's recommended concentrations. Proteins were visualized using HRP conjugated secondary antibodies and chemiluminescence detection. The images were captured by LI-COR C-Digit imaging system (Lincoln, NE, USA).

Transient Transfection

Cells were transfected with plasmids using FuGENE® HD Transfection Reagent according to the manufacturer's protocol. Total proteins were extracted 24 h posterior to transfection.

XTT Assay

The protocol was followed as detailed by He et al. in an earlier publication (12). HEK293 cells were transfected with plasmids for 24 h followed by 40 mM Pi treatment. The XTT assay was performed 24 h after high Pi treatment. The average of specific absorbance from biological duplicates was normalized to the 1 mM Pi-treated group. The fold change of relative absorbances was plotted as the mean absorbance ± SEM using GraphPad Prism version 8 software (San Diego, CA, USA).

Quantitative Proteomics and Phosphoproteomics

HEK293 were treated with physiological (1 mM), pro-survival (10 mM), and pro-death (40 mM) concentrations of Pi for 24 h. Then, the cells were scraped in PBS and the cell pellet was lysed in 8 M urea in 20 mM HEPES buffer pH 8 with phosphatase inhibitors HALT freshly added. The protein concentration of the cell lysates was measured by a BCA assay. Each sample (1 mg of total protein) was reduced with dithiothreitol and alkylated with iodoacetamide before digesting overnight using 200 μg sequencing grade trypsin. Digested peptide samples were desalted

using a C18 solid-phase extraction (SPE) column and dried in a Speedvac centrifugal vacuum concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was dissolved in high-performance liquid chromatography (HPLC) grade water and a quantitative peptide assay was used to measure the peptide concentration. For global proteomics using TMT labels, 25 μg of peptides were taken from each sample and labeled with TMT10plex tags according to the manufacturer's protocol. The TMT labeled samples were desalted and separated using a high pH reversed-phase HPLC method, and the collections were combined into 4 fractions. For phosphoproteomics using TMT, 1 mg of peptides were PO3-enriched using a Hi-select Fe-NTA phospho-enrichment kit. The eluted peptide samples were evaporated in a SpeedVac and desalted using SPE spin columns. The desalted PO3-enriched peptide samples were labeled with TMT labels. The labeled samples were combined without fractionation.

The TMT-labeled samples were analyzed on a ThermoFisher Scientific UltiMate 3000 UHPLC system (ThermoFisher Scientific, Bremen, Germany) interfaced with a ThermoFisher Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, Bremen, Germany). Liquid chromatography (LC) was performed prior to mass spectrometry (MS)/MS analysis for peptide separation. The HPLC column used is a Thermo Scientific™ Acclaim™ PepMap™ 100 C18 reversed-phase capillary chromatography column (Thermo Fisher Scientific, Waltham, MA, USA) 75 μm x 15 cm, 2 μm, 100 Å. Then, 5 μl volumes of the peptide extract were injected and peptides eluted from the column by a 90-min acetonitrile/0.1% formic acid gradient at a flow rate of 0.30 μl/min and introduced to the source of the mass spectrometer online. Nano electrospray ion source was operated at 2.3 kV. The digest was analyzed using the data-dependent multitask capability of the instrument acquiring full scan mass spectra using a Fourier Transform (FT) Orbitrap analyzer to determine peptide molecular weights and higher-energy collisional dissociation (HCD) MS/MS product ion spectra with the Orbitrap FT analyzer (Thermo Fisher Scientific, San Jose, CA, USA) at 38% normalized collision energy (NCE) to determine both the amino acid sequence and the quantities of the isobaric tags. The MS method used in this study was a data-dependent acquisition (DDA) with a 3 s duty cycle. It includes one full scan at a resolution of 120,000 followed by as many MS/MS scans as possible on the most abundant ions in that full scan. The MS/MS HCD scan starts at 110 m/z with a resolution of 30,000. Dynamic exclusion was enabled with a repeat count of 1 and ions within 10 ppm of the fragmented mass were excluded for a duration of 60 s.

The data were analyzed using Proteome Discoverer V2.3 (Thermo Fisher Scientific, Waltham, MA, USA) with the search engine Sequest-HT which is integrated with the Proteome Discoverer software (Thermo Fisher Scientific, Waltham, MA, USA). The protein sequence database used to search the MS/MS spectra was the Uniprot mouse protein database containing 25,035 entries with an automatically generated decoy database (reversed sequences). The protease was set to full activity trypsin with a maximum of two missed cleavages. Oxidation of Methionine and acetylation of protein N-terminus were set as dynamic modifications and carbamidomethylation of cysteine, TMT6plex of Lysine, and peptide N-terminus were set as static modifications. The precursor mass tolerance for these searches was set to 10 ppm and the fragment ion mass tolerance was set to 0.02 Da. Keratins were known contaminants and were excluded from identified proteins. A false discovery rate (FDR) was set to 1% for both peptide and protein identification and calculated using the number of identified peptides/proteins from the decoy database divided by the total number of identified peptides/proteins. Two peptides were required for positive protein identification to decrease the chance of false discovery by a random match.

Relative quantitation of the samples labeled by different isobaric tags was done by the Reporter Ions Quantifier node in Proteome Discoverer using the intensity of the reporter ions from MS/MS scans. The m/z tolerance of the reporter ions was set to 20 ppm, and the ion selection was set to the most confident centroid. Quantitative values were normalized by the total amount of peptide in each label channel. The peptide used for quantification was set to Unique + Razor, and the precursor Co-isolation threshold was set to 50%. Razor-peptides are non-unique peptides and these are assigned to the protein group containing the largest number of other peptides, according to Occam's razor principle.

Bioinformatics

The high-qualified ($P < 0.05$ from three biological replicates) differentially expressed proteins with at least two matching peptides and phosphopeptides with >2-fold change between 1 and 10mM Pi-treated cells, and 1 and 40mM Pi-treated cells, were the input dataset for the bioinformatic analyses.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapping

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map is a manually drawn graphical diagram showing metabolic, signaling, and other molecular interaction/reaction networks (16). The differential hits were uploaded to KEGG Mapper (https://www.genome.jp/kegg/tool/map_pathway1.html) to map specific proteins in KEGG signaling pathways.

HuRI Mapping

HuRI is a reference map of the human binary protein interactome, documenting human "all-by-all" binary protein interactions based on experimental validation (high-throughput yeast two-hybrids) and literature curation (17). The differential hits were uploaded to the Human Reference Interactom (HuRI) database (http://www.interactome-atlas.org/search) to acquire the Pi-associated protein interactome.

Ingenuity Pathway Analysis (IPA)

Ingenuity pathway analysis is a web-based software application that enables analysis, integration, and understanding of data from gene expression, miRNA, and SNP microarrays, as well as metabolomics, proteomics, and RNAseq experiments (18–21). The proteins and protein phosphorylation significantly dysregulated by high Pi treatment identified in the proteome and phosphoproteome were investigated using IPA software [outsource service provided by QIAGEN (Germantown, MD,

USA), https://www.qiagen.com/ingenuity]. This analysis used a pre-made significance test as a cut-off ($p < 0.05$) for all fold change values, to note enrichment in IPA. The analysis examines genes in the dataset known to affect each biological function and compares their direction of change to what is expected from the literature. Phospho-proteomic core analysis is performed to identify significantly associated canonical pathways, predicted upstream regulators, the top predicted diseases and bioFunctions associated with differentially expressed gene set and molecular networks.

Statistics

An unpaired student's *t*-test was used to compare means between control and high Pi-treated groups. A value of $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. GraphPad Prism version 8 software was used to perform the statistics (San Diego, CA, USA).

RESULTS

Proteomic and Phosphoproteomic Profiling of High Pi-treated Cells

To understand the complex networks and functions coordinated by protein phosphorylation, we adopted proteomic and phosphoproteomic platforms to analyze Pi-related protein and protein phosphorylation change globally and quantitatively in HEK293 cells (**Figure 1A**).

The cells were treated with 1 mM (physiological), 10 mM (pro-survival), and 40 mM (pro-death) Pi for 24 h. We applied an abnormally high concentration of Pi in the acute phosphate toxicity cell model to determine the immediate cytotoxic effects, as detailed in our earlier publication (12). The extracted protein was digested and labeled prior to LC-MS/MS analysis. Each treatment has 3 biological replicates. A total of 4,704 proteins were identified with at least two matching peptides (**Supplementary Table 1**), and the quantitation result is given in **Supplementary Table 2**. A total of 5,041 phosphopeptides were identified, within which 4,249 peptides had quantitative values (**Supplementary Table 3**). We thereafter compared the global protein and protein phosphorylation changes between 1 and 10 mM, and 1 and 40 mM Pi-treated groups and set ≥2-fold change and $P \leq 0.05$ ($n = 3$) as cut-off values. Compared to 1mM Pi-treated groups, 10 mM Pi treatment decreased the level of one protein (Calmodulin-3, highlighted in **Supplementary Table 4**) and 20 phosphopeptides from 12 proteins (highlighted in red in **Supplementary Table 5**), increased the abundance of one phospho-peptide (S138) from Alpha-2-HS-glycoprotein (highlighted in yellow in **Supplementary Table 5**). In contrast to 10, 40 mM Pi caused more pronounced global protein expression and protein phosphorylation changes. The treatment resulted in the downregulation of 44 proteins (highlighted in red in **Supplementary Table 6**) and upregulation of 18 proteins (highlighted in yellow in **Supplementary Table 6**). It led to 560 underrepresented phosphopeptides from 171 proteins (highlighted in red in **Supplementary Table 7**) and 19 overrepresented phosphopeptides from 14 proteins (highlighted in yellow in **Supplementary Table 7**). The differential expressed proteins and phosphopeptides are summarized in **Figure 1B**.

Bioinformatic Analysis of High Pi-perturbed Cell Signaling Networks

The differentially expressed proteins and protein phosphorylation with high quality (as described at Bioinformatics) are the input dataset for the following bioinformatic analyses.

KEGG Mapping

The analysis mapped differential protein and phosphorylation hits between 1 and 40 mM Pi-treated cells to enriched pathways, including spliceosome, complement, and coagulation cascades, Rap1 signaling pathway for proteome, and spliceosome, RNA transport, and pathways in cancer for phosphoproteome. High Pi impairment of spliceosome assembly is exemplified in **Supplementary Figure 1A**.

HuRI Mapping

The analysis identified protein-protein interaction networks of 40mM Pi-mediated reduction of protein expression and protein phosphorylation. The molecular interaction networks are involved in RNA splicing by proteomics and phosphoproteomics (**Supplementary Figure 1B**).

IPA

Canonical Pathways

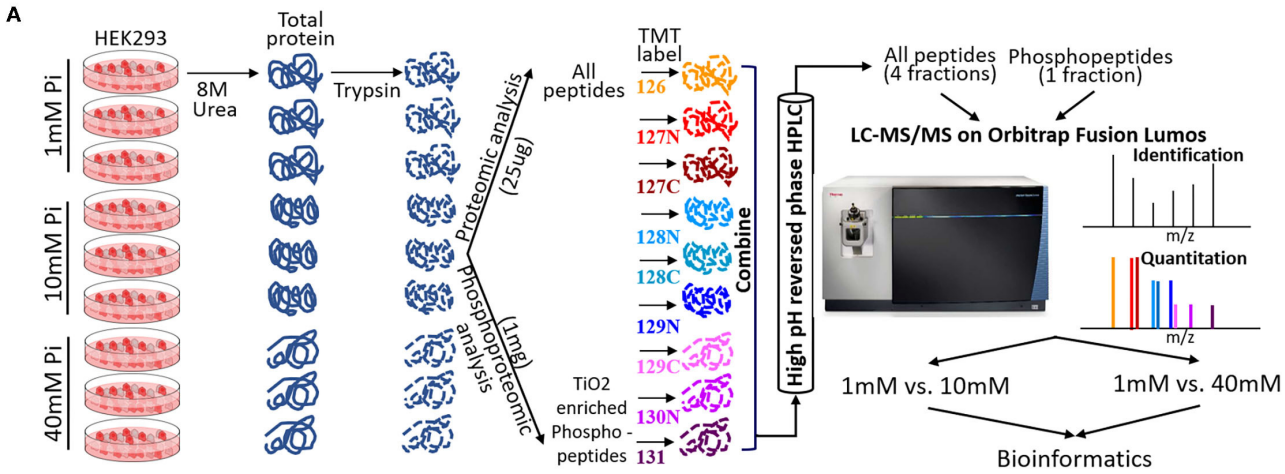
The core analysis identified the IPA canonical pathways that were significantly enriched from the dataset. Fisher exact test was used to determine significant p-values and the association of the dataset with canonical pathways. In addition, pathways were predicted to be activated or inhibited based on the entire dataset provided to IPA. The analysis identified significantly dysregulated pathways in 40mM Pi-treated cells. A Heatmap of the comparison analysis showed significant inactivation of EIF2 signaling by proteomic analysis and spliceosomal cycle by phosphoproteomic analysis (**Figure 2A**). Specific pathways, such as EIF2 signaling (**Figure 2B**) and spliceosomal cycle (**Figure 2C**), were colored using the Molecular activity predictor in IPA.

Upstream Regulators

Ingenuity pathway analysis (IPA) core analysis allows for predicting upstream regulators that may be responsible for the gene expression changes observed in the dataset based on the information from the IPA knowledge base. Network maps for specific upstream regulators were colored by MAP and overlaid with specific canonical pathways. **Supplementary Figure 2A** displays high Pi downregulated key regulators of RNA splicing (SRPK1/2, CLK1) and cell signaling (CSNK2A1, CDK1/6). The affected regulating networks are depicted in **Supplementary Figures 2A–H**.

Disease and Function Analysis

The Diseases and Functions Analysis identifies downstream effects that are expected to increase or decrease, given the observed gene expression changes in the dataset. It is based on the expected causal effects, derived from the literature compiled



B

Groups	Proteomics	Phosphoproteomics
1mM vs. 10mM	volcano plot Down-regulated: Calmodulin-3 *P*=0.0001 10/1=0.35	Down-regulated: 12 proteins 20 phosphopeptides *P* ≤ 0.05 10/1≤ 0.5 Up-regulated: 1 protein 1 phosphopeptide *P*= 0.0108 10/1=2.46 Alpha-2-HS-glycoprotein (S138)
1mM vs. 40mM	Down-regulated: **44** *P* ≤ 0.05 40/1≤ 0.5 Calmodulin-3 included (40/1=0.26) Up-regulated: **18** *P* < 0.05 40/1 ≥ 2	Down-regulated: 171 proteins 560 phosphopeptides *P* ≤ 0.05 40/1≤ 0.5 Up-regulated: 14 p-proteins 19 phosphopeptides *P* ≤ 0.05 40/1 ≥ 2

FIGURE 1 | Comparison of protein abundance and protein phosphorylation between non-treated and high Pi-treated cells by quantitative proteomics and phosphoproteomics. **(A)** Experimental flow chart. **(B)** Summary of differential hits between groups. The embedded volcano plots demonstrate statistical significance (P-value, y-axis) vs. magnitude of change (fold change, x-axis).

in the Ingenuity Knowledge Base, between genes and functions. The analysis examines genes in the dataset that are known to affect functions and uses the expected causal effects of the genes derived from the literature to issue a prediction for each function, based on the direction of change in gene expression. The z-score captures the direction of change. In line with our previous findings (12), proteomics and phosphoproteomics revealed that 10mM Pi treatment increased cell viability and decreased cell death/apoptosis, while 40mM Pi treatment enhanced cell death and mitigated cell survival (**Supplementary Figure 3**).

Network Analysis

Network view displays an interactive graphical representation of the interrelationships between molecules. The analysis demonstrated complicated molecular networks regulating splicing and processing of RNA in 40mM Pi-treated cells by phosphoproteomics (**Supplementary Figure 4**).

Literature-Based High Pi-impacted Cellular Processes

In parallel, the differential hits resulting from different concentrations of Pi treatment by proteomics and phosphoproteomics were searched against published data. As shown in **Supplementary Figure 5**, the literature search revealed that the dysregulation of protein expression and phosphorylation by high Pi suppresses Calcium signaling, CREB signaling, cell cycle progression, and pre-mRNA alternative splicing. It promotes TGF-β signaling, ER stress, and apoptosis. Cross-talk among the Pi-rewired signaling pathways is demonstrated in

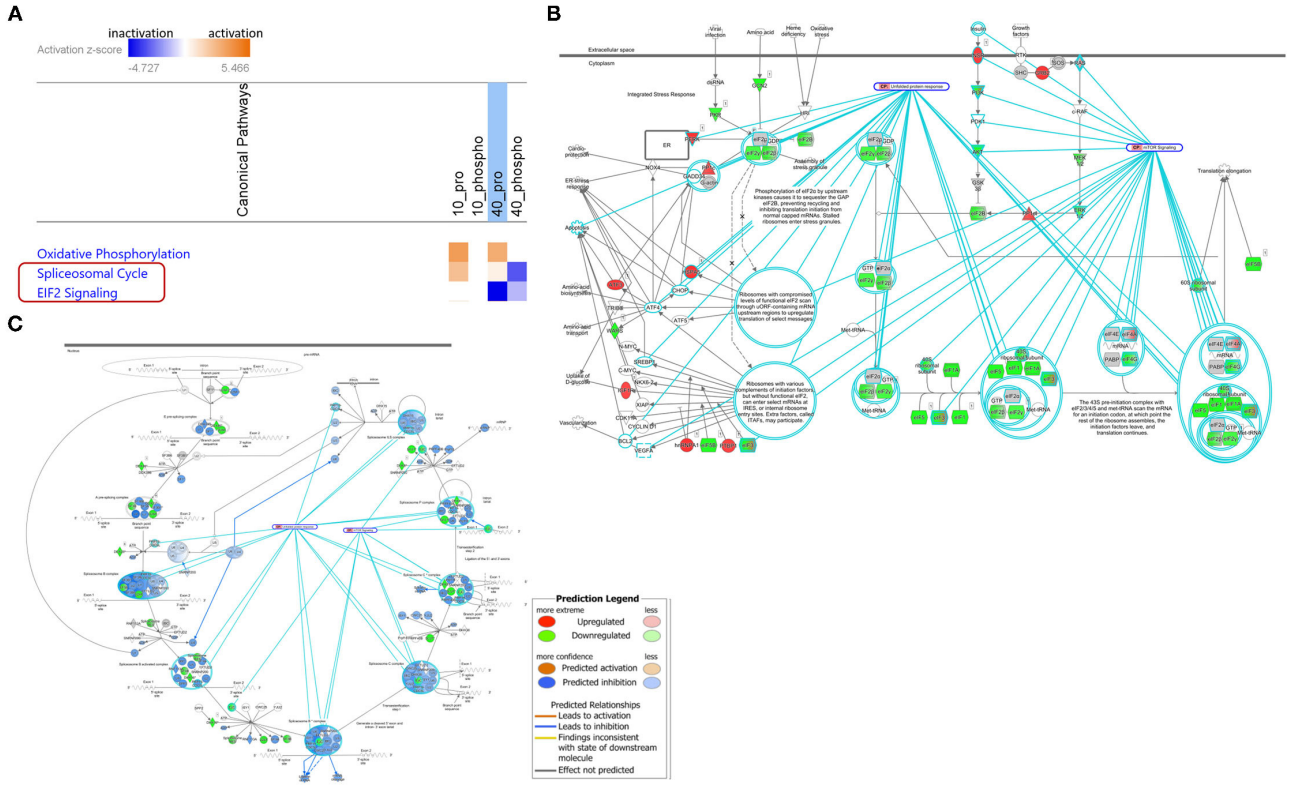


FIGURE 2 | High Pi-dysregulated significant pathways by IPA Canonical Pathways analysis. **(A)** Heatmap of the comparison analysis. Orange: pathways with positive z-scores; Blue: pathways with negative z-scores; White: pathways that have a z-score of 0, indicating that the differential gene expression data did not allow for a clear determination of the activity prediction. 10_Pro: 10 mM Pi treatment by proteomic analysis; 10_Phospho: 10 mM Pi treatment by phosphoproteomic analysis; 40_Pro: 40 mM Pi treatment by proteomic analysis; 40_Phospho: 40mM Pi treatment by phosphoproteomic analysis. EIF2 signaling **(B)** and spliceosomal cycle **(C)** pathways were colored using the Molecular activity predictor. Molecules in red and green indicate high Pi upregulated and downregulated protein expression or phosphorylation, respectively.

Figure 3A. Notably, mRNA alternative splicing is the most extensively affected by high Pi treatment (**Figure 3B**).

High Pi-mediated the Dysregulation of MRNA Splicing

Bioinformatics (**Figure 2**, **Supplementary Figures 1**, **5**) and literature searching (**Figure 3**) congruently show the high Pi-elicited differentially expressed and phosphorylated proteins enriched in pathways that regulate pre-mRNA splicing and spliceosome assembly. Among the 40mM Pi downregulated proteins and protein phosphorylation, 12 out of 18 (66.7%) proteins and 63 out of 187 (33.7%) phosphorylated proteins participate in RNA splicing. To validate the proteomic and phosphoproteomic data, we examined the expression and phosphorylation of RNA splicing effectors, especially SR proteins (SRSFs) and SR protein kinases (SRPK), and their master regulators, CSNK2A1 and JUN (as indicated in **Supplementary Figures 2, 4, 5**), PP1C (22) and MYC (23) (as noted in literature). WB analysis verified universal decline of SRSFs' expression (SRSF1, SRSF4, and SRSF10) and phosphorylation (SRSF1, SRSF3, SRSF4, SRSF5, and SRSF6) induced by excessive extracellular Pi (**Figure 4A**). We observed a slight molecular weight (Mw) shift (possibly due to high Pi-mediated dephosphorylation) of SRSF9 in 40mM Pi-treated cells (**Figure 4B**). We also observed reduced SRPK1, but not SRPK2, in high Pi-treated cells (**Figures 4A,C**). The high Pi-suppressed SRSFs and SRPK1 were abolished by the inhibition of Pi intake with phosphonoformic acid (PFA), an inhibitor of sodium/phosphate (Na/Pi) co-transporters (24). Regarding the upstream regulators of SRSFs and SRPKs, we validated increased phosphorylation of c-JUN at Serine (S) 63 and S73 and detected decreased expression of MYC induced by high Pi (**Figure 4B**). However, we found no expression changes in CSNK2A1 and PP1C in the cultured cells exposed to elevated Pi (**Figure 4A**).

High Pi-induced Cell Death Is Independent of Aberrant MRNA Splicing

Western blot (WB) analysis displayed 40mM Pi suppressed splicing of Caspase-2 by showing decreased Caspase-2 long isoform (Casp2L) compared to Caspase-2 short isoform (Casp2S), but no splicing alterations in Caspase-9 nor Bcl-x (**Figure 5A**).

As global proteomic and phosphoproteomic profiling, in line with WB verification, demonstrated a high Pi-mediated

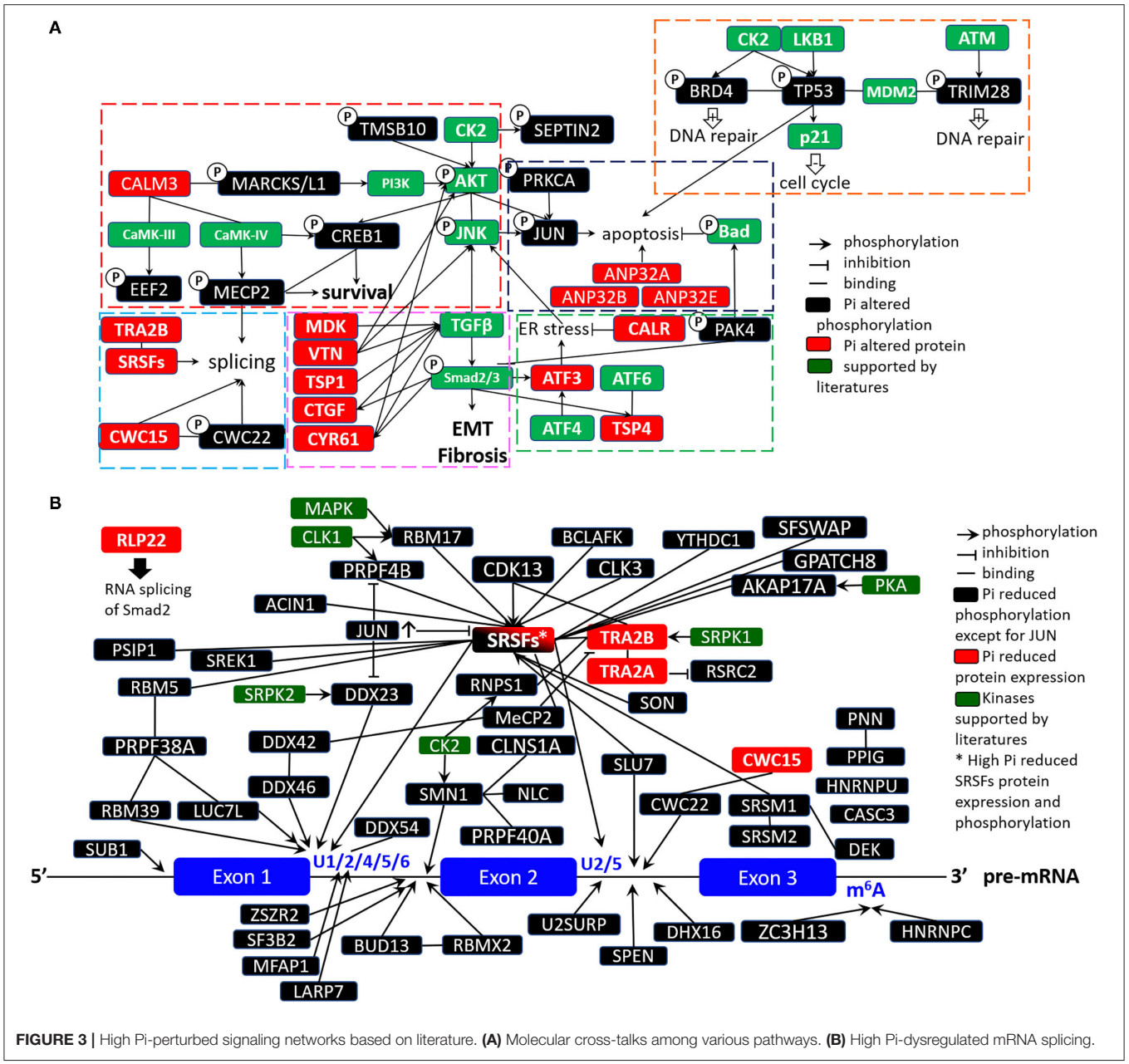


FIGURE 3 | High Pi-perturbed signaling networks based on literature. **(A)** Molecular cross-talks among various pathways. **(B)** High Pi-dysregulated mRNA splicing.

decrease in the expression and phosphorylation of key mRNA splicing regulators, we wanted to know if the complementation of those regulators could rescue the cells cultured in a high Pi medium. HEK293 cells were transfected with plasmids encoding wild-type (WT) SR proteins (SRSF1-7, SRSF11, SRF12, TRA2A, and TRA2B) fused with Flag tag. WB analysis confirmed the successful over-expression of each regulator at correct Mws by using an antibody against the Flag tag (**Figure 5B**). After 24 h following transfection, cells were treated with 40mM Pi for another 24 h followed by XTT analysis of cell survival. XTT analysis showed that high Pi-induced cell death could not be significantly prevented by over-expressing SR proteins (**Figure 5C**), suggesting minor roles of defective mRNA splicing in excess Pi-mediated cell damage.

DISCUSSION

Use of Phosphoproteomics to Study High Pi-rewired Signaling Networks

We previously showed high phosphate-mediated damage in mice kidneys (25), and in cells that originated from kidneys, such as HEK293 (12). The *in vitro* study showed that extracellular increased Pi elicited a profound change in protein phosphorylation, which is one type of post-translational modification (PTM). It is the core driving force for cell signaling and orchestrates several cellular processes (26). Intracellular signaling usually involves reversible protein phosphorylation regulated by the activity of kinase that uses ATP as a substrate to phosphorylate signaling molecules and

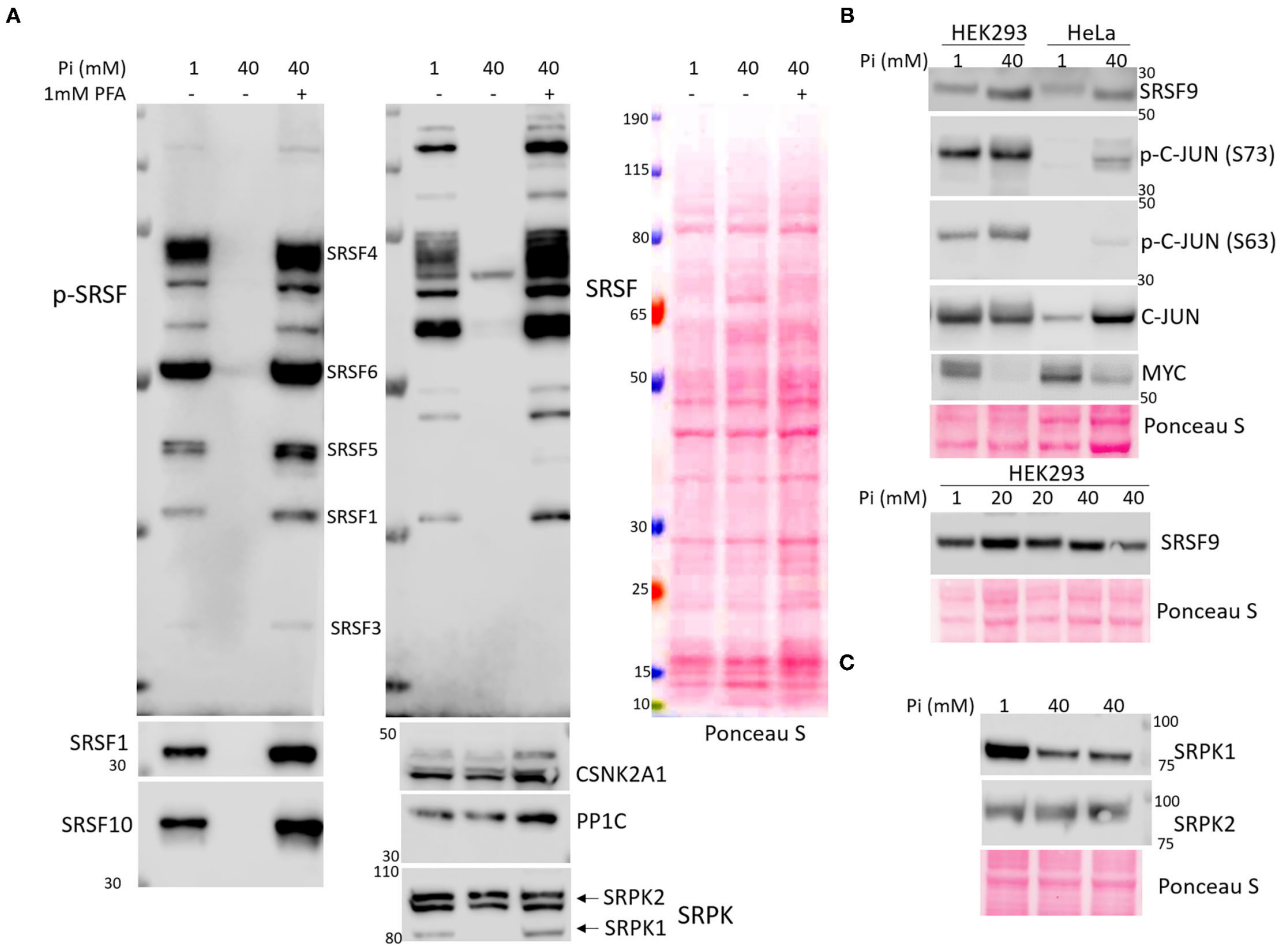


FIGURE 4 | Elevated extracellular phosphate-mediated dysregulation of the effectors in mRNA alternative splicing process by Western blot (WB) analysis. HEK293 and HeLa cells were grown to 80–90% confluence followed by treatment with increased NaH_2PO_4 (Pi) for 24 h. In the PFA treated group **(A)**, HEK293 cells were pre-treated with 1mM PFA for 0.5 h followed by 40mM Pi treatment in the presence of PFA for 24 h. Total protein (30 μg) extracted from each treatment was used for WB analysis with the primary anti-phospho SRSFs, SRSFs, CSNK2A1, PP1C, SRPK (recognizing both SRPK1 and SRPK2) antibodies in **(A)**, anti-phospho c-JUN (S63), phospho c-JUN (S73), c-JUN, SRSF9, and MYC antibodies in **(B)**, and anti-SRPK1and SRPK2 antibodies in **(C)**. The Ponceau S stain of the membrane was used as a loading control.

the activity of phosphatase that catalyzes the transfer of the Pi from a phosphoprotein to a water molecule (27). Pi may serve as an important regulator of phosphatase in different organisms (28). Hence, the alteration of extracellular Pi may affect the activity of phosphatase and regulate the overall cell signaling network. Beck's group established the investigation of phosphate-controlled cellular response by quantitative proteomics (2, 29, 30). They used cleavable isotope-coded affinity tag reagents to identify and quantitate protein expression differences in phosphate-treated murine MC3T3-E1 osteoblast cells. They found a nearly two-fold increased abundance of Cyclin D1 when cells treated with 10mM NaH_2PO4 for 24 h were compared to Na_2SO4 treated control cells (30). However, these studies focused on Pi-related transcriptomics and proteomics instead of PTMs, such as phosphorylation. Therefore, global phosphorylation dynamics perturbed by extracellular phosphate remain elusive. The phosphoproteomic strategy has been proven to successfully analyze a drug or pathogen-induced overall change in phosphorylation events occurring in a cell at different time phases (31–37). A phosphoproteomics-based investigation of the global phosphorylation and protein abundance landscape of phosphate toxicity can help us understand the connections between the known Pi-related cell signaling pathways and expand our view of phosphate-associated signaling networks that induce direct cytotoxicity. Thus, we adopted quantitative proteomic and phosphoproteomic platforms to analyze Pi-mediated protein and protein phosphorylation change (**Figure 1A**). The analysis revealed significant changes in both global protein expression and protein phosphorylation induced by extremely high concentrations of extracellular Pi, especially for 40 mM Pi-treated cells (**Figure 1B**).

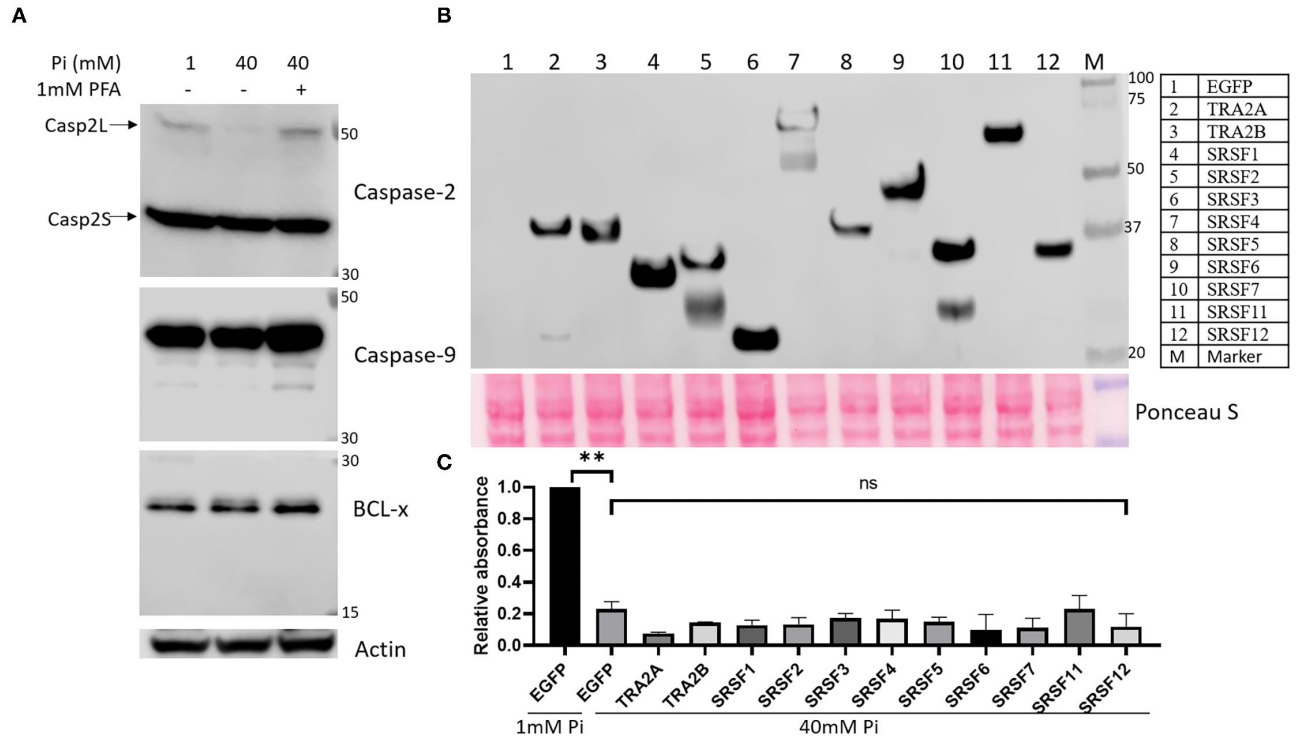


FIGURE 5 | (A) High Pi treatment reduces alternative splicing of Caspase-2 but has no effect on Caspase-9 and BCL-x splicing in HEK293 cells. Actin was used as a loading control. **(B)** Over-expression of SR proteins. HEK293 cells were transfected with pIRES2-EGFP (negative control) and pcDNA3.1-Flag vectors harboring WT full-length cDNA sequence of human SRSF1-7, SRSF11, SRF12, TRA2A, and TRA2B. Cells were harvested 24 h after transfection, and 15μg total protein was analyzed by WB with anti-Flag primary antibody. PageRuler^{TM} Plus Prestained 10-250 kDa Protein Ladder was loaded as protein molecular weight marker. Ponceau S stain of the membrane was used as a loading control. **(C)** WT SR protein complementation does not prevent high Pi-induced cell death by XTT analysis. After being transfected with plasmids for 24 h, HEK293 cells were treated with 1 mM and 40 mM Pi for 24 h followed by XTT assay. The data were represented as means ± SEM from two independent experiments. An unpaired Student t-test was used to compare means between 1 and 40 mM Pi-treated cells transfected with the pIRES2-EGFP plasmid, and 40 mM Pi-treated cells transfected with pIRES2-EGFP plasmid vs. vectors encoding SR proteins. $^{**}P < 0.01$; ns, not significant.

Bioinformatics and Literature Searching Reveals the Cross-Talk Among High Pi-perturbed Cell Signaling Pathways

To acquire an extensive and in-depth view of high Pi-dysregulated cellular processes, we harnessed different bioinformatic tools to study Pi-altered pathways (by KEGG mapping and IPA), protein-protein interaction network (by HuRI mapping), and master regulators (by IPA). These bioinformatic analyses revealed elevated Pi-rewired Rap1 signaling and EIF2 signaling pathways (**Figure 2A**), as well as high Pi-mediated dysregulation of pre-mRNA alternative splicing and the spliceosomal cycle (**Supplementary Figure 1**, **Figure 2**). Further literature search confirmed not only excess Pi-perturbed mRNA splicing, but also displayed high Pi-dysregulated calcium signaling, CREB signaling, cell cycle progression, TGF-β signaling, ER stress, and apoptosis. The latter three biological processes induced by high Pi were also validated in our earlier study (12). Moreover, Pi-related cellular processes and pathways synergistically contribute to high Pi's cytotoxicity by extensive and intricate cross-talk (**Figure 3A**).

Pi Cytotoxicity and Defective mRNA Splicing

We focused on high Pi-mediated aberrant mRNA splicing because both bioinformatics and literature review (38) revealed high Pi caused differential protein expression and phosphorylation highly enriched in the pre-mRNA splicing process. To the best of our knowledge, there is no study focusing on phosphate's toxic effects on RNA splicing. The assembly of the spliceosome and the subsequent splicing requires serial phosphorylation and dephosphorylation of essential factors known as SR proteins (38). Impressively, proteomic analysis revealed that the majority of SR protein family members (SRSF1-8,12 and TRA2A/B) showed lower abundance and hypo-phosphorylation in 40mM Pi-treated cells compared to 1mM Pi-treated cells. Kinases are known to phosphorylate the RS (Arginine/Serine)-rich regions of SR proteins and other spliceosomal proteins include members of the Cdc2-like kinase family (CLK1-3, PRPF4B) and SRPK family (SRPK1-3), CDK13, and DNA topoisomerase 1 (39). WB analysis verified the reduction in protein expression and phosphorylation in several SR proteins and demonstrated low expression of one

of the key kinases of SR proteins, SRPK1 (**Figure 4**). Since dephosphorylation by phosphatase (mainly PP1 and PP2A) in SR proteins is also required for splicing activity (40), we tested PP1's expression in high-Pi treated cells. However, WB did not detect a significant change in the PP1 level (**Figure 4A**). Beyond kinases and phosphatases, splicing effectors can also be transcriptionally modulated. Katiyar et al. reported that c-JUN could directly impair mRNA splicing by downregulating the expression of over 50 genes controlling mRNA processing and splicing, including SRSFs (41). Oncoprotein MYC could transcriptionally upregulate the expression of the SR protein splicing factor, SRSF1, in lung cancer cells, which triggered mRNA splicing of a series of kinases and facilitated oncogenic signaling (23). Indeed, we found increased phosphorylation of JUN and a reduced amount of MYC in elevated Pi-treated cells (**Figure 4B**). We applied the HeLa cell line, a widely used homogenous experimental cell line in the published research works, in the verification study to ensure the consistency and the reproducibility of WB data. Therefore, we used HEK293 cells as primary and HeLa cells as secondary cell-based model systems in this study. Taken together, our findings suggest increased phosphate exposure gives rise to defective mRNA splicing in cultured cells.

Next, we wanted to determine whether high Pi-associated alteration of mRNA splicing contributes to phosphate cytotoxicity. Excessive Pi-induced apoptosis is one of the fundamental mechanisms of Pi-related tissue damage (11, 12). The alternative pre-mRNA splicing regulated programmed cell death has been well documented (42–44). As summarized by Schwerk and Schulze-Osthoff (43), several pro-/anti-apoptotic effectors are regulated by alternative splicing that generates different protein isoforms with different and sometimes even opposite functions during apoptosis. For instance, SR proteins, SRSF1 and SRSF2, promote skipping of exon 9 in the *Caspase-2* gene, causing an increased expression of pro-apoptotic Capase-2 long isoform (Casp2L) and thus enhancing apoptosis. In contrast, hnRNP facilitates the inclusion of exon 9 in *Caspase-2,* generating a premature stop codon and that leads to the expression of pro-survival Capase-2 short isoform (Casp2S) (45). Consistently, we found elevated Pi-induced depression of SRSF1, SRSF2, and the subsequent decrease of pro-apoptotic Casp2L (**Figure 5A**), indicating an anti-apoptotic role of high Pi-dysregulated mRNA splicing. Researchers also found that the apoptosis inducer, ceramide, could dephosphorylate SR proteins by activating PP1 phosphatase, which mediated the increase of pro-apoptotic splicing variants of Caspase 9 and Bcl-x(s) (46). However, we did not detect excess Pi-mediated alterations in the alternative splicing of Caspase-9 and Bcl-x (**Figure 5A**). The over-expression of SR proteins could not prevent high Pi-induced cell death (**Figure 5B**), suggesting high Pi-mediated suppression of SR proteins plays a minor role in excessive Pi-induced apoptosis. Collectively, this data indicates cell damage caused by excess Pi is not primarily ascribed to the changes in mRNA alternative splicing.

Limitations

We previously showed the effects of phosphate toxicity on kidney tissues (25) and cells obtained from the kidney (HEK293) (12). Hence, we used HEK293 cells as primary cells to investigate cell signaling networks rewired by elevated phosphate in this study. However, since different cell lines' responses to increased extracellular Pi may vary (13, 15, 47–49), the proteomic and phosphoproteomic landscapes in high environmental Pi from one cell line may not be applicable to other cell lines. Systematic analysis of other lines derived from various tissues cultured in medium with different concentrations of Pi is desired to globally profile Pi-associated proteomic and phosphoproteomic signatures. This study focuses on acute Pi-mediated cytotoxicity by exposing cells with a high concentration of Pi for 24 h. Chronic effects (for instance, 3–7 days treatment) of elevated Pi may generate varying results on proteomics and phosphoproteomics. Again, in our study, we used the non-physiologic concentration of Pi to document the immediate cytotoxic effects of Pi by exposing the cells to the toxic range of Pi. We believe such an approach is likely to provide mechanistic insights into acute cytotoxicity.

We did not observe the rescue effects with the over-expression of plasmid-encoded WT SR proteins in high Pi-treated cells. The transfection of a single target may cause it. Several SR proteins may play combinational roles in regulating mRNA alternative splicing in high Pi-treated HEK293 cells. Therefore, co-transfection of multiple SR proteins in excessive Pi-treated cells may assist the understanding of the relationship between RNA splicing and apoptosis in the context of phosphate toxicity.

CONCLUSION

Underlying the broad spectrum of phosphate cytotoxicity is the intricate high phosphate-perturbed cell signaling networks and cellular processes as revealed by quantitative proteomic and phosphoproteomic analyses. The bioinformatics and functional assays determined abnormal pre-mRNA splicing as a novel mechanism of phosphate-induced cytotoxicity. An in-depth study of how aberrant alternative splicing contributes to phosphate toxicity will open another window to fully understand the high phosphate-related pathologies, and likely to provide therapeutic clues to manage phosphate toxicity-associated organ damages.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the PRIDE repository, accession number PXD026301, and doi: 10.6019/PXD026301.

AUTHOR CONTRIBUTIONS

PH and MR: conceptualization. RH and EB: methodology. PH and LL: software. RH and PH: validation. PH: formal analysis, writing—original draft preparation, supervision, project administration, and funding acquisition. AJ, SK, and BW:

investigation. LL and BW: resources. EB, AJ, and SK: data curation. RH and MR: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by LECOM internal Seed Grants, grant number COM-20-21. The Fusion Lumos instrument was purchased *via* an NIH shared instrument grant, 1S10OD023436-01.

ACKNOWLEDGMENTS

We appreciate Dr. Margo Wolfe's assistance in proofreading the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.765391/full#supplementary-material

REFERENCES

1. Michigami T, Kawai M, Yamazak M, Ozono K. Phosphate as a signaling molecule and its sensing mechanism. *Physiol Rev.* (2018) 98:2317–48. doi: 10.1152/physrev.00022.2017
2. Camalier CE, Yi M, Yu LR, Hood BL, Conrads KA, Lee YJ, et al. An integrated understanding of the physiological response to elevated extracellular phosphate. *J Cell Physiol.* (2013) 228:1536–50. doi: 10.1002/jcp.24312
3. Goodson JM, Shi P, Razzaque MS. Dietary phosphorus enhances inflammatory response: a study of human gingivitis. *J Steroid Biochem Mol Biol.* (2019) 188:166–71. doi: 10.1016/j.jsbmb.2019.01.023
4. Goodson JM, Shi P, Mumena CH, Haq A, Razzaque MS. Dietary phosphorus burden increases cariogenesis independent of vitamin D uptak. *J Steroid Biochem Mol Biol.* (2017) 167:33–8. doi: 10.1016/j.jsbmb.2016.10.006
5. Cancela AL, Santos RD, Titan SM, Goldenstein PT, Rochitte CE, Lemos PA, et al. Phosphorus is associated with coronary artery disease in patients with preserved renal function. *PLoS ONE.* (2012) 7:e36883. doi: 10.1371/journal.pone.0036883
6. Mancini FR, Affret A, Dow C, Balkau B, Clavel-Chapelon F, Bonnet F, et al. High dietary phosphorus intake is associated with an increased risk of type 2 diabetes in the large prospective E3N cohort study. *Clin Nutr.* (2018) 37:1625–30. doi: 10.1016/j.clnu.2017.07.025
7. Razzaque MS. Phosphate toxicity: new insights into an old problem. *Clin Sci.* (2011) 120:91–7. doi: 10.1042/CS20100377
8. Marks J, Debnam ES, Unwin RJ. The role of the gastrointestinal tract in phosphate homeostasis in health and chronic kidney disease. *Curr Opin Nephrol Hypertens.* (2013) 22:481–7. doi: 10.1097/MNH.0b013e3283621310
9. Brown RB, Razzaque MS. Phosphate toxicity and tumorigenesis. *Biochim Biophys Acta Rev Cancer.* (2018) 1869:303–9. doi: 10.1016/j.bbcan.2018.04.007
10. Giachelli CM. The emerging role of phosphate in vascular calcification. *Kidney Int.* (2009) 75:890–7. doi: 10.1038/ki.2008.644
11. Di Marco GS, Hausberg M, Hillebrand U, Rustemeyer P, Wittkowski W, Lang D, et al. Increased inorganic phosphate induces human endothelial cell apoptosis *in vitro*. *Am J Physiol Renal Physiol.* (2008) 294:F1381–7. doi: 10.1152/ajprenal.00003.2008
12. He P, Mann-Collura O, Fling J, Edara N, Hetz R, Razzaque MS. High phosphate actively induces cytotoxicity by rewiring pro-survival and pro-apoptotic signaling networks in HEK293 and HeLa cells. *Faseb J.* (2021) 35:e20997. doi: 10.1096/fj.202000799RR
13. Chang SH, Yu KN, Lee YS, An GH, Beck GR, Colburn NH, et al. Elevated inorganic phosphate stimulates Akt-ERK1/2-Mnk1 signaling in human lung cells. *Am J Respir Cell Mol Biol. (*2006) 35:528–39. doi: 10.1165/rcmb.2005-0477OC
14. Jin H, Xu CX, Lim HT, Park SJ, Shin JY, Chung YS, et al. High dietary inorganic phosphate increases lung tumorigenesis and alters Akt signaling. *Am J Respir Crit Care Med.* (2009) 179:59–368. doi: 10.1164/rccm.200802-306OC
15. Yamazaki M, Ozono K, Okada T, Tachikawa K, Kondou H, Ohata Y, et al. Both FGF23 and extracellular phosphate activate Raf/MEK/ERK pathway via FGF receptors in HEK293 cells. *J Cell Biochem.* (2010) 111:1210–21. doi: 10.1002/jcb.22842
16. Kanehisa M, Sato Y. KEGG Mapper for inferring cellular functions from protein sequences. *Protein Sci.* (2020) 29:28–35. doi: 10.1002/pro.3711
17. Luck K, Kim DK, Lambourne L, Spirohn K, Begg BE, Bian W, et al. A reference map of the human binary protein interactome. *Nature.* (2020) 580:402–8. doi: 10.1038/s41586-020-2188-x
18. Rahm M, Merl-Pham J, Adamski J, Hauck SM. Time-resolved phosphoproteomic analysis elucidates hepatic 11,12-Epoxyeicosatrienoic acid signaling pathways. *Prostaglandins Other Lipid Mediat.* (2020) 146:106387. doi: 10.1016/j.prostaglandins.2019.106387
19. Alvarez-Salamer C, Castillo-Gonzalez R, Pastor-Fernandez G, Mariblanca IR, Pino J, Cibrian D, et al. IL-23 signaling regulation of pro-inflammatory T-cell migration uncovered by phosphoproteomics. *PLoS Biol.* (2020) 18:e3000646. doi: 10.1371/journal.pbio.3000646
20. Schafer A, Gjerga E, Welford RW, Renz I, Lehembre F, Groenen PM, et al. Elucidating essential kinases of endothelin signalling by logic modelling of phosphoproteomics data. *Mol Syst Biol.* (2019) 15:e8828. doi: 10.15252/msb.20198828
21. Zadora PK, Chumduri C, Imami K, Berger H, Mi Y, Selbach M, et al. Integrated phosphoproteome and transcriptome analysis reveals chlamydia-induced epithelial-to-mesenchymal transition in host cells. *Cell Rep.* (2019) 26:1286–302 e8. doi: 10.1016/j.celrep.2019.01.006
22. Mermoud JE, Cohen PT, Lamond AI. Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism, *Embo J.* (1994) 13:5679–88. doi: 10.1002/j.1460-2075.1994.tb06906.x
23. Das S, Anczukow O, Akerman M, Krainer AR. Oncogenic splicing factor SRSF1 is a critical transcriptional target of MYC. *Cell Rep.* (2012) 1:110–7. doi: 10.1016/j.celrep.2011.12.001
24. Szczepanska-Konkel M, Yusufi AN, VanScoy M, Webster SK, Dousa TP. Phosphonocarboxylic acids as specific inhibitors of Na+-dependent transport of phosphate across renal brush border membrane. *J Biol Chem.* (1986) 261:6375–83. doi: 10.1016/S0021-9258(19)84572-6
25. Nakatani T, Sarraj B, Ohnishi M, Densmore MJ, Taguchi T, Goetz R, et al. *In vivo* genetic evidence for klotho-dependent, fibroblast growth factor 23 (Fgf23) -mediated regulation of systemic phosphate homeostasis. *Faseb J.* (2009) 23:433–41. doi: 10.1096/fj.08-114397
26. Riley NM, Coon JJ. Phosphoproteomics in the age of rapid and deep proteome profiling. *Anal Chem.* (2016) 88:74–94. doi: 10.1021/acs.analchem.5b04123
27. Cheng HC, Qi RZ, Paudel H, Zhu HJ. Regulation and function of protein kinases and phosphatases. *Enzyme Res.* (2011) 2011:794089. doi: 10.4061/2011/794089
28. Dick CF, Dos-Santos AL, Meyer-Fernandes JR. Inorganic phosphate as an important regulator of phosphatases. *Enzyme Res.* (2011) 2011:103980. doi: 10.4061/2011/103980
29. Conrads KA, Yi M, Simpson KA, Lucas DA, Camalier CE, Yu LR, et al. A combined proteome and microarray investigation of inorganic phosphate-induced pre-osteoblast cells. *Mol Cell Proteomics.* (2005) 4:1284–96. doi: 10.1074/mcp.M500082-MCP200
30. Conrads KA, Yu LR, Lucas DA, Zhou M, Chan KC, Simpson KA, et al. Quantitative proteomic analysis of inorganic phosphate-induced murine MC3T3-E1 osteoblast cells. *Electrophoresis.* (2004) 25:1342–52. doi: 10.1002/elps.200405892
31. Mohideen F, Paulo JA, Ordureau A, Gygi SP, Harper JW. Quantitative phospho-proteomic analysis of TNFα/NFκB signaling reveals a role for RIPK1 phosphorylation in suppressing necrotic cell death. *Mol Cell Proteomics.* (2017) 16:1200–16. doi: 10.1074/mcp.M117.068189

32. Wiredja DD, Tabler CO, Schlatzer DM, Li M, Chance MR, Tilton JC. Global phosphoproteomics of CCR5-tropic HIV-1 signaling reveals reprogramming of cellular protein production pathways and identifies p70-S6K1 and MK2 as HIV-responsive kinases required for optimal infection of CD4+ T cells. *Retrovirology.* (2018) 15:44. doi: 10.1186/s12977-018-0423-4
33. Mandal A, Mandal S, Park MH. Global quantitative proteomics reveal up-regulation of endoplasmic reticulum stress response proteins upon depletion of eIF5A in HeLa cells. *Sci Rep.* (2016) 6:25795. doi: 10.1038/srep25795
34. Yángüez E, Hunziker A, Dobay MP, Yildiz S, Schading S, Elshina E, et al. Phosphoproteomic-based kinase profiling early in influenza virus infection identifies GRK2 as antiviral drug target. *Nat Commun.* (2018) 9:3679. doi: 10.1038/s41467-018-06119-y
35. Batth TS, Papetti M, Pfeiffer A, Tollenaere MAX, Francavilla C, Olsen JV. Large-scale phosphoproteomics reveals Shp-2 phosphatase-dependent regulators of pdgf receptor signaling. *Cell Rep.* (2018) 22:2784–96. doi: 10.1016/j.celrep.2018.02.038
36. Wiredja DD, Ayati M, Mazhar S, Sangodkar J, Maxwell S, Schlatzer D, et al. Phosphoproteomics profiling of nonsmall cell lung cancer cells treated with a novel phosphatase activator. *Proteomics.* (2017) 17. doi: 10.1002/pmic.201700214
37. Bouhaddou M, Memon D, Meyer B, White KM, Rezelj VV, Marrero MC, et al. The global phosphorylation landscape of SARS-CoV-2 infection. *Cell.* (2020) 182:685–712.e19. doi: 10.1016/j.cell.2020.06.034
38. Wilkinson ME, Charenton C, Nagai K. RNA splicing by the spliceosome. *Annu Rev Biochem.* (2020) 89:359–88. doi: 10.1146/annurev-biochem-091719-064225
39. Zhou Z, Fu XD. Regulation of splicing by SR proteins and SR protein-specific kinases. *Chromosoma.* (2013) 122:191–207. doi: 10.1007/s00412-013-0407-z
40. Mermoud JE, Cohen P, Lamond AI. Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing. *Nucleic Acids Res.* (1992) 20:5263–9. doi: 10.1093/nar/20.20.5263
41. Katiyar S, Jiao X, Addya S, Ertel A, Covarrubias Y, Rose V, et al. Mammary gland selective excision of c-jun identifies its role in mRNA splicing. *Cancer Res.* (2012) 72:1023–34. doi: 10.1158/0008-5472.CAN-11-3647
42. Paronetto MP, Passacantilli I, Sette C. Alternative splicing and cell survival: from tissue homeostasis to disease. *Cell Death Differ.* (2016) 23:1919–29. doi: 10.1038/cdd.2016.91
43. Schwerk C, Schulze-Osthoff K. Regulation of apoptosis by alternative pre-mRNA splicing. *Mol Cell.* (2005) 19:1–13. doi: 10.1016/j.molcel.2005.05.026
44. Lin JC, Tsao MF, Lin YJ. Differential impacts of alternative splicing networks on apoptosis. *Int J Mol Sci.* (2016) 17:2097. doi: 10.3390/ijms17122097
45. Jiang ZH, Zhang WJ, Rao Y, Wu, JY. Regulation of Ich-1 pre-mRNA alternative splicing and apoptosis by mammalian splicing factors. *Proc Natl Acad Sci U S A.* (1998) 95:9155–60. doi: 10.1073/pnas.95.16.9155
46. Chalfant CE, Rathman K, Pinkerman RL, Wood RE, Obeid LM, Ogretmen B, et al. *De novo* ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1. *J Biol Chem.* (2002) 277:12587–95. doi: 10.1074/jbc.M112010200
47. Beck GR Jr, Moran E, Knecht N. Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. *Exp Cell Res.* (2003) 288:288–300. doi: 10.1016/S0014-4827(03)00213-1
48. Camalier CE, Young MR, Bobe G, Perella CM, Colburn NH, Beck GR. Elevated phosphate activates N-ras and promotes cell transformation and skin tumorigenesis. *Cancer Prev Res.* (2010) 3:359–70. doi: 10.1158/1940-6207.CAPR-09-0068
49. Lin Y, McKinnon KE, Ha SW, Beck GR. Inorganic phosphate induces cancer cell mediated angiogenesis dependent on forkhead box protein C2 (FOXC2) regulated osteopontin expression. *Mol Carcinog.* (2015) 54:926–34. doi: 10.1002/mc.22153

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.

frontiers in Nutrition

SYSTEMATIC REVIEW
published: 01 February 2022
doi: 10.3389/fnut.2021.810494

Associations of the Dietary Iron, Copper, and Selenium Level With Metabolic Syndrome: A Meta-Analysis of Observational Studies

Jun Ding[1], *Qi Liu*[2], *Ze Liu*[2], *Hongbin Guo*[2,3], *Jieyu Liang*[2,3] *and Yi Zhang*[2,3]*

[1] Changsha Social Work College, Changsha, China, [2] Department of Orthopaedics, Xiangya Hospital, Central South University, Changsha, China, [3] National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, China

OPEN ACCESS

Edited by:
Mohammed S. Razzaque, Lake Erie College of Osteopathic Medicine, United States

Reviewed by:
Farhana Akter, Chittagong Medical College, Bangladesh
Rahnuma Ahmad, Medical College for Women and Hospital, Bangladesh

***Correspondence:**
Yi Zhang
zhangyi0205@csu.edu.cn

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

Received: 07 November 2021
Accepted: 21 December 2021
Published: 01 February 2022

Citation:
Ding J, Liu Q, Liu Z, Guo H, Liang J and Zhang Y (2022) Associations of the Dietary Iron, Copper, and Selenium Level With Metabolic Syndrome: A Meta-Analysis of Observational Studies. Front. Nutr. 8:810494. doi: 10.3389/fnut.2021.810494

Background: Epidemiological studies have investigated the associations of dietary iron, copper, and selenium level with metabolic syndrome (MetS). However, their results are conflicting. This meta-analysis of observational study was, therefore, employed to investigate the associations above.

Methods: A comprehensive literature search was employed using PubMed, Web of Science, Embase, and Scopus database up to October 2021 (no restriction was set for the initiate time). The pooled relative risk (RR) of MetS for the highest vs. lowest dietary iron, copper, and selenium level was estimated, respectively.

Results: A total of 14 observational studies (55,131 participants) were identified as meeting the inclusion criteria. Specifically, 7 studies were related to the dietary iron level. The overall multivariable adjusted RR demonstrated that the dietary iron level was positively associated with MetS (RR = 1.27, 95% CI: 1.12–1.44; $p < 0.001$). With regard to the dietary copper level, 7 studies were included for meta-analysis. The overall multivariable adjusted RR showed that the dietary copper level was inversely associated with MetS (RR = 0.85, 95% CI: 0.78–0.93; $p < 0.001$). In addition, 4 studies were specified for the dietary selenium level. The overall multivariable adjusted RR indicated that the dietary selenium level was inversely associated with MetS (RR = 0.77, 95% CI: 0.63–0.95; $p = 0.01$) as well.

Conclusion: Our results suggest that the dietary iron level is positively associated with MetS, whereas a negative association between the dietary copper and selenium level and

MetS is obtained. Further large well-designed prospective cohort studies are warranted to elaborate on the issues examined in this study.

Keywords: dietary iron level, dietary copper level, dietary selenium level, metabolic syndrome, meta-analysis, observational studies

INTRODUCTION

Elevated waist circumference, blood pressure, fasting blood glucose, triglycerides, and decreased high-density lipoprotein cholesterol (at least three of the five above metabolic abnormalities) are considered as the presence of metabolic syndrome (MetS) (1). MetS is a well-known attributable risk to diabetes, stroke, and coronary heart disease epidemic (2–4). Moreover, MetS increases the incidence of atherosclerotic cardiovascular disorder and complication that decreases longevity (5). However, the subjects suffering from MetS are progressively growing worldwide and the current global prevalence of MetS is between 11.6 and 62.5% (6). Although the etiology of MetS is not well understood yet, dietary factors are considered to be involved in MetS (7–11).

Micronutrients are important factors for cellular and biochemical functions (release of energy for synthesis and movement) (12). Iron, copper, and selenium are considered to be significant micronutrients and their dietary sources are meat, seeds, heme, tea, milk, nuts, cereals, eggs, fish, and so on (13–18). Iron is one of the most abundant elements, which plays a significant role in various cellular processes, such as iron-dependent signaling, cellular respiration, DNA replication and synthesis, nucleic acid repair, and energy metabolism (19–21). Iron consumption, uptake, transfer, and storage are involved to maintain iron homeostasis (22). However, excess iron leads to inflammation and tissue damage, produces hydroxyl radicals (Haber–Weiss–Fenton reactions), which cause oxidative damage to cellular components (lipids, proteins, and DNA) (23, 24). On the contrary, copper, a component of extracellular superoxide dismutase (25), is essential for iron uptake and signaling in eukaryotic organisms, energy metabolism, reactive oxygen species, and detoxification (26). In addition, copper plays an essential role in mitochondrial function and signaling involving mitophagy, bioenergetics, and dynamics, which affect cell fate by metabolic reprogramming (26). Selenium, also an essential micronutrient, is necessary to maintain the different cellular functions, such as signaling transduction pathways and immune-endocrine function (18). Moreover, selenium incorporates into selenoproteins and selenium-dependent enzymes (e.g., glutathione peroxidases), which are involved in intracellular redox regulation and modulation (27). Since oxidative stress and inflammation play a significant role in the pathophysiology of MetS (28), the dietary iron, copper, and selenium level is considered to be closely related to MetS.

A number of observational studies have been employed to investigate the associations of the dietary iron, copper, and selenium level with MetS (29–42). However, their results are still conflicting. Thus, this meta-analysis of observational studies is employed to further investigate the above associations. It is hypothesized that the dietary iron level is positively associated with MetS, whereas the dietary copper and selenium level is inversely associated with MetS.

MATERIALS AND METHODS

Search Strategy

Our meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (43). The PubMed, Web of Science, Embase, and Scopus electronic databases were searched up to October 2021 (no restriction was set for the initiate time) by using a combination of keywords that related to MetS ("metabolic syndrome"), iron ("iron," "Fe"), copper ("copper," "Cu"), and selenium ("selenium," "Se"). No language restriction was set in the search strategy. We screened the titles and abstracts of all the articles and then read the full articles to identify the eligible studies.

Study Selection

The titles, abstracts, and full texts of all the retrieved studies were reviewed by two researchers independently. Disagreements were resolved by discussions. The included studies were required to meet the following criteria: (1) the study design is an observational study; (2) the outcomes include the associations of the dietary iron, copper, and selenium level with MetS; and (3) the relative risk (RR) or odds ratio (OR) with 95% CI was reported. The exclusion criteria were listed as follows: (1) duplicated or irrelevant articles; (2) reviews, letters, or case reports; (3) randomized controlled trials; and (4) non-human studies.

Data Extraction

The data were extracted by two researchers independently and disagreements were resolved by discussions. The information about the first author and year of publication, location, age, gender, sample size, study design, adjustments, exposure, category of exposure, effect estimates, and diagnostic criteria of MetS was collected. The corresponding effect estimates with 95% CIs for the highest vs. lowest dietary iron, copper, and selenium level and MetS was extracted (adjusted for the maximum number of confounding variables).

Quality Assessment

The Newcastle–Ottawa Scale (NOS) criteria for non-randomized studies were employed to assess the quality of each included study. The NOS is based on three broad perspectives: (1) the selection process of the study cohorts; (2) the comparability among the different cohorts; and (3) the identification of exposure or outcome of the study cohorts. Disagreements

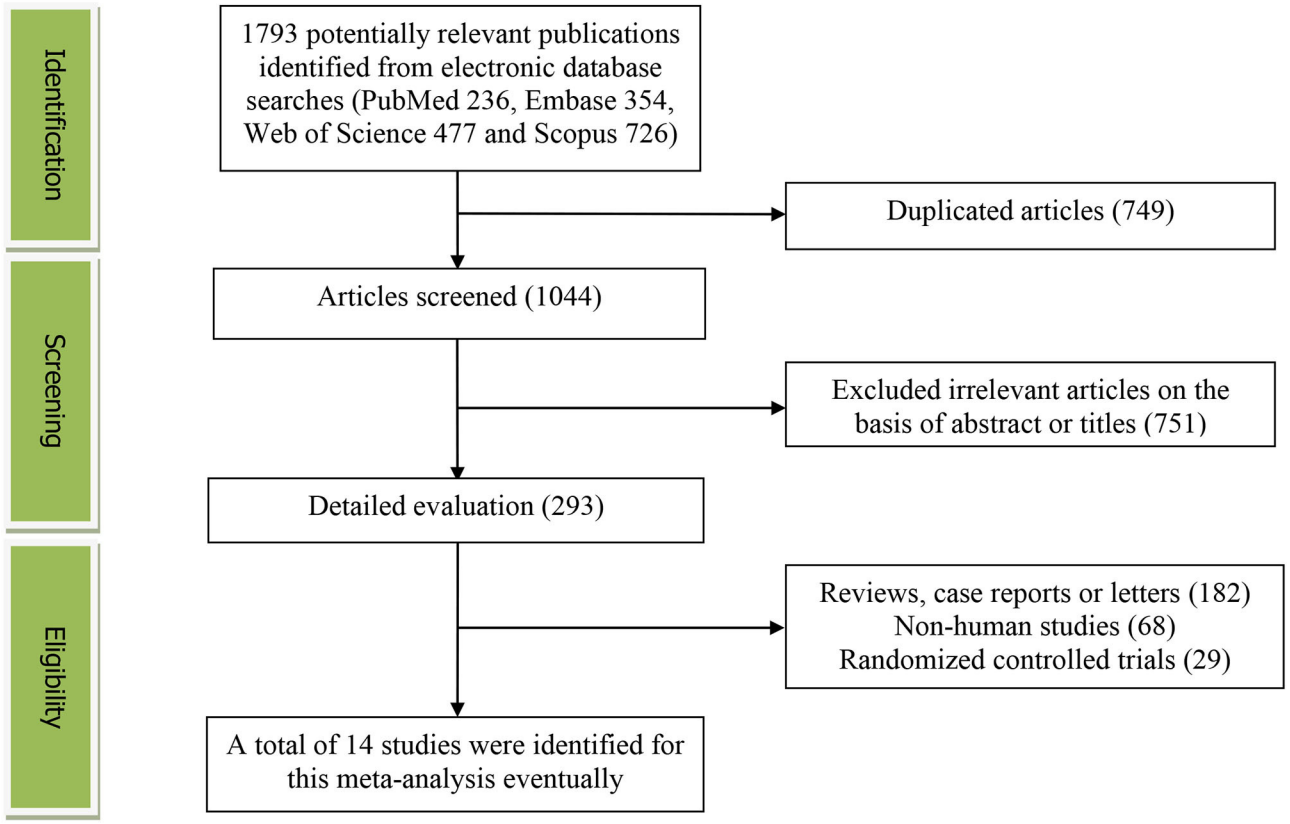


FIGURE 1 | The detailed flow diagram of the study identification and selection in this meta-analysis.

with respect to the methodological quality were resolved by mutual consultation.

Statistical Analyses

The RR for MetS was the outcome measure in this meta-analysis. The I^2 statistic, which measures the percentage of total variation across studies due to heterogeneity, was examined ($I^2 > 50\%$ was considered as heterogeneity). If significant heterogeneity was observed among the studies, a random-effects model was used; otherwise, a fixed-effects model was accepted. Begg's test was employed to assess the publication bias (44). A p-value of < 0.05 was considered statistically significant. Moreover, subgroup analysis for study design, diagnostic criteria of MetS, sample size, exposure assessment, type of iron, and the population was employed.

RESULTS

Study Identification and Selection

Figure 1 presents the detailed flow diagram of the study identification and selection. A total of 1,793 potentially relevant articles (PubMed: 236, Embase: 354, Web of Science: 477, and Scopus: 726) were retrieved during the initial literature search. After eliminating 749 duplicated articles, 1,044 articles were screened according to the titles and abstracts and 751 irrelevant studies were removed. Then, 182 reviews, case reports, or letters; 68 non-human studies; and 29 randomized controlled trial studies were excluded, respectively. Eventually, a total of 14 studies were identified for this meta-analysis.

Study Characteristics

Table 1 presents the main characteristics of the included studies. These studies were published between 2010 and 2021. Eight studies were performed in Asian countries [Korea (31), China (32, 35, 37, 39, 41), and Iran (33, 40)]. The other 6 studies were conducted in Brazil (29, 38, 42), USA (30), Spain (36), and Columbia (34), respectively. Except for the study by Bruscato (only female) (29), both the male and female participants were considered. The sample size ranged from 284 to 15,051 for a total of 55,131. The dietary iron, copper, and selenium level were assessed by a food-frequency questionnaire (FFQ) in 3 studies (35, 37, 40) and 24 h or 3-day recall method in 11 studies (29–34, 36, 38, 39, 41, 42). The criteria for MetS were the National Cholesterol Education Program-Adult Treatment Panel III (NCEP ATP III) (31, 32, 38–41), the International Diabetes Federation (IDF) (29, 33, 37), and the American Heart Association (AHA) (30, 35, 36) in 6, 3, and 3 studies, respectively. Moreover, some other criteria (34, 42) were also employed for adolescents.

TABLE 1 | Characteristics of the individual studies included in this meta-analysis.

References	Location	Age years	Gender	Sample size	Study design	Adjustments	Exposure	Category of exposure	Effect estimates	Diagnostic criteria of MetS	NOS
Bruscato et al. (29)	Brazil	>60	Female	284	Cross-sectional	Age, smoking, years of education, physical activity and dietary fiber.	24 h recall	Iron		IDF	7
								Quartiles 1	1.00		
								Quartiles 2	0.65 (0.30, 1.38)		
								Quartiles 3	1.33 (0.63, 2.83)		
								Quartiles 4	0.72 (0.30, 1.72)		
Otto et al. (30)	US	45–84	Both	3,828	Cohort	Energy intake, age, sex, race-ethnicity, education, study center, alcohol intake, physical activity, BMI, fiber intake, cigarette smoking, dietary supplement use, the ratio of polyunsaturated fat intake: saturated fat intake and antioxidant intake	24 h recall	Iron		AHA	8
								Quintiles 1	1.00		
								Quintiles 2	1.07 (0.85, 1.36)		
								Quintiles 3	1.02 (0.80, 1.30)		
								Quintiles 4	1.28 (1.01, 1.63)		
								Quintiles 5	1.06 (0.81, 1.40)		
Choi et al. (31)	Korea	>19	Both	5,136	Cross-sectional	Age, energy intake and alcohol frequency	24 h recall	Copper		NCEP ATP III	8
								Male			
								Quartiles 1	1.00		
								Quartiles 2	0.98 (0.70, 1.37)		
								Quartiles 3	0.85 (0.60, 1.20)		
								Quartiles 4	0.90 (0.60, 1.37)		
								Female			
								Quartiles 1	1.00		
								Quartiles 2	1.07 (0.80, 1.43)		
								Quartiles 3	1.02 (0.74, 1.40)		
								Quartiles 4	0.86 (0.58, 1.27)		
Li et al. (32)	China	18–65	Both	550	Cross-sectional	Age and sex	3 days recall	Copper		NCEP ATP III	7
								Quartiles 1	1.00		
								Quartiles 2	0.75 (0.45, 1.24)		
								Quartiles 3	0.65 (0.39, 1.07)		
								Quartiles 4	0.61 (0.36, 1.01)		
								Selenium			
								Quartiles 1	1.00		
								Quartiles 2	1.38 (0.89, 2.45)		
								Quartiles 3	0.81 (0.54, 1.49)		
								Quartiles 4	0.82 (0.46, 1.30)		
Motamed et al. (33)	Iran	35–65	Both	3,800	Cross-sectional	Sex, age, physical activity level, smoking, past medical history, energy intake and BMI	24 h recall	Iron		IDF	7
								Quintiles 1	1.00		
								Quintiles 2	1.15 (0.90, 1.40)		
								Quintiles 3	1.24 (0.90, 1.50)		
								Quintiles 4	1.24 (1.00, 1.50)		
								Quintiles 5	1.12 (0.90, 1.40)		
								Copper			
								Quintiles 1	1.00		
								Quintiles 2	1.25 (1.00, 1.50)		
								Quintiles 3	1.33 (1.06, 1.60)		
								Quintiles 4	1.16 (0.90, 1.40)		
								Quintiles 5	1.15 (0.90, 1.40)		
								Selenium			
								Quintiles 1	1.00		

(Continued)

TABLE 1 | Continued

References	Location	Age years	Gender	Sample size	Study design	Adjustments	Exposure	Category of exposure	Effect estimates	Diagnostic criteria of MetS	NOS
								Quintiles 2	0.96 (0.70, 1.10)		
								Quintiles 3	0.85 (0.60, 1.04)		
								Quintiles 4	0.92 (0.70, 1.10)		
								Quintiles 5	0.78 (0.60, 0.90)		
Suarez-Ortegón et al. (34)	Colombia	11–16	Both	1,311	Cross-sectional	Age, BMI, socioeconomic status, and intakes of fat, carbohydrates, protein and ascorbic acid	24 h recall	Copper Males Tertiles 1 Tertiles 2 Tertiles 3 Females Tertiles 1 Tertiles 2 Tertiles 3	 1.00 / 0.84 (0.32, 2.19) 1.00 / 0.77 (0.30, 1.82)	Ferranti	7
Wei et al. (35)	China	>18	Both	2,069	Cross-sectional	Age, sex, cigarette smoking, alcohol drinking, nutritional supplementary, activity level, dietary energy intake, fiber intake and protein intake	FFQ	Selenium Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4	 1.00 0.60 (0.43, 0.86) 0.82 (0.58, 1.17) 0.72 (0.46, 1.14)	AHA	7
Bulka et al. (36)	Spain	18–74	Both	15,051	Cross-sectional	Energy intake, age, gender, and Hispanic/Latino background	24 h recall	Copper Below EAR Normal	 1.00 0.81 (0.69, 0.95)	AHA	8
Qu et al. (37)	China	20–75	Both	9,108	Cross-sectional	Age, sex, BMI, physical activity, drinking, and smoking	FFQ	Copper Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4	 1.00 0.95 (0.82, 1.11) 0.85 (0.74, 0.99) 0.81 (0.70, 0.94)	IDF	8
Zhu et al. (39)	China	>18	Both	3,099	Cross-sectional	Age, sex, income, physical activity level, intentional physical exercise, smoking status, alcohol use and dietary total energy intake	24 h and 3 days recall	Iron Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4	 1.00 1.37 (1.06, 1.78) 1.47 (1.11, 1.94) 1.59 (1.15, 2.20)	NCEP ATP III	8
Vieira et al. (38)	Brazil	>18	Both	591	Cross-sectional	Physical activity, gender, alcohol consumption, household per capita income, BMI, high-sensitivity C-reactive protein, age, smoking status, race, total energy intake, misreporting, saturated fat and vitamin C intakes	24 h recall	Iron Quintiles 1 Quintiles 2 Quintiles 3 Quintiles 4 Quintiles 5	 1.00 0.83 (0.36, 2.70) 1.34 (0.63, 2.84) 0.52 (0.26, 1.04) 1.14 (0.54, 2.40)	NCEP ATP III	7
Esfandiar et al. (40)	Iran	>18	Both	4,654	Cohort	Age, sex, baseline BMI, educational level, smoking status, total energy intake, fiber, saturated fat, sodium, vitamin C and magnesium intakes	FFQ	Iron Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4	 1.00 0.97 (0.79, 1.19) 1.10 (0.81, 1.49) 2.04 (0.97, 4.28)	NCEP ATP III	7

(Continued)

TABLE 1 | Continued

References	Location	Age years	Gender	Sample size	Study design	Adjustments	Exposure	Category of exposure	Effect estimates	Diagnostic criteria of MetS	NOS
Zhu et al. (41)	China	>18	Both	5,323	Cross-sectional	Age, sex, region, years of education, physical activity level, intended physical exercises, smoking status, alcohol use and daily energy intake, zinc and magnesium	24 h and 3 days recall	Iron Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4	 1.00 1.35 (1.10, 1.65) 1.47 (1.15, 1.88) 1.60 (1.21, 2.11)	NCEP ATP III	8
Batista et al. (42)	Brazil	14–19	Both	327	Cross-sectional	Sex, age, maternal education, family income, physical activity, and alcohol consumption	24 h recall	Copper Tertiles 1 Tertiles 2 Tertiles 3 Selenium Tertiles 1 Tertiles 2 Tertiles 3	 1.00 1.49 (0.56, 4.09) 0.87 (0.28, 2.70) 1.00 2.17 (0.66, 7.36) 0.81 (0.30, 2.19)	Cooks	7

The Relative Risk of MetS for the Highest vs. Lowest Dietary Iron Level

The overall multivariable adjusted RR showed that the dietary iron level was positively associated with MetS (RR = 1.27, 95% CI: 1.12–1.44; $p < 0.001$) (**Figure 2**). No substantial level of heterogeneity was obtained among various studies ($p = 0.097$, $I^2 = 44.1\%$). No evidence of publication bias existed according to the Begg's rank correlation test ($p = 1.000$). **Table 2** presents the results of subgroup analysis. The above findings were confirmed in cross-sectional (RR = 1.31, 95% CI: 1.13–1.52; $p < 0.001$), Asians (RR = 1.44, 95% CI: 1.13–1.83; $p = 0.003$), the NCEP ATP III (RR = 1.59, 95% CI: 1.30–1.93; $p < 0.001$), >1,000 sample sized (RR = 1.34, 95% CI: 1.09–1.65; $p = 0.006$), 24 h or 3-day recall method (RR = 1.25, 95% CI: 1.10–1.42; $p < 0.001$), and non-heme iron (RR = 1.25, 95% CI: 1.08–1.46; $p = 0.004$) studies, respectively.

The Relative Risk of MetS for the Highest vs. Lowest Dietary Copper Level

The overall multivariable adjusted RR showed that the dietary copper level was negatively associated with MetS (RR = 0.85, 95% CI: 0.78–0.93; $p < 0.001$) (**Figure 3**). No substantial level of heterogeneity was obtained among various studies ($p = 0.391$, $I^2 = 5.3\%$). No evidence of publication bias existed according to the Begg's rank correlation test ($p = 0.754$). **Table 3** presents the results of subgroup analysis. The above findings were confirmed in > 1,000 sample sized (RR = 0.86, 95% CI: 0.78–0.94; $p = 0.001$) and adults (RR = 0.85, 95% CI: 0.78–0.93; $p < 0.001$) studies, respectively.

The Relative Risk of MetS for the Highest vs. Lowest Dietary Selenium Level

The overall multivariable adjusted RR showed that the dietary selenium level was negatively associated with MetS (RR = 0.77, 95% CI: 0.63–0.95; $p = 0.01$) (**Figure 4**). No substantial level of heterogeneity was obtained among various studies ($p = 0.985$, $I^2 = 0.0\%$). No evidence of publication bias existed according to the Begg's rank correlation test ($p = 1.000$). **Table 4** presents the results of subgroup analysis. The above findings were confirmed in Asians (RR = 0.77, 95% CI: 0.63–0.95; $p = 0.02$), the NCEP ATP III/IDF (RR = 0.79, 95% CI: 0.62–1.00; $p = 0.05$), >1,000 sample sized (RR = 0.76, 95% CI: 0.61–0.96; $p = 0.02$), 24 h or 3-day recall (RR = 0.79, 95% CI: 0.62–0.99; $p = 0.04$), and adults (RR = 0.77, 95% CI: 0.63–0.95; $p = 0.02$) studies, respectively.

DISCUSSION

A total of 14 observational studies were identified in the present meta-analysis. The pooled results showed that the dietary iron level was positively associated with MetS, whereas a negative association between the dietary copper and selenium level and MetS was obtained.

The pathophysiology of MetS is involved in oxidative stress and inflammation. As a strong pro-oxidant, iron causes oxidative stress and damage to pancreatic beta cells, which decreases the synthesis and secretion of insulin, impairs insulin signaling, and then alters the glucose metabolism (45, 46). Recently, iron-mediated cell death (ferroptosis) has also been reported to induce cardiomyocyte damage and plays an important role in cardiovascular disorders-related pathology (47). On the contrary, copper and selenium are important antioxidants that act against oxidative stress (25, 27). Copper is served as a cofactor of the copper/zinc superoxide dismutase, a protein located in both the cytosol and the mitochondrial inner membrane space to relieve the electron transport chain-generated reactive oxygen species (26). Differently from other metals, selenium works by incorporation into proteins by a cotranslational mechanism (as part of the amino acid selenocysteine) (48). Most selenium proteins participate in antioxidant defense and redox state regulation, particularly the families of glutathione peroxidases

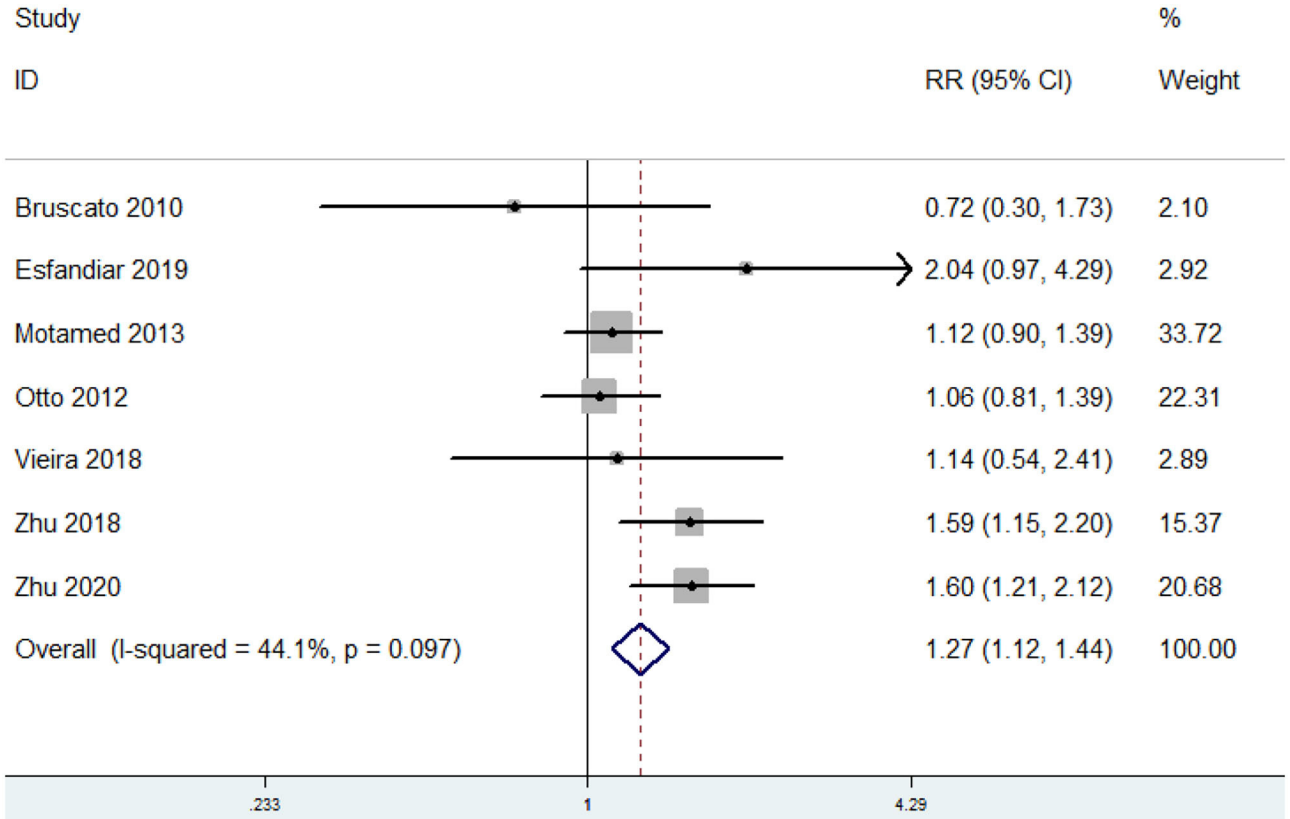


FIGURE 2 | Forest plot of meta-analysis: Overall multi-variable adjusted RR of MetS for the highest vs. lowest dietary iron level.

TABLE 2 | Subgroup analysis of metabolic syndrome (MetS) for the highest vs. lowest dietary iron level category.

Stratification	Number of studies	Pooled RR	95% CI	*P*-value	Heterogeneity
All studies	7	1.27	1.12, 1.44	$P < 0.001$	$P = 0.01$; $I^2 = 44\%$
Study design					
Cross-sectional	5	1.31	1.13, 1.52	$P < 0.001$	$P = 0.12$; $I^2 = 45\%$
Cohort	2	1.34	0.72, 2.47	$P = 0.35$	$P = 0.10$; $I^2 = 62\%$
Race					
Asian	4	1.44	1.13, 1.83	$P = 0.003$	$P = 0.10$; $I^2 = 53\%$
American	3	1.04	0.81, 1.32	$P = 0.77$	$P = 0.69$; $I^2 = 0\%$
Diagnostic criteria of MetS					
NCEP ATP III	4	1.59	1.30, 1.93	$P < 0.001$	$P = 0.75$; $I^2 = 0\%$
Other	3	1.08	0.91, 1.27	$P = 0.37$	$P = 0.62$; $I^2 = 0\%$
Sample size					
<1,000	2	0.94	0.53, 1.66	$P = 0.83$	$P = 0.43$; $I^2 = 0\%$
>1,000	5	1.34	1.09, 1.65	$P = 0.006$	$P = 0.06$; $I^2 = 56\%$
Exposure assessment					
FFQ	1	2.04	0.97, 4.29	/	/
24 h or 3 days recall	6	1.25	1.10, 1.42	$P < 0.001$	$P = 0.10$; $I^2 = 45\%$
Type of iron					
Heme iron	5	0.98	0.79, 1.22	$P = 0.88$	$P = 0.04$; $I^2 = 61\%$
Non-heme iron	5	1.25	1.08, 1.46	$P = 0.004$	$P = 0.18$; $I^2 = 36\%$

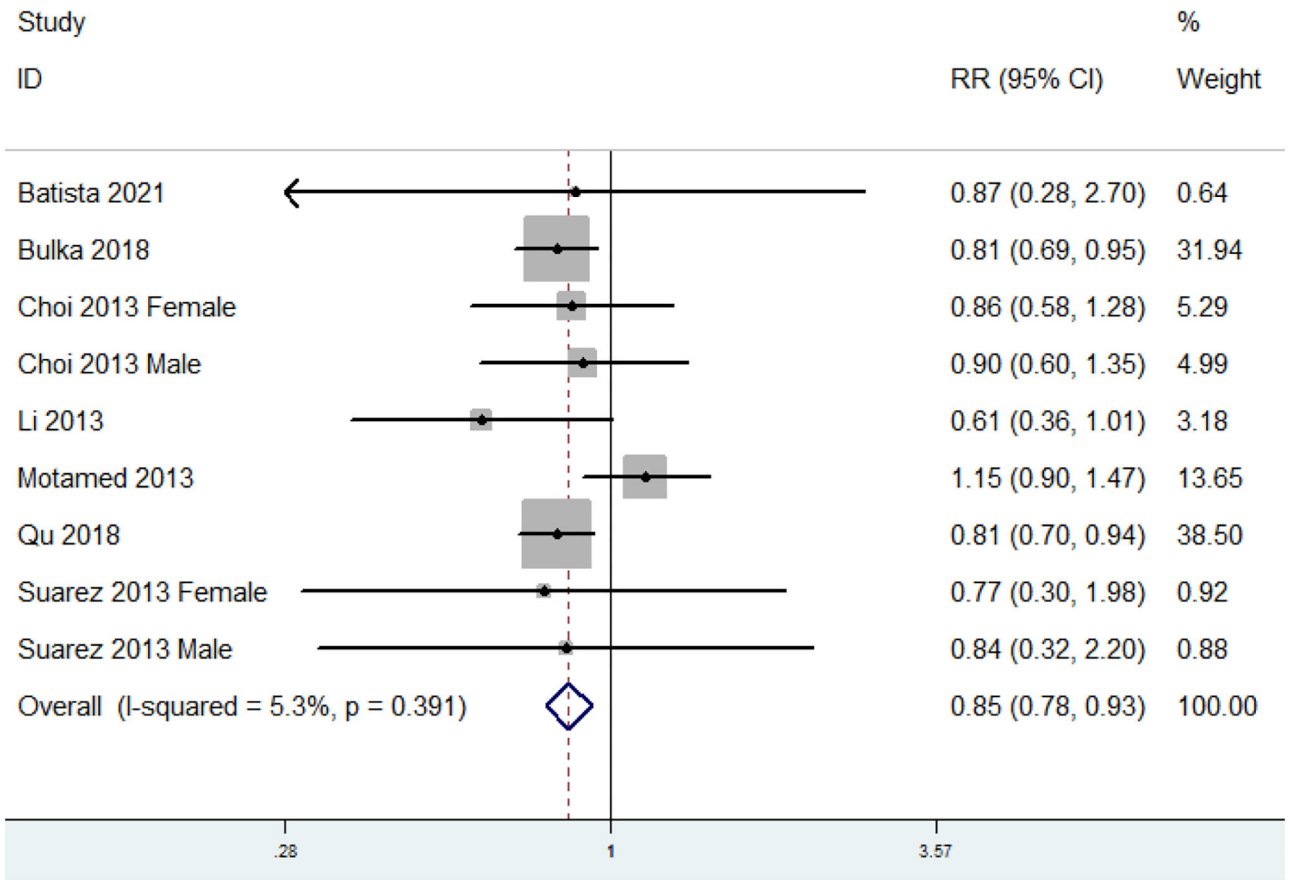


FIGURE 3 | Forest plot of meta-analysis: Overall multi-variable adjusted RR of MetS for the highest vs. lowest dietary copper level.

TABLE 3 | Subgroup analysis of MetS for the highest vs. lowest dietary copper level category.

Stratification	Number of studies	Pooled RR	95% CI	*P*-value	Heterogeneity
All studies	7	0.85	0.78, 0.93	$P < 0.001$	$P = 0.39$; $I^2 = 5\%$
Study design					
Cross-sectional	7	0.85	0.78, 0.93	$P < 0.001$	$P = 0.39$; $I^2 = 5\%$
Cohort	/	/	/	/	/
Race					
Asian	4	0.87	0.78, 0.97	$P = 0.01$	$P = 0.10$; $I^2 = 49\%$
American	3	0.81	0.69, 0.95	$P = 0.008$	$P = 1.00$; $I^2 = 0\%$
Diagnostic criteria of MetS					
NCEP ATP III/IDF	4	0.87	0.78, 0.97	$P = 0.01$	$P = 0.10$; $I^2 = 49\%$
Other	3	0.81	0.69, 0.95	$P = 0.008$	$P = 1.00$; $I^2 = 0\%$
Sample size					
<1,000	2	0.64	0.40, 1.02	$P = 0.06$	$P = 0.57$; $I^2 = 0\%$
>1,000	5	0.86	0.78, 0.94	$P = 0.001$	$P = 0.35$; $I^2 = 10\%$
Exposure assessment					
FFQ	1	0.81	0.70, 0.94	/	/
24 h or 3 days recall	6	0.87	0.78, 0.98	$P = 0.02$	$P = 0.35$; $I^2 = 10\%$
Population					
Adults	5	0.85	0.78, 0.93	$P < 0.001$	$P = 0.14$; $I^2 = 41\%$
Adolescents	2	0.82	0.46, 1.46	$P = 0.50$	$P = 0.99$; $I^2 = 0\%$

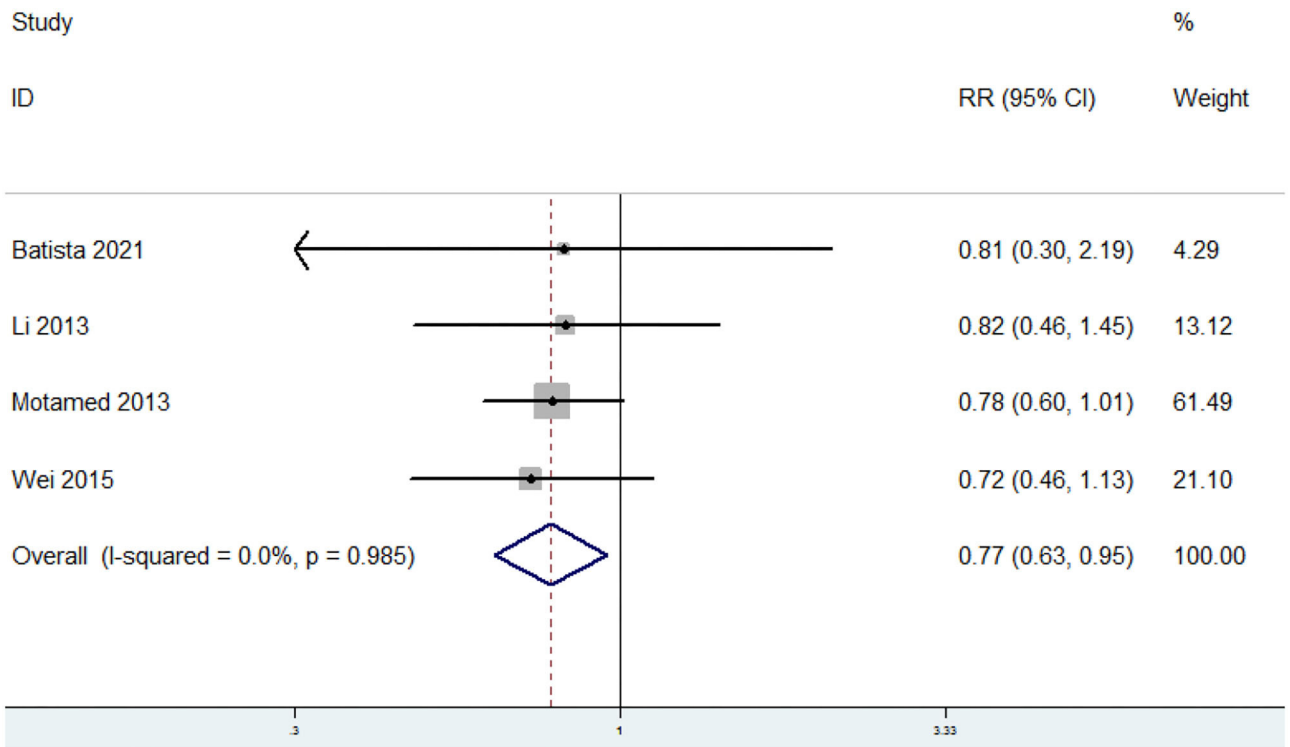


FIGURE 4 | Forest plot of meta-analysis: Overall multi-variable adjusted RR of MetS for the highest vs. lowest dietary selenium level.

TABLE 4 | Subgroup analysis of MetS for the highest vs. lowest dietary selenium level category.

Stratification	Number of studies	Pooled RR	95% CI	*P*-value	Heterogeneity
All studies	4	0.77	0.63, 0.95	$P = 0.01$	$P = 0.99$; $I^2 = 0\%$
Study design					
Cross-sectional	4	0.77	0.63, 0.95	$P = 0.01$	$P = 0.99$; $I^2 = 0\%$
Cohort	/	/	/	/	/
Race					
Asian	3	0.77	0.63, 0.95	$P = 0.02$	$P = 0.93$; $I^2 = 0\%$
American	1	0.81	0.30, 2.19	/	/
Diagnostic criteria of MetS					
NCEP ATP III/IDF	2	0.79	0.62, 1.00	$P = 0.05$	$P = 0.88$; $I^2 = 0\%$
Other	2	0.73	0.49, 1.10	$P = 0.14$	$P = 0.83$; $I^2 = 0\%$
Sample size					
<1,000	2	0.82	0.50, 1.34	$P = 0.42$	$P = 0.98$; $I^2 = 0\%$
>1,000	2	0.76	0.61, 0.96	$P = 0.02$	$P = 0.76$; $I^2 = 0\%$
Exposure assessment					
FFQ	1	0.72	0.46, 1.13	/	/
24 h or 3 days recall	3	0.79	0.62, 0.99	$P = 0.04$	$P = 0.99$; $I^2 = 0\%$
Population					
Adults	3	0.77	0.63, 0.95	$P = 0.02$	$P = 0.93$; $I^2 = 0\%$
Adolescents	1	0.81	0.30, 2.19	/	/

and thioredoxin reductases (48). Copper deficiency is associated with the increased high-density lipoprotein (HDL) cholesterol level in rats (49) and blood cholesterol levels in humans (50). Similarly, selenium is also considered to prevent high-fat diet-induced hyperglycemia and liver damage in rats (51) and type 2 diabetes mellitus and cardiovascular disease in humans (52).

The above may significantly account for the major findings of this study.

Ferritin, a ubiquitous intracellular protein, is important in the regulation of iron homeostasis (53). Similarly, a meta-analysis of the observational study suggested that the increased ferritin level is positively associated with MetS (54). Moreover, iron chelation therapy (reduce serum ferritin level) was associated with improved serum glucose and HDL levels (55). The phlebotomy with a reduction of body iron could significantly improve cardiovascular risk and glycemic control in patients with MetS (56). The above findings are decent supplements to ours.

Heme iron exists in most animal foods, whereas the rest in animal or plant food is non-heme iron (57). Non-heme iron is absorbed less efficiently than heme iron (∼5% non-heme iron and 25% heme iron are absorbed from diet) (41). The epidemiological data indicate that the effect of heme and non-heme iron on MetS may vary from the regional variety (39, 40) and heme iron alone cannot reflect the iron status (58, 59). Indeed, our results with regard to heme and non-heme iron are quite different. The specification of the iron subtype should be considered in further study.

Interestingly, our findings are only confirmed in the NCEP ATP III/IDF diagnostic criteria and > 1,000 sample-sized studies. It is speculated that MetS diagnostic criteria or sample size may influence the results of this study. Moreover, the negative relationship between the dietary iron and selenium and MetS is only specified in the 24 h or 3-day recall method and adult population. Although the number of studies for FFQ and adolescents is limited, the recall method might be precise to reflect the issues and some age-related differences with the dietary pathology of MetS cannot be fully excluded. In addition, the issue of race should also be noted. The corresponding findings only exist in Asians, but not in Americans (with regard to the dietary iron and selenium). Our results suggest that the potential effect of racial variation should not be ignored either.

Our findings can be incorporated into the daily lives of subjects suffering from MetS. The programs to build awareness with collaboration between physicians and nutritionists should be encouraged in the future. For example, reinforce the dietary education in MetS subjects: avoid the dietary iron overdose or copper/selenium deficiency. Nevertheless, the toxicity of excess copper intake should also be emphasized. Excess copper intake is reported to induce oxidative stress, damage to the mitochondrial, contributes to apoptosis, DNA damage, and inflammatory responses (60, 61). Therefore, careful validation by randomized controlled trial/prospective cohort study is still needed before its clinical application.

This study has several strengthens. To begin with, this is the first meta-analysis of observational studies on the associations of the dietary iron, copper, and selenium level with MetS. In addition, the included studies are analyzed based on the adjusted results and large samples. Moreover, the limited heterogeneity level may reflect the decent reliability of our results. Finally, our findings might provide significant information to better consider the dietary effects on MetS.

The limitations of this study should also be acknowledged. First, only 2 prospective cohort studies were identified totally due to the limited relevant literature (causal relationships could not be obtained). Second, the classification of exposure varies greatly among individuals. Third, the adjusted factors and definition of MetS were not uniform. Forth, one included study has combined the data for the dietary iron and red meat as a whole (40). These limitations may weaken the significance of this study.

CONCLUSION

Our results suggest that the dietary iron level is positively associated with MetS, whereas a negative association between the dietary copper and selenium level and MetS is obtained. Further large well-designed prospective cohort studies are warranted to elaborate on the issues examined in this study.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YZ and JD conceived the idea and drafted this manuscript and selected and retrieved relevant papers. ZL and QL performed the statistical analysis. JL and HG assessed each study. YZ was the guarantor of the overall content. All the authors revised and approved the final version of the manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (82102581), the National Postdoctoral Science Foundation of China (2021M693562), the Provincial Outstanding Postdoctoral Innovative Talents Program of Hunan (2021RC2020), the Provincial Natural Science Foundation of Hunan (2019JJ40517), the Young Investigator Grant of Xiangya Hospital, Central South University (2020Q14), and the Fuqing Postdoc Program of Xiangya Hospital, Central South University (176).

REFERENCES

1. Zhang Y, Zhang D-Z. Relationship between serum zinc level and metabolic syndrome: a meta-analysis of observational studies. *J Am Coll Nutr*. (2018) 37:708–15. doi: 10.1080/07315724.2018.1463876
2. Gurka MJ, Guo Y, Filipp SL, DeBoer MD. Metabolic syndrome severity is significantly associated with future coronary heart disease in Type 2 diabetes. *Cardiovascular Diabetol*. (2018) 17:17. doi: 10.1186/s12933-017-0647-y
3. DeBoer MD, Gurka MJ, Golden SH, Musani SK, Sims M, Vishnu A, et al. Independent associations between metabolic syndrome severity and future

coronary heart disease by sex and race. *J Am Coll Cardiol.* (2017) 69:1204–5. doi: 10.1016/j.jacc.2016.10.088
4. Decker JJ, Norby FL, Rooney MR, Soliman EZ, Lutsey PL, Pankow JS, et al. Metabolic syndrome and risk of ischemic stroke in atrial fibrillation: ARIC. *Study Stroke.* (2019) 50:3045–50. doi: 10.1161/STROKEAHA.119.025376
5. Rochlani Y, Pothineni NV, Kovelamudi S, Mehta JL. Metabolic syndrome: pathophysiology, management, and modulation by natural compounds. *Ther Adv Cardiovasc Dis.* (2017) 11:215–25. doi: 10.1177/1753944717711379
6. Ranasinghe P, Mathangasinghe Y, Jayawardena R, Hills AP, Misra A. Prevalence and trends of metabolic syndrome among adults in the Asia-pacific region: a systematic review. *BMC Public Health.* (2017) 17:101. doi: 10.1186/s12889-017-4041-1
7. Zhang Y, Zhang D-Z. Relationship between nut consumption and metabolic syndrome: a meta-analysis of observational studies. *J Am Coll Nutr.* (2019) 38:499–505. doi: 10.1080/07315724.2018.1561341
8. Zhang Y, Zhang D-Z. Associations of vegetable and fruit consumption with metabolic syndrome. A meta-analysis of observational studies. *Public Health Nutr.* (2018) 21:1693–703. doi: 10.1017/S1368980018000381
9. Guo H, Ding J, Liang J, Zhang Y. Associations of whole grain and refined grain consumption with metabolic syndrome. A meta-analysis of observational studies. *Front Nutr.* (2021) 8:695620. doi: 10.3389/fnut.2021.695620
10. Guo H, Ding J, Liang J, Zhang Y. Association of red meat and poultry consumption with the risk of metabolic syndrome: a meta-analysis of prospective cohort studies. *Front Nutr.* (2021) 8:691848. doi: 10.3389/fnut.2021.691848
11. Guo H, Ding J, Liu Q, Li Y, Liang J, Zhang Y. Vitamin C and metabolic syndrome: a meta-analysis of observational studies. *Front Nutr.* (2021). 8:728880. doi: 10.3389/fnut.2021.728880
12. Mahan LK, Escott Stump S, Reymond JL. *Krause's Food and the Nutrition Care Process*. 13th ed. St Louis, MO: Elsevier (2012).
13. Szudzik M, Lipiński P, Jończy A, Mazgaj R, Pieszka M, Kamyczek M, et al. Long-term effect of split iron dextran/hemoglobin supplementation on erythrocyte and iron status, growth performance, carcass parameters, and meat quality of polish large white and 990 line pigs. *Biol Trace Elem Res.* (2020) 196:472–80. doi: 10.1007/s12011-019-01950-w
14. Mari S, Bailly C, Thomine S. Handing off iron to the next generation: how does it get into seeds and what for? *Biochem J.* (2020) 477:259–74. doi: 10.1042/BCJ20190188
15. Staroń R, Lipiński P, Lenartowicz M, Bednarz A, Gajowiak A, Smuda E, et al. Dietary hemoglobin rescues young piglets from severe iron deficiency anemia: duodenal expression profile of genes involved in heme iron absorption. *PLoS ONE.* (2017) 12:e0181117. doi: 10.1371/journal.pone.0181117
16. Podwika W, Kleszcz K, Krośniak M, Zagrodzki P. Copper, manganese, zinc, and cadmium in tea leaves of different types and origin. *Biol Trace Elem Res.* (2018) 183:389–95. doi: 10.1007/s12011-017-1140-x
17. Puchkova LV, Babich PS, Zatulovskaia YA, Ilyechova EY, Di Sole F. Copper metabolism of newborns is adapted to milk ceruloplasmin as a nutritive source of copper: overview of the current. *Data Nutr.* (2018) 10:1591. doi: 10.3390/nu10111591
18. Gorini F, Sabatino L, Pingitore A, Vassalle C. Selenium: an element of life essential for thyroid function. *Molecules.* (2021) 26:7084. doi: 10.3390/molecules26237084
19. Torti SV, Torti FM. Iron and cancer: more ore to be mined. *Nat Rev Cancer.* (2013) 13:342–55. doi: 10.1038/nrc3495
20. Jung M, Mertens C, Tomat E, Brüne B. Iron as a central player and promising target in cancer progression. *Int J Mol Sci.* (2019) 20:273. doi: 10.3390/ijms20020273
21. Munoz M, Garcia-Erce JA, Remacha AF. Disorders of iron metabolism. Part 1: Molecular basis of iron homoeostasis. *J Clin Pathol.* (2011) 64:281–6. doi: 10.1136/jcp.2010.079046
22. Abbaspour N, Hurrell R, Kelishadi R. Review on iron and its importance for human health. *J Res Med Sci.* (2014) 19:164–74.
23. Galaris D, Barbouti A, Pantopoulos K. Iron homeostasis and oxidative stress: an intimate relationship. *Biochim Biophys Acta Mol Cell Res.* (2019) 1866:118535. doi: 10.1016/j.bbamcr.2019.118535
24. Hansen JB, Moen IW, Mandrup-Poulsen T. Iron: the hard player in diabetes pathophysiology. *Acta Physiol.* (2014) 210:717–32. doi: 10.1111/apha.12256
25. Ma X, Jiang S, Yan S, Li M, Wang C, Pan Y, et al. Association between copper, zinc, iron, and selenium intakes and TC/HDL-C ratio in US adults. *Biol Trace Elem Res.* (2020) 197:43–51. doi: 10.1007/s12011-019-01979-x
26. Ruiz LM, Libedinsky A, Elorza AA. Role of copper on mitochondrial function and metabolism. *Front Mol Biosci.* (2021) 8:711227. doi: 10.3389/fmolb.2021.711227
27. Ding J, Zhang Y. Relationship between the circulating selenium level and stroke: a meta-analysis of observational studies. *J Am Coll Nutr.* (2021) 1–9. doi: 10.1080/07315724.2021.1902880
28. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. *World Allergy Organ J.* (2012) 5:9–19. doi: 10.1097/WOX.0b013e3182439613
29. Bruscato NM, da Costa Vieira JL, do Nascimento NM, Canto ME, Stobbe JC, Gottlieb MG, et al. Dietary intake is not associated to the metabolic syndrome in elderly women. *N Am J Med Sci.* (2010) 2:182–8.
30. Otto MCO, Alonso A, Lee D-H, Delclos GL, Bertoni AG, Jiang R, et al. Dietary intakes of zinc and heme iron from red meat, but not from other sources, are associated with greater risk of metabolic syndrome and cardiovascular disease. *J Nutr.* (2012) 142:526–33. doi: 10.3945/jn.111.149781
31. Choi M-K, Bae Y-J. Relationship between dietary magnesium, manganese, and copper and metabolic syndrome risk in Korean adults: the Korea National Health and Nutrition Examination Survey (2007-2008). *Biol Trace Elem Res.* (2013) 156:56–66. doi: 10.1007/s12011-013-9852-z
32. Li Y, Guo H, Wu M, Liu M. Serum and dietary antioxidant status is associated with lower prevalence of the metabolic syndrome in a study in Shanghai, China. *Asia Pac J Clin Nutr.* (2013) 22:60–8. doi: 10.6133/apjcn.2013.22.1.06
33. Motamed S, Ebrahimi M, Safarian M, Ghayour-Mobarhan M, Mouhebati M, Azarpazhouh M, et al. Micronutrient intake and the presence of the metabolic syndrome. *N Am J Med Sci.* (2013) 5:377–85. doi: 10.4103/1947-2714.114171
34. Suarez-Ortegón MF, Ordoñez-Betancourth JE, Aguilar-de Plata C. Dietary zinc intake is inversely associated to metabolic syndrome in male but not in female urban adolescents. *Am J Hum Biol.* (2013) 25:550–4. doi: 10.1002/ajhb.22408
35. Wei J, Zeng C, Gong Q-y, Li X-x, Lei G-h, Yang T-b. Associations between dietary antioxidant intake and metabolic syndrome. *PLoS ONE.* (2015) 10:e0130876. doi: 10.1371/journal.pone.0130876
36. Bulka CM, Daviglus ML, Sotres-Alvarez D, Durazo-Arvizu RA, Mossavar-Rahmani Y, Persky VW, et al. Dietary minerals and metabolic syndrome: results from the hispanic community health study/study of latinos (HCHS/SOL). *Circulation.* (2018) 137:AP246. doi: 10.1161/circ.137.suppl_1.p246
37. Qu R, Jia Y, Liu J, Jin S, Han T, Na L. Dietary flavonoids, copper intake, and risk of metabolic syndrome in Chinese adults. *Nutrients.* (2018) 10:991. doi: 10.3390/nu10080991
38. Vieira DAS, Sales CH, Cesar CLG, Marchioni DM, Fisberg RM. Influence of haem, non-haem, and total iron intake on metabolic syndrome and its components: a population-based study. *Nutrients.* (2018) 10:314. doi: 10.3390/nu10030314
39. Zhu Z, Wu F, Lu Y, Wu C, Wang Z, Zang J, et al. Total and nonheme dietary iron intake is associated with metabolic syndrome and its components in Chinese men and women. *Nutrients.* (2018) 10:1663. doi: 10.3390/nu10111663
40. Esfandiar Z, Hosseini-Esfahani F, Mirmiran P, Habibi-Moeini A-L, Azizi F. Red meat and dietary iron intakes are associated with some components of metabolic syndrome: Tehran Lipid and Glucose Study. *J Transl Med.* (2019) 17:313. doi: 10.1186/s12967-019-2059-0
41. Zhu Z, He Y, Wu F, Zhao L, Wu C, Lu Y, et al. The associations of dietary iron, zinc and magnesium with metabolic syndrome in China's mega cities. *Nutrients.* (2020) 12:659. doi: 10.3390/nu12030659
42. Batista CC, Nascimento LM, de Sá Lustosa LCR, Rodrigues BGM, Campelo V, de Macêdo Gonçalves Frota K. Metabolic syndrome in adolescents and antioxidant nutrient intake: a cross-sectional study. *Rev Assoc Med Bras.* (2021) 67:918–25. doi: 10.1590/1806-9282.20200733
43. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *BMJ.* (2009) 339:b2700. doi: 10.1136/bmj.b2700

44. Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics.* (1994) 50:1088–101. doi: 10.2307/2533446
45. Rajpathak SN, Crandall JP, Wylie-Rosett J, Kabat GC, Rohan TE, Hu FB. The role of iron in type 2 diabetes in humans. *Biochim Biophys Acta.* (2009) 1790:671–81. doi: 10.1016/j.bbagen.2008.04.005
46. Fernandez-Real JM, McClain D, Manco M. Mechanisms linking glucose homeostasis and iron metabolism toward the onset and progression of type 2 diabetes. *Diabetes Care.* (2015) 38:2169–76. doi: 10.2337/dc14-3082
47. Wu X, Li Y, Zhang S, Zhou X. Ferroptosis as a novel therapeutic target for cardiovascular disease. *Theranostics*. (2021) 11:3052–9. doi: 10.7150/thno.54113
48. Roman M, Jitaru P, Barbante C. Selenium biochemistry and its role for human health. *Metallomics*. (2014) 6:25–54. doi: 10.1039/C3MT00185G
49. Lefevre M, Keen CL, Lönnerdal B, Hurley LS, Schneeman BO. Copper deficiency-induced hypercholesterolemia: effects on HDL subfractions and hepatic lipoprotein receptor activity in the rat. *J Nutr.* (1986) 116:1735–46. doi: 10.1093/jn/116.9.1735
50. Salonen JT, Salonen R, Korpela H, Suntionen S, Tuomilehto J. Serum copper and the risk of acute myocardial infarction: a prospective population study in men in Estern Finland. *Am J Epidemiol.* (1991) 134:268–76. doi: 10.1093/oxfordjournals.aje.a116080
51. Gao Y, Xu Y, Ruan J, Yin J. Selenium affects the activity of black tea in preventing metabolic syndrome in high-fat diet-fed Sprague-Dawley rats. *J Sci Food Agric*. (2020) 100:225–34. doi: 10.1002/jsfa.10027
52. Wang X-L, Yang T-B, Wei J, Lei G-H, Zeng C. Association between serum selenium level and type 2 diabetes mellitus: a non-linear dose-response meta-analysis of observational studies. *Nutr J*. (2015) 15:48. doi: 10.1186/s12937-016-0169-6
53. Cook JD, Lipschitz DA, Miles LE, Finch CA. Serum ferritin as a measure of iron stores in normal subjects. *Am J Clin Nutr.* (1974) 27:681–7. doi: 10.1093/ajcn/27.7.681
54. Cooksey RC, Jones D, Gabrielsen S, Huang J, Simcox JA, Luo B, et al. Dietary iron restriction or iron chelation protects from diabetes and loss of beta-cell function in the obese (ob/ob lep-/-) mouse. *Am J Physiol Endocrinol Metab.* (2010) 298:E1236–43. doi: 10.1152/ajpendo.00022.2010
55. Houschyar KS, Ludtke R, Dobos GJ, Kalus U, Broecker-Preuss M, Rampp T, et al. Effects of phlebotomy-induced reduction of body iron stores on metabolic syndrome: results from a randomized clinical trial. *BMC Med.* (2012) 10:54. doi: 10.1186/1741-7015-10-54
56. Geissler C, Singh M. Iron, meat and health. *Nutrients*. (2011) 3:283–316. doi: 10.3390/nu3030283
57. Monsen ER, Hallberg L, Layrisse M, Hegsted DM, Cook JD, Mertz W, et al. Estimation of available dietary iron. *Am J Clin Nutr.* (1978) 31:134. doi: 10.1093/ajcn/31.1.134
58. He J, Shen X, Fang A, Song J, Li H, Guo M, et al. Association between predominantly plant-based diets and iron status in Chinese adults: a cross-sectional analysis. *Br J Nutr.* (2016) 116:1621–32. doi: 10.1017/S0007114516003639
59. Asakura K, Sasaki S, Murakami K, Takahashi Y, Uenishi K, Yamakawa M, et al. Iron intake does not significantly correlate with iron deficiency among young Japanese women: a cross-sectional study. *Public Health Nutr.* (2009) 12:1373–83. doi: 10.1017/S1368980008004072
60. Huo H, Wang S, Bai Y, Liao J, Li X, Zhang H, et al. Copper exposure induces mitochondrial dynamic disorder and oxidative stress *via* mitochondrial unfolded protein response in pig fundic gland. *Ecotoxicol Environ Saf.* (2021) 223:112587. doi: 10.1016/j.ecoenv.2021.112587
61. Guo H, Wang Y, Cui H, Ouyang Y, Yang T, Liu C, et al. Copper induces spleen damage through modulation of oxidative stress, apoptosis, DNA damage, and inflammation. *Biol Trace Elem Res.* (2022) 669–77. doi: 10.1007/s12011-021-02672-8

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 04 February 2022
doi: 10.3389/fendo.2021.735736

Associations of Triglycerides/High-Density Lipoprotein Cholesterol Ratio With Insulin Resistance, Impaired Glucose Tolerance, and Diabetes in American Adults at Different Vitamin D3 Levels

Yuanyuan Liu[1,2†], Rongpeng Gong[1†], Gang Luo[3†], Jinxia Li[1,2], Qidan Li[1], Lixin Yang[1,2] and Xiaoxing Wei[1,3*]*

[1] Medical College of Qinghai University, Xining, China, [2] Endocrinology Department, Qinghai Provincial People's Hospital, Xining, China, [3] College of Eco-environmental Engineering, Qinghai University, Xining, China

OPEN ACCESS

Edited by:
Mor-Li Hartman,
The Forsyth Institute, United States

Reviewed by:
Sepideh Babaniamansour,
University of Michigan, United States
Ishag Adam,
University of Khartoum, Sudan

***Correspondence:**
Lixin Yang
yanglx282@sina.com
Xiaoxing Wei
weixiaoxing@tsinghua.org.cn

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

Specialty section:
This article was submitted to Clinical Diabetes, a section of the journal Frontiers in Endocrinology

Received: 03 July 2021
Accepted: 24 December 2021
Published: 04 February 2022

Citation:
Liu Y, Gong R, Luo G, Li J, Li Q, Yang L and Wei X (2022) Associations of Triglycerides/High-Density Lipoprotein Cholesterol Ratio With Insulin Resistance, Impaired Glucose Tolerance, and Diabetes in American Adults at Different Vitamin D3 Levels. Front. Endocrinol. 12:735736. doi: 10.3389/fendo.2021.735736

Background: Previous studies have shown that vitamin D3 (VD3) may be a protective factor for diabetes mellitus (DM), while triglycerides/high-density lipoprotein (TG/HDL) may be a risk factor for diabetes. However, no existing study has elucidated the interaction between TG/HDL and VD3. Therefore, this work aimed to investigate the relationships of TG/HDL with insulin resistance (IR), impaired glucose tolerance (IGT), and DM at different VD3 levels.

Methods: With the use of the data from five National Health and Nutrition Examination Survey (NHANES) cycles, a total of 2,929 males and 3,031 females were divided into 4 groups according to their VD3 levels. Logistic regression was performed to observe the associations of TG/HDL ratio with IR, IGT, and DM in different groups.

Results: The relationships of TG/HDL with IR, IGT, and DM showed a threshold effect, with the cutoff values of 1.094, 1.51, and 1.11, respectively. On both sides of the cutoff values, the correlation was first weakened and then enhanced with the increase in VD3 levels.

Conclusion: TG/HDL is a risk factor for IR, IGT, and DM. Both too low and too high levels of VD3 can strengthen this association, whereas keeping VD3 at a reasonable level helps to reduce the associations of TG/HDL with IR, IGT, and DM.

Keywords: TG/HDL ratio, diabetes mellitus, insulin resistance, impaired glucose tolerance (IGT), cross-sectional study

Abbreviations: IR, insulin resistance; IGT, impaired glucose tolerance; DM, diabetes mellitus; EDU, education; SMOK, smoking; BMI, body mass index; ALT, alanine aminotransferase; ALB, albumin; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GGT, γ-glutamyl transpeptidase; UA, uric acid; FPG, fasting plasma glucose; TC, total cholesterol.

1 BACKGROUND

Vitamin D3 (VD3), also called cholecalciferol, is a type of vitamin D (1). VD3 is the precursor of hormones, which have been recently found to participate in numerous regulatory responses in the body (2–4). Studies have shown that VD3 plays a role not only in bone metabolism but also in insulin resistance (IR), impaired glucose tolerance (IGT), and diabetes mellitus (DM) (3–6).

IR is identified as an impaired response to insulin of target tissues and the resulting reduced efficiency of glucose uptake and utilization (7). If left uncontrolled, IR will develop into IGT and even DM. After feeding rats with a high-fat diet (HFD) for 12 weeks, Zhang et al. found that IR appeared in 93.3% of the rats (8). With the dramatic changes in people's living conditions, the morbidity of DM shows an increasing trend in various countries worldwide (9–11). According to the Diabetes Atlas 2019 published by the International Diabetes Federation (IDF), there are 425 million patients with type 2 DM (T2DM) around the world (12). According to the current development trend, it is estimated that there will be 629 million DM patients aged 20–79 by the year 2045, accounting for 10% of the overall population (13). Besides, there will be more patients with IGT and IR.

At present, VD3 and triglycerides/high-density lipoprotein (TG/HDL) are identified as the factors related to IR, IGT, and DM (14–17). However, few existing studies have investigated whether the relationship between TG/HDL ratio and abnormal glucose metabolism is affected by different VD3 levels. Herein, a retrospective analysis was conducted based on the American National Health and Nutrition Examination Survey (NHANES) database, aiming to discover the difference (or evidence) in the associations of TG/HDL ratio with IR, IGT, and DM at different VD3 levels. The findings in this study will make a significant contribution to exploring the clinical prevention and treatment of DM.

2 METHODS

2.1 Research Population and Test Methods

Altogether, 49,696 participants from five periods were selected from the NHANES database from 2009 to 2018. The NHANES project is a subject study strictly formulated by the National Center for Health Statistics (NCHS) to meet the different population representations. NHANES is a persistent project that makes 2 years as a period, and it ensures that the sample is representative of the American population through multilevel and complex sampling design. There are about 5,000 people who receive the sampling survey, which covers diverse aspects like population, social, economy, diet, and health. The laboratory examination section includes medical and physiological tests. All data are collected by professional and trained personnel. NHANES follows a strict standard and protocol to ensure the privacy of each participant, and the information is not used for identification under the U.S. federal law as well.

In this study, strict inclusion and exclusion criteria were applied to select eligible participants. To be specific, participants meeting one or more of the following criteria were excluded: 1) those aged under 18 years; 2) those with no key indicators of insulin, fasting glucose, TG, or HDL; and 3) others (including patients taking drugs that affected blood lipid metabolism, glucose metabolism, and parathyroid metabolism; patients suffering from immunodeficiency, infectious diseases, or malignant tumors; and patients with a recent history of surgery, trauma, severe infection or other stress).

NHANES 2009–2018 covered five periods (2009–2010, 2011–2012, 2013–2014, 2015–2016, and 2017–2018), involving 49,696 participants in total. Among them, 19,342 participants were excluded due to the age of <18 years, 17,313 because of the unavailability of insulin data, 3,374 due to unavailable TG data, and 3,707 because of the lack of HDL data. Finally, 5,960 participants were included in this trial (**Figure 1**).

Ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC-ms/MS) was performed to quantify VD3 levels in human serum samples. After fasting for 9 h, the fasting plasma glucose (FPG) and insulin levels in the enrolled participants were measured *via* venipuncture in the morning. Blood lipid data were provided by the laboratory at the University of Minnesota.

In this study, TG/HDL ratio was used as the independent variable, whereas IR, IGT, and DM were used as the dependent variables. According to the definition of VD3, participants were divided into four groups, including VD3 deficiency group (N1, VD3 < 30 ng/ml), VD3 insufficient group (N2, 30 ng/ml < VD3 ≤ 50 ng/ml), VD3 moderate group (N3, 50 ng/ml < VD3 ≤ 80 ng/ml), and overdose group (N4, VD3 ≥ 80 ng/ml) (**Figure 2**).

2.2 Disease Determination

In this study, the disease determination criterion was strictly formulated based on the international standard.

2.2.1 Insulin Resistance

The clinical definition of IR remains elusive, as no generally accepted test is available for IR (18). In some studies, IR is defined by the steady-state model evaluation formula: fasting glucose insulin (μU/ml) × fasting glucose (mmol/L)/22.5 (19). In other research, IR > 2.6 is regarded as IR in a normal American population, which was taken as the determination criterion in our study (**Figure 2**).

2.2.2 Impaired Glucose Tolerance

In this study, according to relevant questionnaires and laboratory tests, IGT was defined as self-reported DM or FPG ≥ 6.0 mmol/L (type 1 DM, gestational DM, and specific types of DM were excluded) (20) (**Figure 2**).

2.2.3 Diabetes Mellitus

DM is defined based on the American Diabetes Association Standards 2015. In this study, according to relevant questionnaires and laboratory tests, DM was defined as self-reported DM or glycosylated hemoglobin ≥6.5% and FPG ≥ 6.0

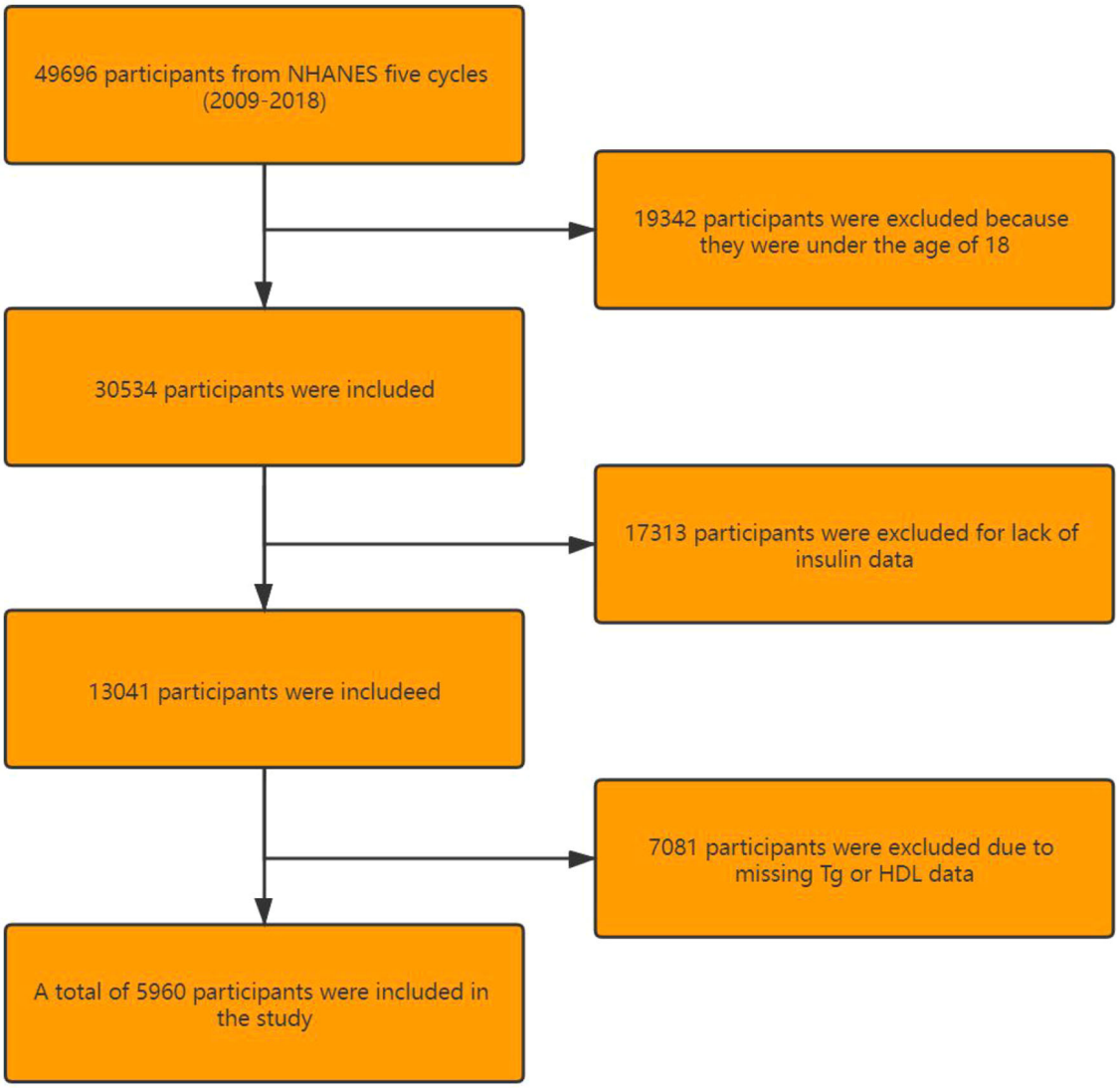


FIGURE 1 | Flowchart of participant selection.

mmol/L (type 1 DM, gestational DM, and specific types of DM were excluded) (21) (**Figure 2**).

2.2.4 Hypertension

In the present work, blood pressure (BP) was measured thrice while the participants were at rest, and the three measurements were averaged to assess whether the participants had hypertension or not. Typically, hypertension was defined as systolic BP (SBP) ≥140 mmHg and/or diastolic BP (DBP) ≥90 mmHg or self-reported hypertension and use of antihypertensive drugs. The definition conformed to the American Heart Association Blood Pressure Guidelines 2017 (22).

2.2.5 Smoking

Participants were classified into three groups according to their different smoking conditions, namely, 1) current smokers (who smoked at least one cigarette a day in the past 30 days), 2) current non-smokers (who smoked an average of less than 1 cigarette per day in the past 30 days or more than 100 cigarettes in their lifetime), and 3) non-smokers (who reported that they smoked less than 100 cigarettes in their lifetime or never smoked). In this study, due to the small number of non-smokers, the current non-smokers and non-smokers were finally combined as non-smokers (23).

2.2.6 Alcohol Use

After the classification of alcohol consumption in previous studies was checked, the participants were finally divided into two groups, including drinkers (who drank more than 12 drinks a year) and non-drinkers (who drank no more than 12 drinks a year) (24).

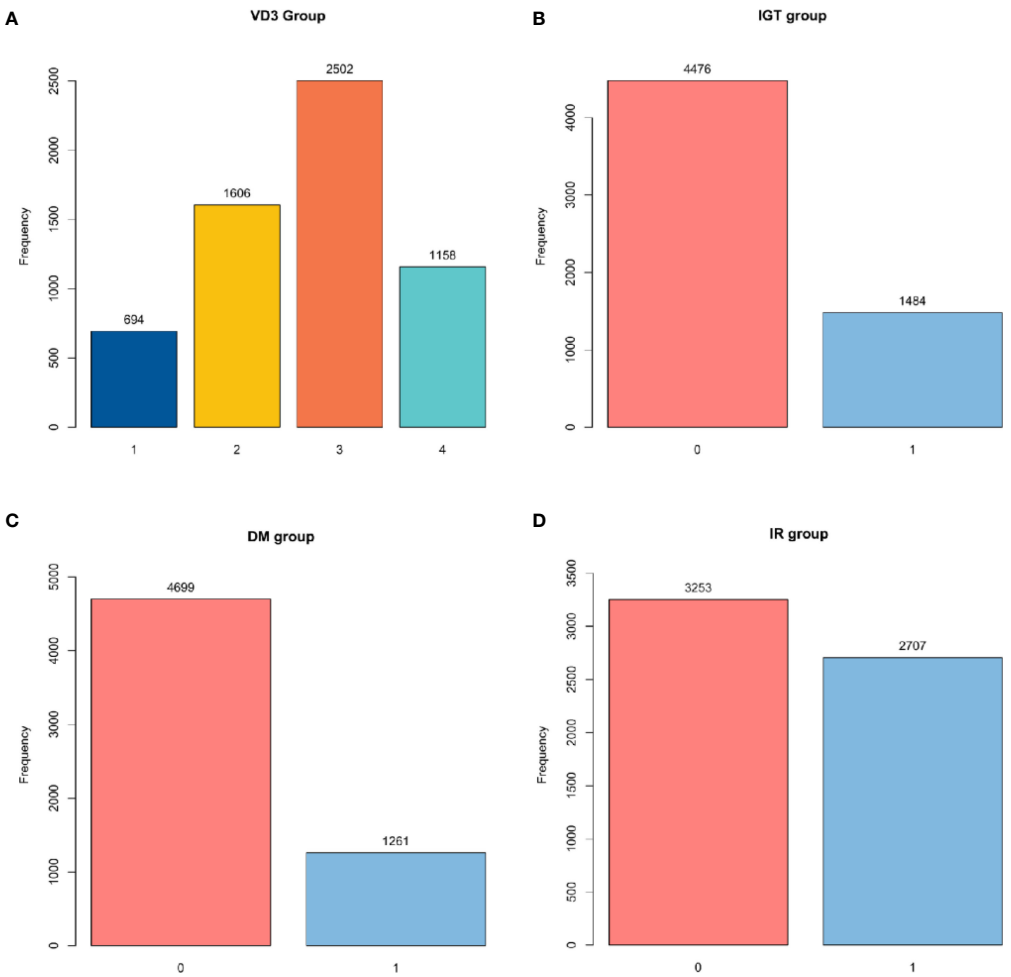


FIGURE 2 | Group diagram of each group. **(A)** Number of participants in different VD3 groups. **(B)** Number of participants with or without IGT. **(C)** Number of participants with or without DM. **(D)** Number of participants with or without IR. VD3, vitamin D3; IGT, impaired glucose tolerance; DM, diabetes mellitus; IR, insulin resistance.

2.2.7 Income and Education

In this study, participants earning more than $100,000 were defined as high income, while those with a junior college education or higher were defined as high education.

3 STATISTICAL ANALYSIS

The NHANES database selects an annual of 5,000 people from a framework of 15 different locations in all counties of the United States to ensure that the data are universal and widespread. The unique multistage probability sampling technique employed by the NHANES database enables the data to better represent the incidence of IR in the U.S. population over the past few years. In this study, samples were collected from the NHANES database at five consecutive periods to make this study more convincing. Besides, data were selected through rigorous comprehensive screening to obtain a more representative sample.

Statistical analysis was performed using R language version 4.0.2, and a two-sided p-value <0.05 indicated statistical significance. Multivariate logistic regression was adopted to analyze the associations of TG/HDL ratio with IR, IGT, and DM, under different VD3 levels in the American adult population. Continuous variables were represented by detailed sample descriptions. Classification variables were expressed as counts and weighted percentages. In addition, the skewed distribution was based on the median and Q1–Q3, whereas the normal distribution was described by the median and SD. Four multivariate logistic regression models and smooth fitting curves were established to analyze the relationships of TG/HDL ratio with IR, IGT, and DM at different VD3 levels. Moreover, two-stage logistic regression and a log-likelihood ratio test were performed to analyze whether there was a non-linear relationship. Multiple imputations were utilized to compensate for the missing variables in this study. To increase the statistical power and avoid bias, the missing data of covariates were removed from this analysis.

A sensitivity analysis was also performed to evaluate whether the generated data differed considerably from the raw data. According to sensitivity analysis, the generated complete data were similar to the raw data. As a result, our following multivariable analyses were carried out using the raw data based on Rubin's guidelines.

3.1 Selection of Covariates

In this study, covariates were screened according to the following criteria, 1) baseline characteristics of the population; 2) variables affecting TG/HDL, IR, IGT, and DM identified in previous studies; 3) the basic model changed by more than 10% after introducing covariates; and 4) experience gained in clinical work.

In line with the abovementioned criteria, gender, age, race, alanine aminotransferase (ALT), aspartate aminotransferase (AST), TG, total cholesterol (TC), γ-glutamyl transpeptidase (GGT), albumin (ALB), HDL, smoking, alcohol use, income, and education were selected as the covariates.

4 RESULTS

4.1 Characteristics of the Participants

A total of 5,960 participants were finally included according to the strict inclusion and exclusion criteria, including 2,929 males (49.1%) and 3,031 females (50.9%). Among these samples, there were significant differences in variables of gender, age, race, smoking, education, hyperuricemia, hypertension, hypercholesterolemia, body mass index (BMI), waist, IR, DM, IGT, ALT, AST, alkaline phosphatase (ALP), total bilirubin (TBIL), blood urea nitrogen (BUN), and GGT ($p < 0.001$) among VD3 groups (N1–N4). Notably, the highest proportions of smoking (79.1%), alcohol use (34.3%), IR (55.6%), IGT (31.7%), and DM (26.9%), together with the highest values of BMI (30.6 ± 8.8) and waist (101.2 ± 20.8) were observed in the N1 group. By contrast, the highest proportions of education (61.3%), income (45.3%), hyperuricemia (23.1%), hypertension (77.0%), and hypercholesterolemia (54.8%), as well as the highest values of AST (23.0, (20.0, 27.0)) and BUN (4.6, (3.9, 6.1)), were seen in the N4 group. The average age of the total samples was 43.0 ± 20.6 years, and the oldest age (52.8 ± 19.9) was observed in the N4 group (**Table 1**).

4.2 Univariate Logistic Analysis

Table 2 presents the results of a univariate analysis to elucidate factors related to IR, IGT, and DM. As a result, the positive factors were age, drinking, hypertension, hyperuricemia, hypercholesterolemia, BMI, waist, TG, ALT, BUN, GGT, and TG/HDL ratio, whereas education, income, smoking, and HDL and VD3 levels were negatively correlated with IR, IGT, and DM (**Table 2**). Among the above variables, TG/HDL ratio showed the most significant relationship with IR among the diverse continuous variables, but its relations with IGT and DM were weaker (**Table 2**).

4.3 Multiple Logistic Regression Models

Four models were constructed to analyze the independent effect of the TG/HDL ratio on IR, IGT, and DM at different VD3 levels. The odds ratios (ORs) and 95% CIs are shown in **Table 3**. The sizes of ORs and 95% CIs were interpreted as the corresponding changes in the incidence rates of IR, IGT, and DM with the increase in TG/HDL ratio by one SD. In the fully adjusted model (Model 4), the ORs of the association between TG/HDL ratio and IR were 2.11 (1.57~2.83), 1.52 (1.29~1.78), 1.28 (1.16~1.41), and 2.16 (1.75~2.67) in the N1–N4 groups, respectively. Meanwhile, the ORs regarding the association between TG/HDL ratio and IGT were 1.41 (1.17~1.70), 1.18 (1.07~1.29), 1.09 (1.02~1.16), and 1.22 (1.05~1.42) in the N1–N4 groups, respectively. The ORs of the association between TG/HDL ratio and DM were 1.31 (1.1~1.55), 1.19 (1.08~1.31), 1.13 (1.06~1.20), and 1.22 (1.05~1.42) in the N1–N4 groups, respectively. In addition, TG/HDL ratio was also related to VD3 levels in IR, IGT, and DM (p for interaction <0.001) (**Table 3** and **Figure 3**). The relatively strongest relationships of TG/HDL with IR, IGT, and DM were observed at the lowest and highest levels of VD3 groups (N1 and N4, **Table 3**).

4.4 Non-Linear Relationships

Here, we analyzed the non-linear relationships of TG/HDL ratio with IR, IGT, and DM at different VD3 levels. Using the fully adjusted Model 4, we discovered different relationships among VD3 groups. The correlation was fitted by logistic regression (**Figure 4**), and the non-linear relationship was approved by double segmentation (**Table 4**). In addition, $p < 0.05$ was obtained upon the log-likelihood ratio test. Thus, two-stage logistic regression was conducted to accurately describe the relationships of TG/HDL ratio with IR, IGT, and DM at different VD3 levels (**Figure 4** and **Table 4**).

4.4.1 Insulin Resistance Group

Based on the two-stage logistic regression models and recursive algorithm, the cutoff value was determined to be 1.094. On the left side of the cutoff value, the ORs and 95% CIs were 5.68 (2.64–12.21), 5.55 (3.27–9.42), 3.50 (2.53–4.84), and 9.55 (3.79–24.06) in the N1–N4 groups, respectively. On the right side of the cutoff value, the ORs and 95% CIs were 1.49 (0.89, 2.48), 1.41 (1.03, 1.93), 1.54 (1.12, 2.11), and 1.84 (1.33, 2.54) in the N1–N4 groups, separately.

4.4.2 Impaired Glucose Tolerance Group

According to the two-stage logistic regression models and recursive algorithm, the cutoff value was set to 1.54. On the left side of the cutoff value, the ORs and 95% CIs were 5.29 (2.94–9.49), 2.68 (1.61–4.45), 2.14 (1.32–3.68), and 1.91 (1.40–2.61) in the N1–N4 groups, respectively. On the right side of the cutoff value, the ORs and 95% CIs were 1.13 (0.63, 2.01), 1.2 (0.85, 1.68), 1.40 (1.1, 1.71), and 0.06 (0.01, 0.46) in the N1–N4 groups, separately.

4.4.3 Diabetes Mellitus Group

In line with the two-stage logistic regression models and recursive algorithm, the cutoff value was 1.11. On the left side of the cutoff value, the ORs and 95% CIs were 7.33 (2.43–22.05), 3.83 (1.04–14.18), 7.13 (2.30–22.08), and 1.33 (0.99–1.77) in the N1–N4 groups, respectively. On the right side of the cutoff value, the ORs and 95% CIs were 1.20 (0.77, 1.85), 1.53 (1.19, 1.97), 1.57 (1.29, 1.94), and 0.54 (0.11, 2.65) in the N1–N4 groups, respectively.

TABLE 1 | Basic characteristics of the participants.

Variables	Total (n = 5,960)	N1 (n = 694)	N2 (n = 1,606)	N3 (n = 2,502)	N4 (n = 1,158)	p-Value
Gender, n (%)						< 0.001
Male	2,929 (49.1)	305 (43.9)	786 (48.9)	1,376 (55)	462 (39.9)	
Female	3,031 (50.9)	389 (56.1)	820 (51.1)	1,126 (45)	696 (60.1)	
Age (mean ± SD)	43.0 ± 20.6	41.2 ± 18.9	38.5 ± 19.3	41.9 ± 20.7	52.8 ± 19.9	<0.001
Race (n (%))						<0.001
Mexican American	804 (13.5)	89 (12.8)	299 (18.6)	350 (14)	66 (5.7)	
Other Hispanics	576 (9.7)	36 (5.2)	156 (9.7)	297 (11.9)	87 (7.5)	
Non-Hispanic white	2,331 (39.1)	116 (16.7)	357 (22.2)	1,096 (43.8)	762 (65.8)	
Non-Hispanic black	1,311 (22.0)	342 (49.3)	497 (30.9)	370 (14.8)	102 (8.8)	
Other race	938 (15.7)	111 (16)	297 (18.5)	389 (15.5)	141 (12.2)	
Alcohol use (n (%))						0.095
No	4,113 (69.0)	456 (65.7)	1,082 (67.4)	1,762 (70.4)	813 (70.2)	
Yes	1,842 (30.9)	238 (34.3)	522 (32.5)	738 (29.5)	344 (29.7)	
SMOK (n (%))						<0.001
No	1,786 (30.0)	145 (20.9)	448 (27.9)	761 (30.4)	432 (37.3)	
Yes	4,174 (70.0)	549 (79.1)	1,158 (72.1)	1,741 (69.6)	726 (62.7)	
EDU (n (%))						<0.001
No higher education	2,668 (44.8)	339 (48.8)	749 (46.6)	1,132 (45.2)	448 (38.7)	
Higher education	3,292 (55.2)	355 (51.2)	857 (53.4)	1,370 (54.8)	710 (61.3)	
INCOME (n (%))						<0.001
No more than $100,000	3,657 (61.4)	495 (71.3)	1,037 (64.6)	1,492 (59.6)	633 (54.7)	
More than $100,000	2,303 (38.6)	199 (28.7)	569 (35.4)	1,010 (40.4)	525 (45.3)	
HUA (n (%))						<0.001
No	4,849 (81.4)	536 (77.2)	1,307 (81.4)	2,116 (84.6)	890 (76.9)	
Yes	1,111 (18.6)	158 (22.8)	299 (18.6)	386 (15.4)	268 (23.1)	
Hbp (n (%))						<0.001
No	1,889 (31.7)	181 (26.1)	581 (36.2)	861 (34.4)	266 (23)	
Yes	4,071 (68.3)	513 (73.9)	1,025 (63.8)	1,641 (65.6)	892 (77)	
HTC (n (%))						<0.001
No	3,405 (57.1)	408 (58.8)	1,024 (63.8)	1,450 (58)	523 (45.2)	
Yes	2,555 (42.9)	286 (41.2)	582 (36.2)	1,052 (42)	635 (54.8)	
BMI, (mean ± SD)	28.0 ± 7.1	30.6 ± 8.8	28.5 ± 7.4	27.4 ± 6.5	27.1 ± 6.3	<0.001
Waist (mean ± SD)	95.8 ± 17.7	101.2 ± 20.8	96.1 ± 18.2	94.5 ± 16.9	94.8 ± 15.8	<0.001
Insulin resistance (n (%))						<0.001
No	3,253 (54.6)	308 (44.4)	780 (48.6)	1,407 (56.2)	758 (65.5)	
Yes	2,707 (45.4)	386 (55.6)	826 (51.4)	1,095 (43.8)	400 (34.5)	
Diabetes (n (%))						<0.001
No	4,699 (78.8)	507 (73.1)	1,257 (78.3)	2,036 (81.4)	899 (77.6)	
Yes	1,261 (21.2)	187 (26.9)	349 (21.7)	466 (18.6)	259 (22.4)	
Prediabetes (n (%))						<0.001
No	4,476 (75.1)	474 (68.3)	1,211 (75.4)	1,945 (77.7)	846 (73.1)	
Yes	1,484 (24.9)	220 (31.7)	395 (24.6)	557 (22.3)	312 (26.9)	
ALT (median (IQR))	19.0 (15.0, 26.0)	19.0 (14.0, 25.0)	19.0 (15.0, 27.0)	20.0 (15.0, 27.0)	20.0 (16.0, 25.0)	0.017
AST (median (IQR))	22.0 (19.0, 27.0)	21.0 (18.0, 26.0)	22.0 (19.0, 26.0)	22.0 (19.0, 27.0)	23.0 (20.0, 27.0)	<0.001
ALP (median (IQR))	66.0 (54.0, 84.0)	68.0 (55.0, 85.0)	68.0 (55.0, 87.0)	66.0 (54.0, 85.0)	63.0 (51.0, 77.0)	<0.001
TBIL (median (IQR))	12.0 (8.6, 13.7)	10.3 (8.6, 13.7)	10.3 (8.6, 13.7)	12.0 (8.6, 13.7)	12.0 (8.6, 13.7)	<0.001
BUN (median (IQR))	4.3 (3.2, 5.4)	3.6 (2.9, 4.6)	3.9 (3.2, 4.6)	4.3 (3.6, 5.4)	4.6 (3.9, 6.1)	<0.001
TC (median (IQR))	4.7 (4.0, 5.4)	4.6 (4.0, 5.4)	4.6 (4.0, 5.4)	4.6 (4.0, 5.4)	4.9 (4.2, 5.6)	<0.001
UA (median (IQR))	315.2 (261.7, 374.7)	324.2 (255.8, 386.6)	315.2 (255.8, 374.7)	315.2 (261.7, 368.8)	309.3 (261.7, 374.7)	0.426
GGT (median (IQR))	17.0 (13.0, 26.0)	18.0 (13.2, 31.0)	18.0 (13.0, 27.0)	17.0 (12.0, 25.0)	17.0 (13.0, 26.0)	<0.001
FPG (median (IQR))	5.2 (4.8, 5.7)	5.2 (4.8, 5.8)	5.2 (4.8, 5.7)	5.2 (4.8, 5.6)	5.2 (4.8, 5.7)	0.092
TG/HDL (median (IQR))	0.8 (0.5, 1.3)	0.8 (0.5, 1.3)	0.7 (0.5, 1.3)	0.8 (0.5, 1.4)	0.8 (0.5, 1.3)	0.071

EDU, education; SMOK, smoking; BMI, body mass index; ALT, alanine aminotransferase; ALB, albumin; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GGT, γ-glutamyl transpeptidase; UA, uric acid; FPG, fasting plasma glucose; TC, total cholesterol; TG/HDL, triglycerides/high-density lipoprotein.

5 DISCUSSION

In this study, 5,960 participants were recruited to analyze the independent associations of TG/HDL ratio with IR, IGT, and DM at different VD3 levels. After the influencing factors were adjusted, TG/HDL ratio was related to IR, IGT, and DM at varying degrees at different VD3 levels (p for interaction <0.05). Specifically, stronger relationships were observed in the N1 and N4 groups (**Table 3**). It suggests that the too low or too high VD3 levels possibly strengthen the associations of TG/HDL ratio with IR, IGT, and DM.

In a study carried out on Hispanic and African Americans, the associations of TG/HDL ratio with IR, β-cell function, and DM were investigated. After the influencing factors were adjusted, TG/HDL ratio was associated with IR in the non-Caucasian populations, and a higher TG/HDL ratio was related

TABLE 2 | Univariate analysis of IR, IGT, and DM.

Variables	IR		IGT		DM	
	OR (95% CI)	p-Value	OR (95% CI)	p-Value	OR (95% CI)	p-Value
Age	1.01 (1~1.01)	<0.001	1.06 (1.05~1.06)	<0.001	1.06 (1.06~1.06)	<0.001
Gender						
Male	1		1		1	
Female	0.99 (0.9~1.1)	0.89	0.79 (0.7~0.89)	<0.001	0.85 (0.75~0.96)	0.011
Education						
No higher education	1		1		1	
Higher education	0.8 (0.72~0.89)	<0.001	0.65 (0.58~0.74)	<0.001	0.62 (0.55~0.7)	<0.001
Income						
No more than $100,000	1		1		1	
More than $100,000	0.75 (0.68~0.84)	<0.001	0.74 (0.66~0.84)	<0.001	0.72 (0.63~0.82)	<0.001
Alcohol use						
No	1		1		1	
Yes	1.25 (1.12~1.4)	<0.001	1.15 (1.02~1.31)	0.028	1.22 (1.07~1.39)	0.003
Smoking						
No	1		1		1	
Yes	0.93 (0.83~1.04)	0.195	0.74 (0.65~0.84)	<0.001	0.76 (0.67~0.87)	<0.001
Hyperuricemia						
No	1		1		1	
Yes	2.6 (2.27~2.98)	<0.001	2.43 (2.11~2.79)	<0.001	2.32 (2.01~2.67)	<0.001
Hypertension						
No	1		1		1	
Yes	1.71 (1.53~1.91)	<0.001	6.56 (5.43~7.91)	<0.001	7.58 (6.1~9.42)	<0.001
Hypercholesterolemia						
No	1		1		1	
Yes	1.73 (1.56~1.92)	<0.001	4.14 (3.65~4.7)	<0.001	4.48 (3.91~5.13)	<0.001
BMI	1.18 (1.17~1.2)	<0.001	1.1 (1.09~1.11)	<0.001	1.09 (1.08~1.1)	<0.001
Waist	1.07 (1.06~1.07)	<0.001	1.05 (1.05~1.06)	<0.001	1.05 (1.05~1.05)	<0.001
TC	1.04 (0.99~1.09)	0.097	1.13 (1.07~1.19)	<0.001	1.11 (1.05~1.17)	<0.001
TG	2.06 (1.91~2.21)	<0.001	1.48 (1.39~1.57)	<0.001	1.44 (1.36~1.53)	<0.001
HDL	0.15 (0.13~0.18)	<0.001	0.41 (0.35~0.49)	<0.001	0.41 (0.34~0.49)	<0.001
TG/HDL	2.09 (1.94~2.25)	<0.001	1.3 (1.24~1.36)	<0.001	1.28 (1.22~1.34)	<0.001
ALT	1.02 (1.02~1.02)	<0.001	1.01 (1.01~1.01)	<0.001	1.01 (1.01~1.01)	<0.001
AST	1 (1~1.01)	0.174	1 (1~1.01)	0.005	1 (1~1.01)	0.032
GGT	1.01 (1.01~1.01)	<0.001	1.01 (1.01~1.01)	<0.001	1.01 (1.01~1.01)	<0.001
ALP	1 (1~1)	<0.001	1 (1~1)	<0.001	1 (1~1)	<0.001
BUN	1.03 (1.01~1.06)	0.009	1.32 (1.27~1.36)	<0.001	1.32 (1.27~1.36)	<0.001
UA	1.01 (1~1.01)	<0.001	1.01 (1~1.01)	<0.001	1.01 (1~1.01)	<0.001

IR, insulin resistance; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GGT, γ-glutamyl transpeptidase; UA, uric acid; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein.

to lower insulin sensitivity in the Hispanic and African American populations (25). Wang et al. also confirmed that TG/HDL ratio was an independent risk factor for DM in the Singapore Chinese (26). In addition, Gong et al. studied more than 100,000 Chinese cohorts and discovered that a higher TG/HDL ratio was positively correlated with the occurrence of IGT and DM after the influencing factors were adjusted (27). These investigations are consistent with our results.

This study further evaluated the effect of VD3 levels on the relationships of TG/HDL ratio with IR, IGT, and DM. As a result, too high or too low VD3 levels promoted the abovementioned relationships. The N3 group (ordinary VD3 levels) showed the weakest correlations of TG/HDL with IR, IGT, and DM. This indicates that, compared with VD3 abundance and deficiency, maintaining the reasonable VD3 levels is more effective in reducing the associations of TG/HDL ratio with IR, IGT, and DM. In addition, we found that such associations were strengthened, not weakened as expected, in the N4 group. This demonstrates that excessive (high level of) VD3 strengthens the associations of TG/HDL with IR, IGT, and DM. The main reason is that excessive VD3 can lead to abnormalities in calcium and phosphorus metabolism as well as VD3 toxicity in the body, thus lowering the protective effect of VD3 on the body. Therefore, this should be further validated by more clinical studies and randomized controlled trials (RCTs).

As reported in previous studies, high TG/HDL ratios lead to reduced retention of fatty acids; therefore, more fatty acids are transported to the liver for TG synthesis, and more free fatty acids will be formed accordingly (28), while higher free fatty acid levels have been identified as a risk factor for T2DM. By combining with insulin, the excess free fatty acids prevent the secretion of a normal amount of insulin from achieving the desired glucose-lowering effect. As a result, pancreatic β-cells are stimulated to secrete more insulin, which thus leads to IR and ultimately the development of DM (29). In addition, TG-rich lipoproteins accelerate the production of leptin, angiotensinogen, tumor necrosis factor-alpha (TNF-a), interleukin 6 (IL-6),

TABLE 3 | Multiple regression analysis of different VD3 levels.

Outcomes	Model 1		Model 2		Model 3		Model 4		*p* for interaction
	OR (95% CI)	p-Value	OR (95% CI)	p-Value	OR (95% CI)	p-Value	OR (95% CI)	p-Value	
IR group									<0.001
N1 (<30)	2.33 (1.83~2.96)	<0.001	2.63 (2.02~3.43)	<0.001	2.02 (1.55~2.64)	<0.001	2.11 (1.57~2.83)	<0.001	
N2 (30–50)	2.01 (1.73~2.34)	<0.001	2.23 (1.89~2.63)	<0.001	1.52 (1.31~1.78)	<0.001	1.52 (1.29~1.78)	<0.001	
N3 (50–80)	1.87 (1.68~2.07)	<0.001	1.88 (1.69~2.09)	<0.001	1.31 (1.18~1.44)	<0.001	1.28 (1.16~1.41)	<0.001	
N4 (>80)	3.27 (2.69~3.98)	<0.001	3.22 (2.63~3.94)	<0.001	2.23 (1.81~2.74)	<0.001	2.16 (1.75~2.67)	<0.001	
IGT group									<0.001
N1 (<30)	1.54 (1.33~1.8)	<0.001	1.63 (1.36~1.94)	<0.001	1.46 (1.21~1.75)	<0.001	1.41 (1.17~1.70)	<0.001	
N2 (30–50)	1.31 (1.19~1.43)	<0.001	1.29 (1.17~1.43)	<0.001	1.18 (1.08~1.3)	<0.001	1.18 (1.07~1.29)	0.001	
N3 (50–80)	1.18 (1.11~1.26)	<0.001	1.19 (1.1~1.27)	<0.001	1.08 (1.01~1.15)	0.016	1.09 (1.02~1.16)	0.007	
N4 (>80)	1.6 (1.39~1.84)	<0.001	1.59 (1.37~1.85)	<0.001	1.28 (1.1~1.48)	0.001	1.22 (1.05~1.42)	0.009	
DM group									<0.001
N1 (<30)	1.43 (1.24~1.65)	<0.001	1.51 (1.27~1.78)	<0.001	1.37 (1.15~1.62)	<0.001	1.31 (1.1~1.55)	0.002	
N2 (30–50)	1.29 (1.18~1.41)	<0.001	1.31 (1.19~1.44)	<0.001	1.2 (1.09~1.32)	<0.001	1.19 (1.08~1.31)	<0.001	
N3 (50–80)	1.18 (1.1~1.26)	<0.001	1.22 (1.13~1.32)	<0.001	1.11 (1.04~1.2)	0.002	1.13 (1.06~1.20)	<0.001	
N4 (>80)	1.51 (1.32~1.73)	<0.001	1.53 (1.31~1.78)	<0.001	1.27 (1.09~1.47)	0.002	1.22 (1.05~1.42)	0.01	

Model 1: non-adjusted. Model 2: adjusted for gender, age, and race. Model 3: Model 2 + adjusted for ALT, AST, TG, TC, GGT, ALB, and HDL. Model 4: Model 3 + adjusted for smoking, alcohol use, income, and education.

VD3, vitamin D3; IR, insulin resistance; IGT, impaired glucose tolerance; DM, diabetes mellitus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglycerides; TC, total cholesterol; GGT, γ-glutamyl transpeptidase; ALB, albumin; HDL, high-density lipoprotein.

fibrinogen activator inhibitor 1, transforming growth factor B (TGF-b), lipocalin, and resistin (29). These factors are the risk factors for the development of IR or DM, at least at the experimental level. VD3 can bind to some free fatty acids (30) to alleviate certain unfavorable effects of fatty acids. This was also represented in results from the N1–N3 groups, where the associations of TG/HDL ratio with IR, IGT, and DM were weakened as VD3 levels increased (**Tables 3** and **4**). As a kind of fat-soluble vitamin, VD3 is mainly obtained by irradiating the skin with sunlight (31). Studies have reported that phototherapy and VD3 supplementation can ameliorate IR and inflammation in a rat model of non-alcoholic fatty liver disease (NAFLD) induced by a special diet (32). Based on these results and our observation, we suppose that VD3 is beneficial to normal metabolism, and safe sunlight exposure can be an appropriate way to increase VD3 levels for human health (33).

Certainly, some of the results in this study were different from those of previous studies. Such discrepancies may be explained from the following aspects. 1) The study populations were different. 2) The adjusted variables were different, and a more adequate adjustment strategy was adopted in this study. 3) The associations of TG/HDL ratio with IR, IGT, and DM were analyzed at different VD3 levels in this study. Notably, the clinical strength of this study is as follows. We found that within a reasonable range of VD3 (0–80 ng/ml), increasing VD3 levels attenuated the associations of TG/HDL ratio with IR, IGT, and DM. This provides new evidence for clinical guidance on the appropriate range of VD3 levels in patients. Meanwhile, we discovered that when VD3 levels were

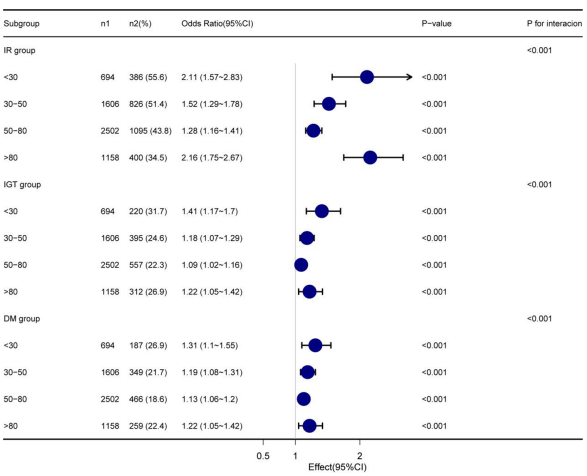


FIGURE 3 | Subgroup analysis based on the multivariate logistic regression analysis of the association between TG/HDL ratio and IR, IGT, and DM. TG/HDL, triglycerides/high-density lipoprotein; IR, insulin resistance; IGT, impaired glucose tolerance; DM, diabetes mellitus.

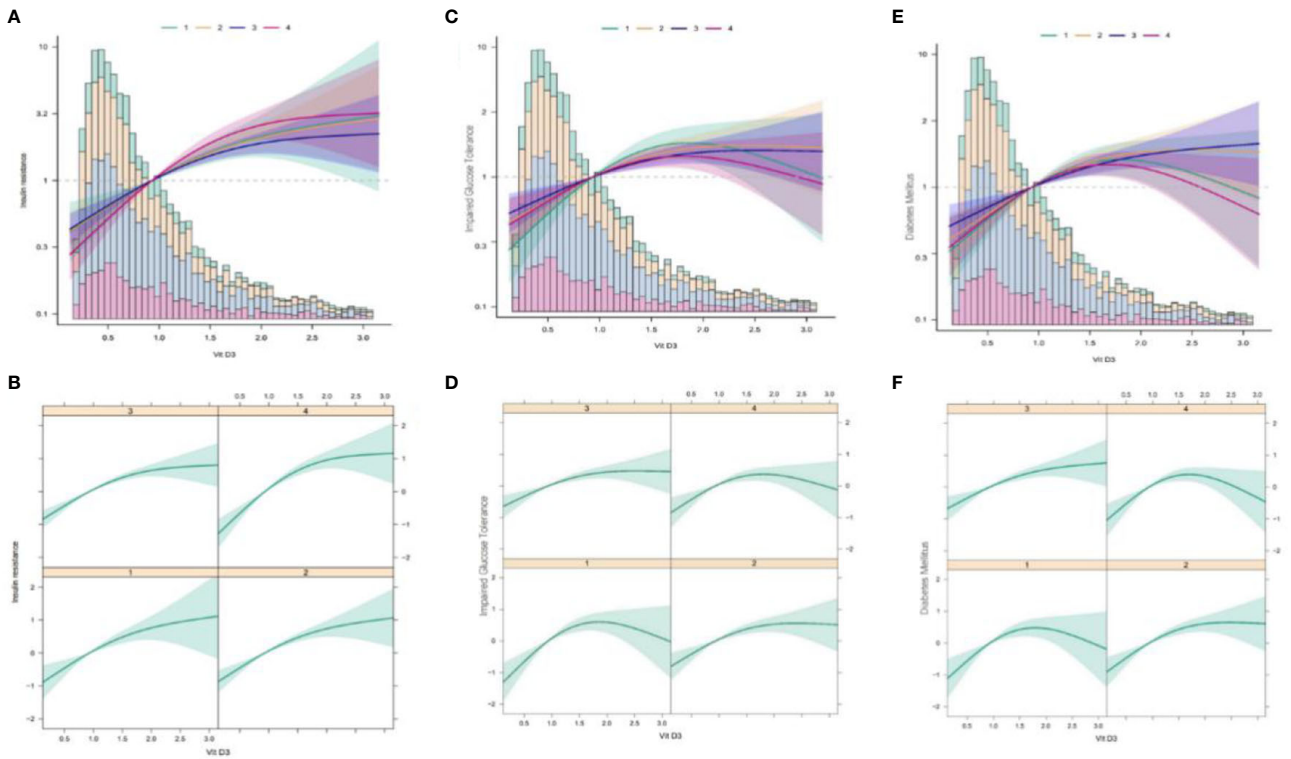


FIGURE 4 | Multivariate logistic regression analysis of the associations of TG/HDL ratio with IR, IGT, and DM in different VD3 groups. TG/HDL, triglycerides/high-density lipoprotein; IR, insulin resistance; IGT, impaired glucose tolerance; DM, diabetes mellitus; VD3, vitamin D3. **(A)** The association between TG/HDL and IR at different VD3 levels (Total). **(B)** The association between TG/HDL and IR at different vD3 levels (Separate). **(C)** The association between TG/HDL and IGT at different VD3 levels (Total). **(D)** The association between TG/HDL and IGT at different vD3 levels (Separate). **(E)** The association between TG/HDL and DM at different VD3 levels (Total). **(F)** The association between TG/HDL and DM at different VD3 levels (Separate). TG/HDL, triglycerides/high-density lipoprotein; IR, insulin resistance; VD3, vitamin D3; IGT, impaired glucose tolerance; DM, diabetes mellitus.

TABLE 4 | Threshold analysis of TG/HDL on the incident of IR, IGT, and DM in the NHANES study, 2009–2018.

Outcomes	N1 (VD3 < 30)		N2 (30 ≤ VD3 < 50)		N3 (50 ≤ VD3 < 80)		N4 (80 ≤ VD3)	
	OR (95% CI)	p-Value	OR (95% CI)	p-Value	OR (95% CI)	p-Value	OR (95% CI)	p-Value
IR group								
Cutoff value	1.16 (1.14, 1.18)		1.10 (1.08, 1.12)		1.34 (1.31, 1.37)		0.91 (0.88, 0.93)	
TG/HDL < 1.094	5.68 (2.64, 12.21)	<0.001	5.55 (3.27, 9.42)	<0.001	3.50 (2.53, 4.84)	<0.001	9.55 (3.79, 24.06)	<0.001
TG/HDL ≥ 1.094	1.49 (0.89, 2.48)	0.123	1.41 (1.03~1.93)	0.033	1.54 (1.12, 2.11)	0.007	1.84 (1.33, 2.54)	<0.001
Likelihood ratio test	–	<0.001	–	<0.001	–	<0.001	–	<0.001
Non-linear test	–	<0.001	–	<0.001	–	<0.001	–	<0.001
Prediabetes group								
Cutoff value	1.46 (1.43, 1.49)		1.22 (1.19, 1.24)		1.11 (1.09, 1.13)		1.97 (1.93, 2.01)	
TG/HDL < 1.51	5.29 (2.94, 9.49)	<0.001	2.68 (1.61, 4.45)	<0.001	2.14 (1.32, 3.48)	<0.001	1.91 (1.40, 2.61)	<0.001
TG/HDL ≥ 1.51	1.13 (0.63, 2.01)	0.685	1.20 (0.85, 1.68)	0.296	1.40 (1.10, 1.78)	0.007	0.06 (0.01, 0.46)	<0.001
Likelihood Ratio test	–	<0.001	–	<0.001	–	<0.001	–	<0.001
Non-linear test	–	<0.001	–	<0.001	–	<0.001	–	<0.001
Diabetes group								
Cutoff value	1.18 (1.10, 1.14)		0.82 (0.75, 0.89)		0.82 (0.75, 0.89)		1.94 (1.91, 1.97)	
TG/HDL < 1.11	7.33 (2.43, 22.05)	<0.001	3.83 (1.04, 14.18)	<0.001	7.13 (2.30, 22.08)	<0.001	1.33 (0.99, 1.77)	0.057
TG/HDL ≥ 1.11	1.20 (0.77, 1.85)	0.421	1.53 (1.19, 1.97)	<0.001	1.57 (1.29, 1.94)	<0.001	0.54 (0.11, 2.65)	0.444
Likelihood ratio test	–	<0.001	–	<0.001	–	<0.001	–	<0.001
Non-linear test	–	<0.001	–	<0.001	–	<0.001	–	<0.001

Adjusted for age, gender, race, education, BMI, WC (waist circumference), income, smoking, alcohol use, hypertension, UA, ALT, AST, GGT, LDH, and BUN.
TG/HDL, triglycerides/high-density lipoprotein; IR, insulin resistance; IGT, impaired glucose tolerance; DM, diabetes mellitus; VD3, vitamin D3; BMI, body mass index; UA, uric acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; LDH, low-density lipoprotein; BUN, blood urea nitrogen.

too high (≥80 ng/ml) in DM patients, it might strengthen the relations of TG/HDL ratio with IR, IGT, and DM. This indicates that the incidence of abnormal glucose metabolism may increase in people with high TG/HDL ratios when VD3 levels are too high.

Nevertheless, certain limitations should also be noted in this study. 1) This was a cross-sectional study, and the causal relationships of TG/HDL ratio with IR, IGT, and DM were not determined. Therefore, a cohort study is warranted to analyze the accurate relationship. 2) Special populations (like pregnant women and children) were excluded from this study, and whether the results were applicable to these populations remains unknown. Also, there are some noteworthy highlights of this study. First, this study was conducted using the official NHANES database, which is more representative of the entire US population after a complex weighting design. Second, in the design of this study, smoothed fitting curve and two-stage logistic regression were used to accurately analyze the relationships.

6 CONCLUSION

Collectively, our results suggest that in the American population, maintaining too high or too low levels of VD3 can promote the associations of TG/HDL with IR, IGT, and DM, which shed new light on DM research. However, other conditions such as age and sunlight exposure level should be taken into consideration when formulating the appropriate VD3 levels.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. Data can be obtained from the NHANES database (https://www.cdc.gov/nchs/nhanes/).

AUTHOR CONTRIBUTIONS

YL and RG: conceived the idea; YL, RG and GL wrote the manuscript; GL and RG collected and read the literature and revised the article; XW and LY read through and corrected the manuscript. All authors contributed to the article and approved the submitted version. YL is the first author. RG and GL are the co-first author. XW and LY are the corresponding author of this paper.

FUNDING

This work was supported by Grants from the National Key R&D Program of China (2018YFC1311500), the National Natural Science Foundation of China (81860370), the CAS "Light of West China" Program (2019), the General Project of Natural Science Foundation of Qinghai Province (2020-ZJ-930) Effects of high altitude environment and season on vitamin D, bone metabolism factors and muscle volume in adults.

REFERENCES

1. Kulda V. Vitamin D Metabolism. *Vnitr Lek* (2012) 58(5):400–4.
2. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: Modulator of the Immune System. *Curr Opin Pharmacol* (2010) 10(4):482–96. doi: 10.1016/j.coph.2010.04.001
3. Wenclewska S, Szymczak-Pajor I, Drzewoski J, Bunk M, Śliwińska A. Vitamin D Supplementation Reduces Both Oxidative DNA Damage and Insulin Resistance in the Elderly With Metabolic Disorders. *Int J Mol Sci* (2019) 20 (12):2891–907. doi: 10.3390/ijms20122891
4. Lu L, Lu Q, Chen W, Li J, Li C, Zheng Z. Vitamin D(3) Protects Against Diabetic Retinopathy by Inhibiting High-Glucose-Induced Activation of the ROS/TXNIP/NLRP3 Inflammasome Pathway. *J Diabetes Res* (2018) 2018:8193523. doi: 10.1155/2018/8193523
5. Gong R, Xu Z, Wei X. The Association Between Vitamin D3 and Diabetes in Both Hyperuricemia and Non-Hyperuricemia Populations. *Endocrine* (2021) 74(1):90–9. doi: 10.1007/s12020-021-02778-y
6. Yang M, Xu J, Yu J, Yang B, Gan H, Li S, et al. Anti-Inflammatory Effects of 1,25-Dihydroxyvitamin D3 in Monocytes Cultured in Serum From Patients With Type 2 Diabetes Mellitus and Diabetic Nephropathy With Uremia *via* Toll-Like Receptor 4 and Nuclear Factor-κb P65. *Mol Med Rep* (2015) 12 (6):8215–22. doi: 10.3892/mmr.2015.4482
7. Kendall DM, Sobel BE, Coulston AM, Peters Harmel AL, McLean BK, Peragallo-Dittko V, et al. The Insulin Resistance Syndrome and Coronary Artery Disease. *Coron Artery Dis* (2003) 14(4):335–48. doi: 10.1097/01.mca.0000076512.29238.2a
8. Zhang XY, Guo CC, Yu YX, Xie L, Chang CQ. Establishment of High-Fat Diet-Induced Obesity and Insulin Resistance Model in Rats. *Beijing Da Xue Xue Bao Yi Xue Ban* (2020) 52(3):557–63. doi: 10.19723/j.issn.1671-167X.2020.03.024
9. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* (2011) 34(Suppl 1):S62–9. doi: 10.2337/dc11-S062
10. Robert AA, Al Dawish MA. The Worrying Trend of Diabetes Mellitus in Saudi Arabia: An Urgent Call to Action. *Curr Diabetes Rev* (2020) 16(3):204–10. doi: 10.2174/1573399815666190531093735
11. Holt RIG. Diabetes Education, Education and Education. *Diabetes Med* (2017) 34(8):1023–4. doi: 10.1111/dme.13399
12. Whiting DR, Guariguata L, Weil C, Shaw J. IDF Diabetes Atlas: Global Estimates of the Prevalence of Diabetes for 2011 and 2030. *Diabetes Res Clin Pract* (2011) 94(3):311–21. doi: 10.1016/j.diabres.2011.10.029
13. Boulton A. Strengthening the International Diabetes Federation (IDF). *Diabetes Res Clin Pract* (2020) 160:108029. doi: 10.1016/j.diabres.2020.108029
14. Calgaroto NS, Thomé GR, da Costa P, Baldissareli J, Hussein FA, Schmatz R, et al. Effect of Vitamin D3 on Behavioural and Biochemical Parameters in Diabetes Type 1-Induced Rats. *Cell Biochem Funct* (2014) 32(6):502–10. doi: 10.1002/cbf.3044
15. Neelankal John A, Iqbal Z, Colley S, Morahan G, Makishima M, Jiang FX. Vitamin D Receptor-Targeted Treatment to Prevent Pathological Dedifferentiation of Pancreatic β Cells Under Hyperglycaemic Stress. *Diabetes Metab* (2018) 44(3):269–80. doi: 10.1016/j.diabet.2017.07.006
16. Uruska A, Zozulinska-Ziolkiewicz D, Niedzwiecki P, Pietrzak M, Wierusz-Wysocka B. TG/HDL-C Ratio and Visceral Adiposity Index may be Useful in Assessment of Insulin Resistance in Adults With Type 1 Diabetes in Clinical Practice. *J Clin Lipidol* (2018) 12(3):734–40. doi: 10.1016/j.jacl.2018.01.005
17. He J, He S, Liu K, Wang Y, Shi D, Chen X. The TG/HDL-C Ratio Might Be a Surrogate for Insulin Resistance in Chinese Nonobese Women. *Int J Endocrinol* (2014) 2014:105168. doi: 10.1155/2014/105168
18. Bothou C, Beuschlein F, Spyroglou A. Links Between Aldosterone Excess and Metabolic Complications: A Comprehensive Review. *Diabetes Metab* (2020) 46(1):1–7. doi: 10.1016/j.diabet.2019.02.003
19. Salgado AL, Carvalho L, Oliveira AC, Santos VN, Vieira JG, Parise ER. Insulin Resistance Index (HOMA-IR) in the Differentiation of Patients With Non-

Alcoholic Fatty Liver Disease and Healthy Individuals. *Arq Gastroenterol* (2010) 47(2):165–9. doi: 10.1590/S0004-28032010000200009
20. Perry RC, Baron AD. Impaired Glucose Tolerance. Why Is It Not a Disease? *Diabetes Care* (1999) 22(6):883–5. doi: 10.2337/diacare.22.6.883
21. American Diabetes Association. (2) Classification and Diagnosis of Diabetes. *Diabetes Care* (2015) 38 Suppl:S8–s16. doi: 10.2337/dc15-S005
22. Elliott WJ. Systemic Hypertension. *Curr Probl Cardiol* (2007) 32(4):201–59. doi: 10.1016/j.cpcardiol.2007.01.002
23. Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in Obesity Among Adults in the United States, 2005 to 2014. *JAMA* (2016) 315 (21):2284–91. doi: 10.1001/jama.2016.6458
24. Ricci C, Schutte AE, Schutte R, Smuts CM, Pieters M. Trends in Alcohol Consumption in Relation to Cause-Specific and All-Cause Mortality in the United States: A Report From the NHANES Linked to the US Mortality Registry. *Am J Clin Nutr* (2020) 111(3):580–9. doi: 10.1093/ajcn/nqaa008
25. Young KA, Maturu A, Lorenzo C, Langefeld CD, Wagenknecht LE, Chen YI, et al. The Triglyceride to High-Density Lipoprotein Cholesterol (TG/HDL-C) Ratio as a Predictor of Insulin Resistance, β-Cell Function, and Diabetes in Hispanics and African Americans. *J Diabetes Complications* (2019) 33(2):118–22. doi: 10.1016/j.jdiacomp.2018.10.018
26. Wang YL, Koh WP, Talaei M, Yuan JM, Pan A. Association Between the Ratio of Triglyceride to High-Density Lipoprotein Cholesterol and Incident Type 2 Diabetes in Singapore Chinese Men and Women. *J Diabetes* (2017) 9(7):689–98. doi: 10.1111/1753-0407.12477
27. Gong R, Liu Y, Luo G, Liu W, Jin Z, Xu Z, et al. Associations of TG/HDL Ratio With the Risk of Prediabetes and Diabetes in Chinese Adults: A Chinese Population Cohort Study Based on Open Data. *Int J Endocrinol* (2021) 2021:9949579. doi: 10.1155/2021/9949579
28. Haluzík M, Parízková J, Haluzík MM. Adiponectin and Its Role in the Obesity-Induced Insulin Resistance and Related Complications. *Physiol Res* (2004) 53(2):123–9. doi: 10.2307/3434063
29. Bełtowski J. Adiponectin and Resistin–New Hormones of White Adipose Tissue. *Med Sci Monit* (2003) 9(2):Ra55–61.
30. Morton RE. Cholesteryl Ester Transfer Protein and its Plasma Regulator: Lipid Transfer Inhibitor Protein. *Curr Opin Lipidol* (1999) 10(4):321–7. doi: 10.1097/00041433-199908000-00006
31. Wacker M, Holick MF. Sunlight and Vitamin D: A Global Perspective for Health. *Dermatoendocrinol* (2013) 5(1):51–108. doi: 10.4161/derm.24494
32. Nakano T, Cheng Y-F, Lai C-Y, Hsu L-W, Chang Y-C, Deng J-Y, et al. Impact of Artificial Sunlight Therapy on the Progress of Non-Alcoholic Fatty Liver Disease in Rats. *J Hepatol* (2011) 55(2):415–25. doi: 10.1016/j.jhep.2010.11.028
33. Erem AS, Razzaque MS. Vitamin D-Independent Benefits of Safe Sunlight Exposure. *J Steroid Biochem Mol Biol* (2021) 213:105957. doi: 10.1016/j.jsbmb.2021.105957

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.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 29 March 2022
doi: 10.3389/fnut.2022.849747

Dietary Magnesium Intake Affects the Vitamin D Effects on HOMA-β and Risk of Pancreatic β-Cell Dysfunction: A Cross-Sectional Study

Rongpeng Gong[1†], *Yuanyuan Liu*[1,2†], *Gang Luo*[3] *and Lixin Yang*[1,2*]

[1] *Medical College of Qinghai University, Xining, China,* [2] *Endocrinology Department, Qinghai Provincial People's Hospital, Xining, China,* [3] *College of Eco-Environmental Engineering, Qinghai University, Xining, China*

OPEN ACCESS

Edited by:
Mohammed S. Razzaque, Lake Erie College of Osteopathic Medicine, United States

Reviewed by:
Farhana Akter, Chittagong Medical College, Bangladesh
Evelyn Frias-Toral, Catholic University of Santiago de Guayaquil, Ecuador

***Correspondence:**
Lixin Yang
yanglx282@sina.com

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

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

Received: *06 January 2022*
Accepted: *11 February 2022*
Published: *29 March 2022*

Citation:
Gong R, Liu Y, Luo G and Yang L (2022) Dietary Magnesium Intake Affects the Vitamin D Effects on HOMA-β and Risk of Pancreatic β-Cell Dysfunction: A Cross-Sectional Study. Front. Nutr. 9:849747. doi: 10.3389/fnut.2022.849747

Background: Some studies have shown that, the circulating vitamin D (Vit D) concentration in the body exerts a crucial role in regulating the pancreatic β-cell function. Meanwhile, the role of magnesium is important in the synthesis of Vit D, since it is an essential element for activating Vit D. Nevertheless, there remains insufficient studies concerning whether dietary Magnesium intake influences the association between Vit D and risk of pancreatic β-cell dysfunction. Hence, this cross-sectional study aimed to assess the effect of Magnesium intake alterations on the association between serum Vit D levels and the risk of pancreatic β-cell dysfunction.

Methods: This large-scale cross-sectional study involves four cycles of National Health and Nutrition Examination Survey (NHANES) (2007–2014), with totally 4,878 participants. Groups were divided depending on the median daily intake of Magnesium, namely, the low intake group (Magnesium intake $<$ 267 Magnesium/d) and the high intake group (Magnesium intake $\geq$ 267 Magnesium/d). By constructing multiple multivariate linear and logistics regression models, the associations between serum Vit D levels and HOMA-β, as well as between serum Vit D levels and the risk of pancreatic β-cell dysfunction were explored at different Magnesium intakes.

Results: In this cross-sectional study, the serum Vit D level is independently correlated with the HOMA-β index [β: 0.65 (0.40–0.90)] and the risk of pancreatic β-cell dysfunction [OR: 0.95 (0.92–0.98)]. Moreover, such correlations are affected by different dietary Magnesium intakes (P for interaction $<$ 0.001).

Conclusion: According to the results of this study, the dietary Magnesium intake influences the associations of serum Vit D levels with HOMA-β index and pancreatic β-cell dysfunction. Besides, the finding requires validation through more RCT or cohort studies.

Keywords: Magnesium intake, vitamin D, HOMA-β index, pancreatic β-cell, cross-sectional study

BACKGROUND

Pancreatic β-cells refer to a kind of insulin-secreting cells of the pancreas. Both impaired pancreatic β-cell functions and relatively insufficient insulin secretion can increase the blood sugars (1). Based on surveys, the impairment of pancreatic β-cells has become a ubiquitous phenomenon worldwide, and most patients with pancreatic β-cell impairment are highly likely to progress to diabetes without intervention or control (2–4). In addition, it has been reported that one of the main pathogeneses of type 2 diabetes is impairment of pancreatic β-cells. The International Diabetes Federation (IDF) claimed that 629 million people aged 20–79 will be estimated to have diabetes by 2045, occupying ∼10% of the total global population (5, 6). This implies that, there may be more patients with impaired pancreatic β-cells hiding under this population. This is a huge challenge to the world health and medical resources. If the pancreatic β-cell impairment can be detected in advance, it will have a profound significance for the occurrence and prevention of diabetes. At the same time, for medical researchers, preventing the pancreatic β-cell damage and reducing the diabetes incidence are an incumbent responsibility. This highly necessitates the identification of the effects of nutrients on β-cell functionality and the prevention of pancreatic β-cell impairment.

Vitamin D (Vit D), as a kind of lipid-soluble vitamins, has vitamins D3 and D2 as major components (7). According to some research findings, Vit D can increase the blood calcium and phosphorus while regulating the bone metabolism in coordination with parathyroid hormones, exhibiting preferable performance in enhancing immunity, as well as in preventing cancer, cardiovascular and metabolic diseases (8–12). However, its association with pancreatic β-cell functionality remains controversial. Most studies have claimed that Vit D can improve the functionality of pancreatic β-cells and insulin sensitivity, while lowering the risk of type 2 diabetes(T2DM) (12–17). Contrastively, other studies have shown that Vit D does not affect β-cell functionality, nor is it a risk factor of cardiovascular diseases, which is even unrelated to diabetes (18–21). Based on an RCT study conducted by Nielsen et al. among Greenland Inuit in 2015, Vit D may be uncorrelated with the risk of T2DM, albeit the presence of a negative correlation between Vit D levels and pancreatic β-cell functions (22). The reason for these disparities in the foregoing results may be that the pancreatic β-cell functionality is affected concurrently by factors such as race, region, age and gender, one of which is the dietary Magnesium intake.

Magnesium is the fourth most abundant mineral in the human body after calcium, potassium and sodium. Magnesium can activate ∼600 enzymes, which are essential for maintaining cellular stability, synthesizing RNAs and DNAs, repairing cells, and sustaining the cellular antioxidant status (23, 24). There exists a close association between Magnesium and synthesis of Vit D, and previous research has demonstrated that Magnesium is an essential element for activating Vit D (24). Neither vitamin D2 or D3 has bioactivity, which needs to be hydroxylated twice in the liver and kidney, in order to produce active 1,25(OH)2D. The enzymatic activities of hepatic 25-hydroxylase and renal 1α-hydroxylase are both Magnesium-dependent processes (25, 26). Some other studies have revealed that Vit D is transported in the human body in conjunction with carrier proteins, where the transport carrier is Vit D-binding protein and that the activity of such Vit D-binding protein is also a Magnesium-dependent process (27, 28). According to a review by Kostov et al. Magnesium plays a crucial role in regulating the electrical activity of pancreatic β-cells and insulin concentration. This may be attributed to the associations of intracellular Magnesium concentration with the phosphorylation of target cell insulin receptors and downstream signal kinases (29). By summarizing previous studies, Toi et al. (30) concluded that the substantial Magnesium intake is negatively correlated with the risk of pancreatic β-cell impairment.

Can Magnesium, as an activator of Vit D and a regulator of pancreatic β-cell functionality, affect the association between serums Vit D levels and pancreatic β-cells? There have been few similar reports in the existing literature (8, 31, 32). Hence, clinical research concerning the effect of Magnesium intake on the association of serums Vit D levels with the risk of pancreatic β-cell impairment is necessary. In this study, we hypothesize that Magnesium intake can affect such association. The objective is to explore the effect of Magnesium intake on the association between Vit D and risk of pancreatic β-cell impairment by adopting a nationally representative public database of the USA.

METHODOLOGY

Data Source

This large cross-sectional study utilizes four cycles (2007–2014) of data from the National Health & Nutrition Examination Survey (NHANES) database (https://www.cdc.gov/nchs/nhanes/). As a research project related to the diet and health of the US citizens, a multistage stratified probability design is adopted during data collection. Therefore, the samples are representative of the entire US citizens who are not in shelter institutions. These data include demographic, dietary, somatometric, laboratory and questionnaire data. All the NHANES-based study protocols have been approved by the Research Ethics Review Board of National Center for Health Statistics (NCHS). Ethical approval and more detailed information can be found on the Review Board's website (https://www.cdc.gov/nchs/nhanes/irba98.htm) (33).

Study Design and Participants

This study was designed as a cross-sectional study, where the target independent variable was the serum Vit D level at the time of participant testing, and the dependent variable was whether the participants were diagnosed with pancreatic β-cell dysfunction. Depending on the median daily intake of Magnesium, the participants were divided into the low intake group (Magnesium intake $<$ 267 Magnesium/d) ($n = 2,436$) and

Abbreviations: Magnesium, Magnesium; Vit D, Vitamin D; IDF, International Diabetes Federation; T2MD, Type 2 Diabetes; US, United States; FPG, Fasting Plasma Glucose; BMI, Body Mass Index; CDC, Centers for Disease Control; MEC, Mobile Examination Center.

the high intake group (Magnesium intake ≥ 267 Magnesium+/d) ($n = 2,442$) (8).

In the current work, participants aged above 20 who completed interviews and exams at the Mobile Examination Center (MEC) between 2007 and 2014 were recruited. Those who satisfied the following criteria were excluded: (1) Participants lacking information about serum Vit D concentration, fasting plasma glucose (FPG) and insulin measurements. (2) Patients taking drugs that affect glucose or interfere with β-cell functionality. (3) Those with combined hepatobiliary and renal diseases or diseases that affect glucose metabolism. (4) Any history of osteoporosis or other bone metabolism abnormalities. (5) Patients with any recent history of surgery, trauma or serious illness, e.g., a history of stress. (6) Serious diseases, such as malignant tumors (34).

Data Collection

All data were collected by well-trained professionals. The data used in this study included demographics (age, gender, race, education, etc.), anthropometric measurements (height, waist circumference, weight, BMI, etc.), health-related behaviors (smoking and exercise) and biochemical tests (FPG, OGTT, etc.). All information and blood samples were collected in the MEC. The basic information was collated immediately, while the serum samples were scientifically stored and subsequently sent to the NCHS laboratory of The Centers for Disease Control and Prevention (CDC) and designated agencies for analysis (35).

Magnesium Intake Measurement

The Magnesium intake protocol adopted in this study is based on the consensus reached at the expert evaluation program seminars that are regularly held by NHANES. During the large cross-sectional study, the dietary intake was determined by 24 h dietary recall. In this study, data on dietary Magnesium intake in the first 24 h were acquired through MEC's diet recall interviews. In accordance with the median value (267 Magnesium+/day), the daily Magnesium intake was classified as either high or low.

Vit D Measurement

Following the MEC serum sampling, the samples were immediately frozen and stored at−30°C. Then, the samples for Vit D measurement were uniformly transported to the CDC's Environmental Health Laboratory in Atlanta, Georgia. The Vit D level was defined as the sum of Vit D3 and D2. In addition, the ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) was employed as the laboratory analysis method (36).

Diagnosis of Pancreatic β-Cell Dysfunction and HOMA-β

HOMA-β index is considered a good indicator for evaluating the β-cell functionality (37, 38). The computational formula for HOMA-β is: 20×fasting insulin (FINS) level (μU/mL)/FPG level (mmol/L)-3.5(%) (%). In this study, the β-cell dysfunction was identified by whether the HOMA-β index was lower than 75% of participants. Through the calculation, this value was found to be 61.76. Accordingly, the pancreatic β-cells were regarded as dysfunctional when HOMA-β < 61.76, and intact when HOMA-β ≥ 61.76.

Definition of Some Other Variables

Diabetes

By multiplying FPG by 0.056 (rounded to three decimal places), the unit was converted from Magnesium/dl to mmol/l (35). The diagnostic criteria for diabetes included: FPG 7.0 mmol/l, OGTT 11.1 mmol/l, doctor diagnoses, self-reports or diabetes medication intakes.

Races

Mexican Americans, other Hispanics, non-Hispanic whites, non-Hispanic blacks, and other races.

Educational Levels

Middle school, senior high school, university or above.

Smoking

Currently smoking, quit smoking, and never smoked. Participants who smoked ≥100 cigarettes in total in the past and reported that they had smoked for several days or every day during the interviews were considered current smokers. Participants who have smoked <100 cigarettes in the past but do not currently smoke were considered smoking quitters. Participants who smoked <100 cigarettes in the past were considered non-smokers.

Sports Activities

Walking, moderate- and high-intensity activities.

BMI

Calculated based on height and weight. Heights were measured by research staff with an electronic stadiometer (Seca Ltd., Medical Scales and Measurement Systems, Birmingham, UK) to the nearest ml. Weights were measured by research staff on a digital scale (Mettler-Toledo, LLC, Columbus, OH, USA). After the completion of the measurements, pounds were converted into kg. The formula for BMI is: BMI = weight (kg)/height (M2) (39). The dietary data came from diet recall interviews in the first 24 h, including total dietary energy, Vit D, calcium, magnesium, protein and fiber.

Statistical Methods

Every year, NHANES selects 5,000 people from its sampling frames over 15 different locations in all the US counties. Thus, its data is representative of a broad American population. To prevent the deviation and inaccuracy of estimation results caused by oversampling of minority groups, we adopted a weight recommended by NHANES, indicating that all our research analyses below are based on weighted model.

All data were analyzed using R version 4.1.2. Continuous variables were represented through detailed sample descriptions, with an average confidence interval of 95%. Categorical variables were represented by counts and weighted percentages. Skew distributions were based on the medians and Q1–Q3, while normal distributions were described by the mid-values and standard deviations. Inter-group comparisons of continuous

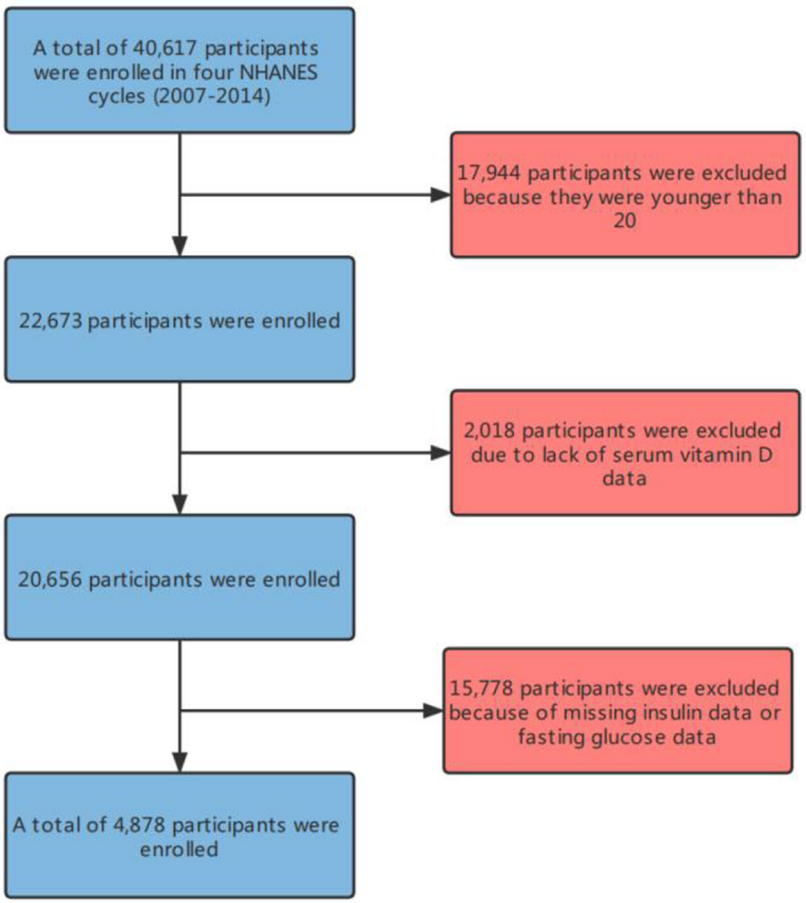


FIGURE 1 | Flowchart of participant selection.

variables were made by normality-based Student *t* test or Mann-Whitney U test, while intra-group comparisons were performed by Fisher's exact probability method. Covariate selection was based on potential confounders that might be associated with the functionalities of Vit D and pancreatic β-cells. Based on previous literature, international standards and relevant clinical experiences, we chose gender, age, race, smoking, BMI, obesity, dietary intake, physical activity and educational level as covariates after comprehensive consideration. The purpose of filling in missing covariates by multiple imputation is to maximize statistical power and minimize bias. Besides, sensitivity analysis was performed to observe whether the generated complete data differed significantly from the original data. The results demonstrate that the multiple imputed data differ statistically insignificantly from the original data ($P > 0.05$). Thus, according to the Rubin criterion, all the results of our multivariate analysis are based on the multiple imputed datasets.

P-values < 0.05 (two-sid Three multivariate linear regression models were built to analyze the association between Vit D and HOMA-β in samples at different Magnesium intakes. Meanwhile, smooth fitting curves were constructed.) were considered statistically significant. In addition, we also developed three multivariate logistic regression models to analyze the association of Vit D with pancreatic β-cell dysfunction under different Magnesium intakes. To ensure the analytical robustness, sensitivity analysis was carried out. Vit D was converted into a categorical variable, and the *P*-value for the trend was calculated. The purpose was to observe whether there was a possibility of non-linearity between Vit D and β-cell functionality when the Vit D level was regarded as a categorical variable.

RESULTS

Description of Basic Demographic Information

In this study, there were 4,878 participants (**Figure 1**) in the four cycles of NHANES (2007–2014). **Table 1** details the basic information of the enrolled participants. Groups were divided depending on the Magnesium intake, namely, the low intake group (<267 Magnesium+/d) and the high intake group (≥267 Magnesium+/d) (**Figure 2**).

For all participants, their mean age was 49.2 ± 17.7 years. The low intake group exhibited a mean age of 50.3

TABLE 1 | Basic information description of participants.

Variables	Total (n = 4,878)	Dietary Magnesium intake (mg/d)		*P*-value
		<267 mg/d (n = 2,436)	≥267 mg/d (n = 2,442)	
Age, mean ± SD	49.2 ± 17.7	50.3 ± 18.4	48.0 ± 16.9	<0.001
BMI, mean ± SD	28.9 ± 6.8	29.2 ± 7.0	28.6 ± 6.6	0.004
FPG, mean ± SD	6.0 ± 1.9	6.0 ± 1.9	5.9 ± 1.9	0.406
OGTT, mean ± SD	7.7 ± 4.3	7.9 ± 4.3	7.4 ± 4.2	<0.001
HOMA-β	97.4 (61.8, 150.4)	100.6 (63.2, 153.4)	94.2 (59.8, 147.4)	0.016
Serum-Vd, median (IQR)	61.4 (44.2, 79.3)	58.6 (41.1, 77.0)	63.8 (48.0, 81.5)	<0.001
Gender, *n* (%)				<0.001
Male	2,353 (48.2)	947 (38.9)	1,406 (57.6)	
Female	2,525 (51.8)	1,489 (61.1)	1,036 (42.4)	
Race, *n* (%)				<0.001
Mexican American	716 (14.7)	294 (12.1)	422 (17.3)	
Other Hispanic	477 (9.8)	243 (10)	234 (9.6)	
Non-Hispanic white	2,307 (47.3)	1,139 (46.8)	1,168 (47.8)	
Non-Hispanic black	913 (18.7)	544 (22.3)	369 (15.1)	
Other races	465 (9.5)	216 (8.9)	249 (10.2)	
Obesity, *n* (%)				0.001
No	3,134 (64.2)	1,510 (62)	1,624 (66.5)	
Yes	1,744 (35.8)	926 (38)	818 (33.5)	
Education, *n* (%)				<0.001
Did not graduate from high school	1,228 (25.2)	688 (28.2)	540 (22.1)	
Graduated from high school	1,068 (21.9)	600 (24.6)	468 (19.2)	
College education or above	2,582 (52.9)	1,148 (47.1)	1,434 (58.7)	
Activity, *n* (%)				0.772
Vigorous work activity	899 (18.4)	464 (19)	435 (17.8)	
Moderate work activity	1,032 (21.2)	505 (20.7)	527 (21.6)	
Walk or bicycle	682 (14.0)	343 (14.1)	339 (13.9)	
Vigorous recreational activities	330 (6.8)	168 (6.9)	162 (6.6)	
Moderate recreational activities	1,935 (39.7)	956 (39.2)	979 (40.1)	
Diabetes, *n* (%)				<0.001
No	3,905 (80.1)	1,892 (77.7)	2,013 (82.4)	
Yes	973 (19.9)	544 (22.3)	429 (17.6)	
Season of examination, *n* (%)				0.327
Winter	2,304 (47.2)	1,133 (46.5)	1,171 (48)	
Summer	2,574 (52.8)	1,303 (53.5)	1,271 (52)	
Dietary factors				
Energy (kcal)	2106.1 ± 10.3	1582.5 ± 6.7	2628.4 ± 15.1	<0.001
Protein (gm)	81.9 ± 42.9	59.1 ± 24.8	104.6 ± 45.0	<0.001
Fiber (gm)	16.7 ± 10.3	10.7 ± 5.1	22.7 ± 10.7	<0.001
Calcium (mg)	920.7 ± 603.4	639.1 ± 342.2	1201.6 ± 672.7	<0.001
Beta cell function is impaired				<0.001
No	3,658 (75.0)	1,856 (76.2)	1,802 (73.8)	
Yes	1,220 (25.0)	580 (23.8)	640 (26.2)	

BMI, Body Mass Index; FPG, Fasting plasma glucose; OGGT, Oral Glucose Tolerance Test.

± 18.4 years, while the high intake group showed a mean age of 48.0 ± 16.9 years, presenting a significant inter-group difference ($P < 0.001$). Among obese population, the proportion of participants increased in the low intake group (35.8%→38%), while decreased in the high intake group (35.8%→33.5%). The BMI, FPG and OGTT levels were all higher in the low intake group than those in the high intake group. Contrastively, the Vit D level in the high intake group was significantly higher than that in the low intake group (**Table 1**).

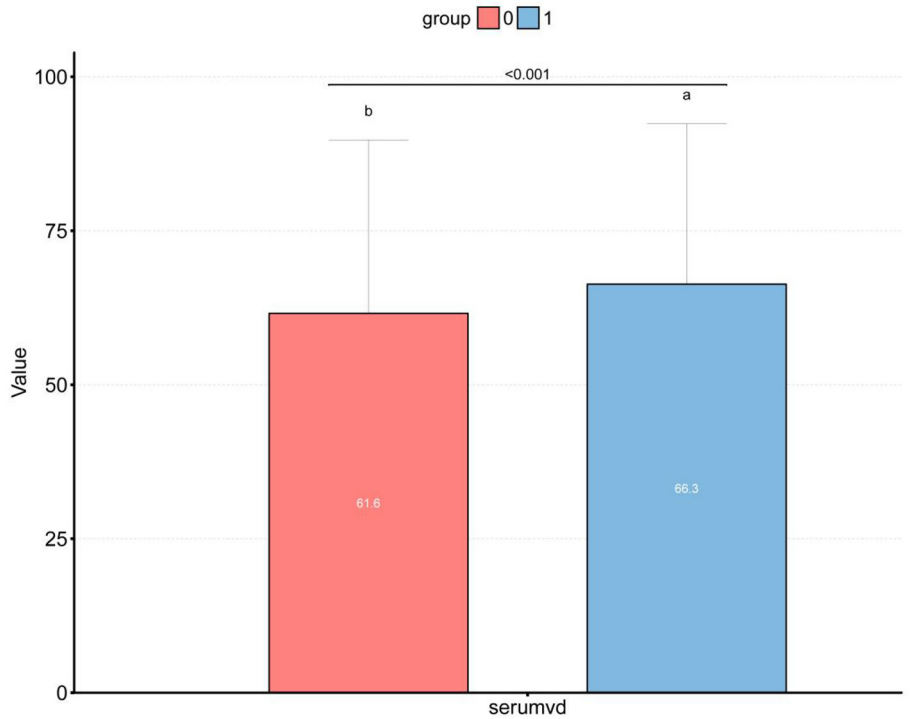


FIGURE 2 | Box plot of the differences between different vitamin D levels in the high and low dietary Magnesium intake groups. **(a)** 1, high dietary Magnesium intake group; **(b)** 0, low dietary Magnesium intake group; $p < 0.001$.

Distribution of Serum Vitamin D in Pancreatic β-Cell Dysfunction Grouped by Magnesium Intake

Figure 2 displays the difference in Vit D level between high and low Magnesium intake groups (61.6 vs. 66.3 nmol/L, $P < 0.001$). Meanwhile, we observed that the Vit D level differed among the β-cell dysfunction-positive and -negative groups ($P < 0.001$), as shown in **Figure 3**. The dysfunction-positive group exhibited significantly lower Vit D levels than the negative group (high Magnesium intake group: 59.7 vs. 68.4 nmol/L, $P < 0001$; low Magnesium intake group: 55.1 vs. 62.0 nmol/L, $P < 0.001$).

Univariate Regression Analysis

Univariate logistic regression was adopted for analyzing which factors might be correlated with the risk of pancreatic β-cell dysfunction. As illustrated in **Table 2**, age, gender, race, obesity, educational level, serum Vit D level and Magnesium intake were all correlated with the risk of pancreatic β-cell dysfunction. Considering the potential influences of the above variables on main results, we controlled these factors as covariates in our main analysis.

Multivariate Regression Analysis

In the present study, three linear regression models were built to analyze the independent correlation between Vit D level and HOMA-β index, and to clarify whether such correlation was influenced by different levels of Magnesium intake. **Table 3** details the effect sizes β and 95% CIs. The model-based effect sizes indicate that for every additional unit of Vit D, the HOMA-β index increases correspondingly. For example, the total effect size in the unadjusted model is 1.38. Every additional unit of Vit D implies a corresponding increase in the HOMA-β index by 0.38. In the high intake group, the effect size β and 95% CI were 0.40 (1.23–1.57), while in the low intake group, the odds ratio (OR) and 95% CI were 0.32 (1.17–1.47). In terms of Model 2, only the sociodemographic data was adjusted, which yielded total effect size β and 95% CI of 0.42 (0.20–0.63). In the high intake group, the OR and 95% CI were 0.44 (0.20–0.63), while in the low intake group, the effect size β and 95% CI were 0.38 (0.20–0.56). For the fully adjusted Model 3, the total effect size β and 95% CI were 0.65 (0.40–0.90). In the high intake group, the OR and 95% CI were 0.64 (0.39–0.89), while in the low intake group, the effect size β and 95% CI were 0.67 (0.40–0.94). Based on the above results, Vit D level and HOMA-β index are independently correlated, and such correlation is affected by the level of Magnesium intake.

In addition, we also built three logistic regression models to analyze the independent correlation between Vit D level and pancreatic β-cell dysfunction, and to clarify whether such correlation was affected by the level of Magnesium intake. **Table 4** details the effect size ORs and 95% CIs. The model-based effect sizes demonstrate that after adjusting the covariates based on the complete model (Model 3), the Vit D level was independently correlated with the pancreatic β-cell dysfunction and was affected

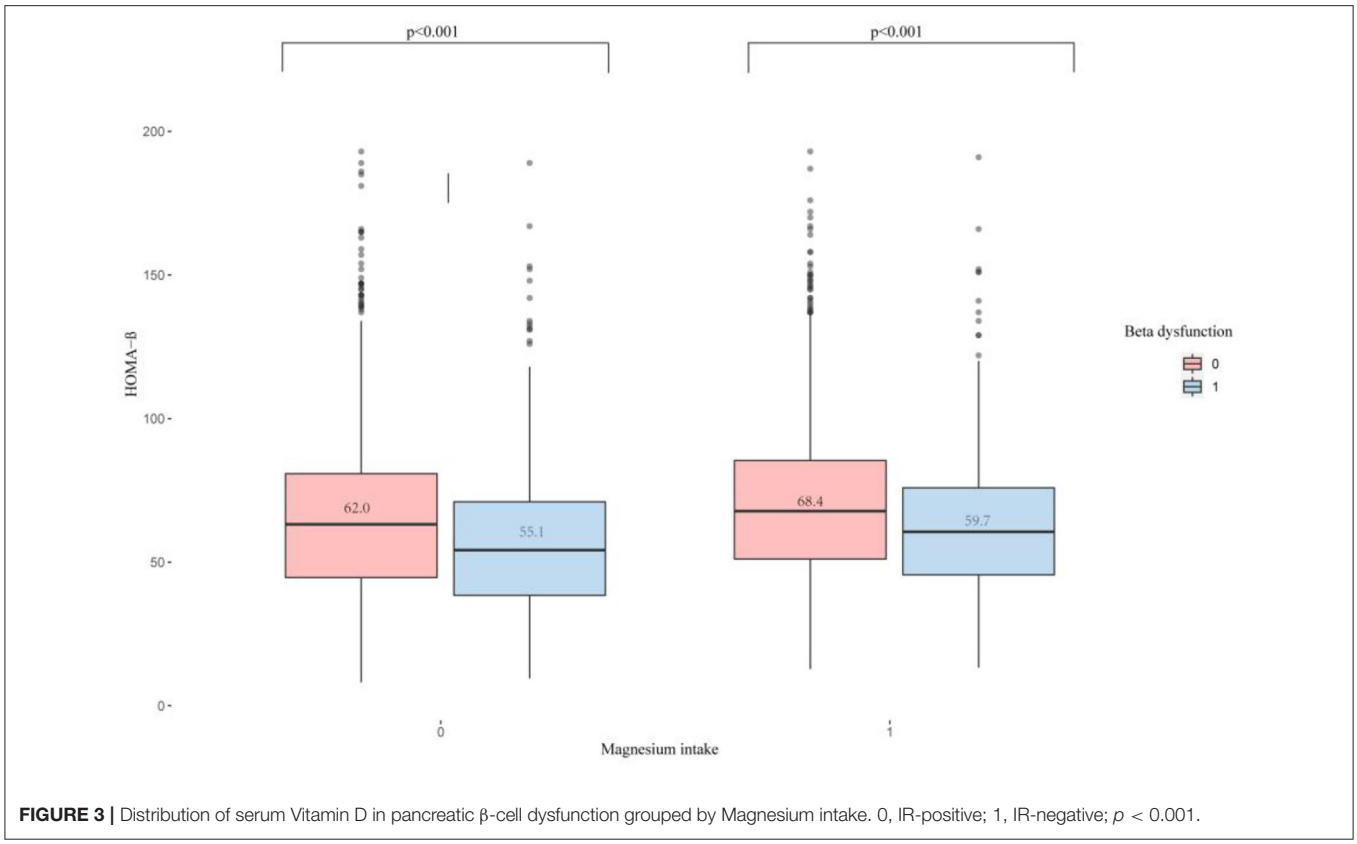


FIGURE 3 | Distribution of serum Vitamin D in pancreatic β-cell dysfunction grouped by Magnesium intake. 0, IR-positive; 1, IR-negative; $p < 0.001$.

by the level of Magnesium intake. The overall effect size OR and 95% CI were 0.95 (0.92–0.98). In the high intake group, the OR and 95% CI were−0.05 (-0.06–0.03), while in the low intake group, the OR and 95% CI were−0.04 (-0.06–0.02).

Curve Fitting Analysis

In this study, we analyzed whether there was a linear correlation between Vit D and HOMA-β index at different levels of Magnesium intake. 1A and 1B display the association between Vit D and HOMA-β index based on multiple linear regression using unadjusted latent variables. 2A and 2B present the association between Vit D and HOMA-β index based on multiple linear regression after adjustment with Model 2. Meanwhile, 3A and 3B display the association between Vit D and HOMA-β index based on multiple linear regression after adjustment with complete model. To sum up, at different levels of Magnesium intake, all the correlations between Vit D level and HOMA-β index were linear (**Figure 4**).

DISCUSSION

In recent decades, the incidence of diabetes has increased tremendously (40, 41). The onset risk has presented an evident upward trend. Most countries and regions have invested substantially in the diagnosis and treatment of this disease (42). The primary factor leading to diabetes is β-cell impairment. Under the trend of growing incidence of diabetes, there are far more people with impaired pancreatic β-cell functionality (43). Therefore, how to effectively prevent β-cell impairment has also become a focus of our concern.

In this study, we built multiple linear regression models to explore the independent correlation between serum Vit D levels and HOMA-β index, and to clarify whether such correlation is affected by the dietary Magnesium intake. Apart from that, multiple logistic regression models were built to analyze the independent correlation between serum Vit D levels and pancreatic β-cell dysfunction, and to clarify whether such independent correlation is affected by the dietary Magnesium intake. After excluding potential confounding factors, it was found in the multivariate regression analysis that the serum Vit D levels was independently correlated with the HOMA-β index and pancreatic β-cell dysfunction, and that such correlations were affected by the level of Magnesium intake. The smooth fitting curves also showed that at different levels of Magnesium intake, a linear correlation was present between the serum Vit D levels and the HOMA-β index. With the increasing level of Vit D, the incidence of pancreatic β-cell dysfunction decreased.

In 2021, Lu et al. (44) conducted a prospective controlled study, finding that after adjusting laboratory indicators, anthropometric data and other factors, the active Vit D supplementation could improve the HOMA-β index in 6 months among diabetic patients. Mattla et al. (45) carried out an RCT

based on the Finnish population. After adjusting for covariates such as gender, age and BMI, the pancreatic β-cells were more severely dysfunctional among those with lower Vit D levels. This may also suggest the presence of a positive correlation between pancreatic β-cell dysfunction and Vit D (45).

TABLE 2 | Association of covariates and impaired pancreatic β-cell function.

Variable	OR (95%CI)	*P*-value
Age	1.02 (1.02–1.02)	<0.001
Gender, *n* (%)		
Male	1	
Female	0.69 (0.61–0.79)	<0.001
Race/ethnicity, n (%)		
Mexican American	1	
Other Hispanic	1.18 (0.89–1.58)	0.254
Non-Hispanic white	1.65 (1.34–2.04)	<0.001
Non-Hispanic black	1.46 (1.15–1.86)	0.002
Other races	1.94 (1.48–2.56)	<0.001
Obesity, *n* (%)		
No	1	
Yes	6.73 (5.9–7.67)	<0.001
Education level, *n* (%)		
Did not graduate from high school	1	
Graduated from high school	0.98 (0.81–1.18)	0.839
College education or above	0.95 (0.81–1.11)	0.48
Smoking status, *n* (%)		
Current smoker	1	
Former smoker	0.93 (0.77–1.13)	0.468
Never smoker	1.06 (0.9–1.25)	0.468
Physical activity, *n* (%)		
Vigorous work activity	1	
Moderate work activity	1.28 (1.04–1.58)	0.02
Walk or bicycle	1.46 (1.16–1.83)	0.001
Vigorous recreational activities	1.23 (0.92–1.65)	0.161
Moderate recreational activities	1.08 (0.89–1.3)	0.439
Season of examination, *n* (%)		
Winter	1	
Summer	0.91 (0.81–1.02)	0.099
Serum-Vd	0.99 (0.99–0.99)	<0.001
Ma-intake	0.99 (0.99–0.99)	<0.001

Data presented are ORs and 95% CIs.

Research has shown that Magnesium is necessary for proper glucose utilization and insulin signaling. Pancreatic β-cells secrete insulin, and such secretion is associated with the ATP-sensitive K+ channel. However, the lack of Magnesium leads to the dysfunction of this channel, which may impair the insulin secretion (29). Takaya et al. review of previous studies found that Magnesium is vitally important for the phosphorylation of insulin receptors and the tyrosine kinase activity in insulin signaling pathways. Inhibiting the intracellular Magnesium concentration might lead to defective activity of enzymes, which also altered the insulin sensitivity by influencing the binding receptor activity or intracellular signal transduction, ultimately resulting in abnormal glucose metabolism (46). A cross-sectional study by Huang et al. among the American population in 2021 demonstrated that Magnesium is an important cofactor for the double hydroxylation of Vit D, and that the activity of Vit D binding receptors is a Magnesium-dependent process. Accordingly, high Magnesium intake can promote the activation of Vit D and increase the transfer of Vit D to target tissues (8). To sum up, Magnesium supplementation may enhance the activity of Vit D, thereby increasing its protective function on pancreatic β-cells.

However, this study still has several limitations. Firstly, due to the sample restrictions, we did not consider some special populations, such as pregnant women and children. It remains unknown whether the results of this study are applicable to these special populations. Certainly, this limitation will be resolved in the future since we will investigate these populations in future studies. Secondly, given the cross-sectional nature of this study, it is impossible to draw the causal association of Vit D level with HOMA-β and pancreatic β-cell function. To analyze this causal association, a cohort study is required in the future. Finally, our dietary data is derived from self-reported 24-h diet recalls, which inevitably has some recall and self-reporting biases. However, the impact is too small to affect our results, since NHANES suppresses these biases by collecting data with professionals and selecting subjects through a multi-stage stratified probability design. Certainly, this study also has certain advantages over others, such as the large sample size

TABLE 3 | Interactive effect of vitamin D and dietary magnesium intake on HOMA-β.

Variable	Model 1			Model 2			Model 3		
	β (95%CI)	*P*-value	*P* for interaction	β (95%CI)	*P*-value	*P* for interaction	β (95%CI)	*P*-value	*P* for interaction
VD	0.38 (0.20–0.55)	<0.001		0.42 (0.20–0.63)	<0.001		0.65 (0.40–0.90)	<0.001	
Magnesium intake group									
<267 mg/day VD	0.32 (0.07–0.47)	<0.001	<0.001	0.38 (0.20–0.56)	<0.001	<0.001	0.64 (0.39– 0.89)	<0.001	<0.001
≥267 mg/day VD	0.40 (0.23–0.57)	<0.001		0.44 (0.20–0.63)	<0.001		0.67 (0.40–0.94)	<0.001	

Model 1, Non-adjusted; Model 2, adjusted age, gender, race; Model 3, Adjusted age, gender, race, obesity, education level, physical activity, smoking status, the season of examination, and dietary calcium intake.

TABLE 4 | Interactive effect of vitamin D and dietary magnesium intake on Beta cell function is impaired.

Variable	Model 1			Model 2			Model 3		
	OR (95%CI)	*P*-value	*P* for interaction	OR (95%CI)	*P*-value	*P* for interaction	OR (95%CI)	*P*-value	*P* for interaction
VD	0.99 (0.99–0.99)	<0.001		0.99 (0.99–0.99)	<0.001		0.95(0.92–0.98)	<0.001	
Magnesium intake group									
<267 mg/day VD	0.99 (0.99–0.99)	<0.001	<0.001	0.99 (0.99–0.99)	<0.001	<0.001	0.96(0.93–0.99)	<0.001	<0.001
≥267 mg/day VD	0.98 (0.97–0.99)	<0.001		0.96 (0.93–0.99)	<0.001		0.94 (0.92–0.96)	<0.001	

Model 1, Non-adjusted; Model 2, Adjusted age, gender and race; Model 3, Adjusted age, gender, race, obesity, education level, physical activity, smoking status, the season of examination, and dietary calcium intake.

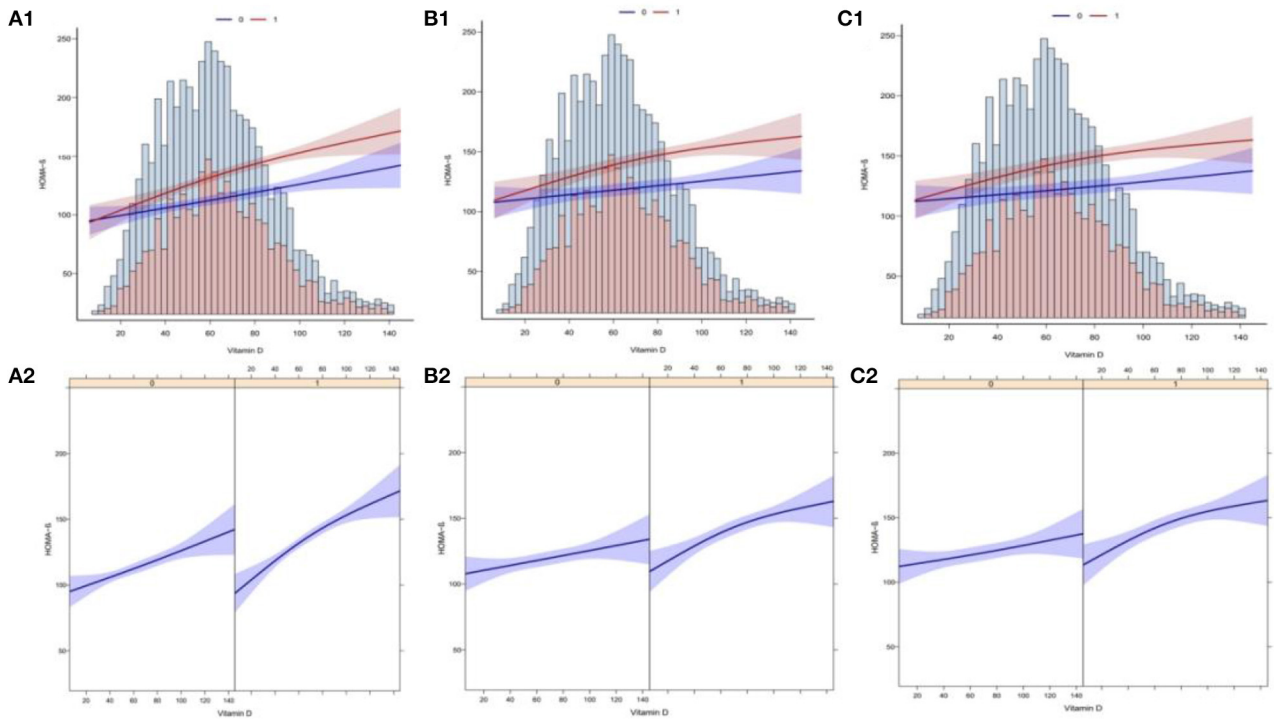


FIGURE 4 | Curve fitting of Vitamin D levels and the risk of HOMA-β index. **(A1)** The association between VD and HOMA-β with no adjustment for covariates (Model 1). **(A2)** The association between VD and HOMA-β with no adjustment for covariates (Model 1). **(B1)** The association between VD and HOMA-β with the adjustment of age, gender and race (Model 2). **(B2)** The association between VD and HOMA-β with the adjustment of age, gender and race (Model 2). **(C1)** The association between VD and HOMA-β with the adjustment other covariates (Model 3). **(C2)** The association between VD and HOMA-β with the adjustment other covariates (Model 3).

of participants. Moreover, we adopted an advanced statistical method (multiple imputation) for processing the missing data, in order to maximize the statistical power of results and minimize the errors.

CONCLUSION

After adjusting for potential confounding factors, this study finds that among the US adult population, Vit D level is independently associated with the HOMA-β index and pancreatic β-cell dysfunction, and that the Magnesium intake enhances such association. Meanwhile, we also know that Vit D influences normal glucose metabolism. Besides, safe sun exposure also provides a good way for increasing the concentration of circulating Vit D and benefiting the health. As a result, among the numerous ways to enhance the concentration of circulating Vit D, increasing the intake of dietary magnesium or safe sun exposure would prove an effective way. This finding offers a new insight for the clinical research. However, we cannot determine their causality given

the cross-sectional nature of this study. Hence, more RCTs or cohort studies are required in the future to confirmed the obtained finding.

DATA AVAILABILITY STATEMENT

Data can be obtained from the NHANES database (https://www.cdc.gov/nchs/nhanes/).

AUTHOR CONTRIBUTIONS

RG and YL conceived the idea and wrote the manuscript. GL and RG collected, read the literature, and revised the article. LY and YL read through and corrected the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by General Project of Natural Science Foundation of Qinghai Province (2020-ZJ-930), Effects of high altitude environment and season on vitamin D, bone metabolism factors and muscle volume in adults.

ACKNOWLEDGMENTS

Thanks to YL for his contribution to this research, at the same time, thanks to GL for her contribution in writing. I am also grateful to my supervisor LY for his guidance on this article.

REFERENCES

1. Memon B, Abdelalim EM. Stem cell therapy for diabetes: beta cells versus pancreatic progenitors. *Cells*. (2020) 9:283. doi: 10.3390/cells9020283
2. Han W, Eizirik DL. The pancreatic beta cells: still much to be learned. *Semin Cell Dev Biol.* (2020) 103:1–2. doi: 10.1016/j.semcdb.2020.04.020
3. Rutter GA, Pullen TJ, Hodson DJ, Martinez-Sanchez A. Pancreatic β-cell identity, glucose sensing and the control of insulin secretion. *Biochem J.* (2015) 466:203–18. doi: 10.1042/BJ20141384
4. Campbell JE, Newgard CB. Mechanisms controlling pancreatic islet cell function in insulin secretion. *Nat Rev Mol Cell Biol.* (2021) 22:142–58. doi: 10.1038/s41580-020-00317-7
5. Rose KJ, Petrut C, L'Heveder R, de Sabata S. IDF Europe's position on mobile applications in diabetes. *Diabetes Res Clin Pract.* (2019) 149:39–46. doi: 10.1016/j.diabres.2017.08.020
6. Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW, et al. IDF diabetes atlas: global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Res Clin Pract*. (2018) 138:271–81. doi: 10.1016/j.diabres.2018.02.023
7. Sizar O, Khare S, Goyal A, Bansal P, Givler A. *Vitamin D Deficiency*, in *StatPearls* 2021. Treasure Island, FL: StatPearls Publishing Copyright © 2021, StatPearls Publishing LLC.
8. Huang W, Ma X, Liang H, Li H, Chen J, Fang L, et al. Dietary magnesium intake affects the association between serum vitamin D and type 2 diabetes: a cross-sectional study. *Front Nutr.* (2021) 8:763076. doi: 10.3389/fnut.2021.763076
9. Carlberg C, Velleuer E. Vitamin D and the risk for cancer: a molecular analysis. *Biochem Pharmacol.* (2021) 196:114735. doi: 10.1016/j.bcp.2021.114735
10. Grübler MR, März W, Pilz S, Grammer TB, Trummer C, Müllner C, et al. Vitamin-D concentrations, cardiovascular risk and events - a review of epidemiological evidence. *Rev Endocr Metab Disord.* (2017) 18:259–72. doi: 10.1007/s11154-017-9417-0
11. Muñoz-Torres M, Aguado P, Daudén E, Carrascosa JM, Rivera R. Osteoporosis and psoriasis. *Actas Dermosifiliogr.* (2019) 110:642–52. doi: 10.1016/j.ad.2019.02.005
12. Danescu LG, Levy S, Levy J. Vitamin D and diabetes mellitus. *Endocrine.* (2009) 35:11–7. doi: 10.1007/s12020-008-9115-5
13. Wei Z, Yoshihara E, He N, Hah N, Fan W, Pinto AFM, et al. Vitamin D switches BAF complexes to protect β cells. *Cell.* (2018) 173:1135–49. doi: 10.1016/j.cell.2018.04.013
14. Szymczak-Pajor I, Sliwińska A. Analysis of association between vitamin D deficiency and insulin resistance. *Nutrients.* (2019) 11:794. doi: 10.3390/nu11040794
15. Patil MB, Ragav ED. A clinical, biochemical profile of Type-2 diabetes in women with special reference to vitamin-D Status in obese and non-obese. *J Assoc Physicians India.* (2018) 66:21–4.
16. Infante M, Ricordi C, Sanchez J, Clare-Salzler MJ, Padilla N, Fuenmayor V, et al. Influence of vitamin D on islet autoimmunity and beta-cell function in type 1 diabetes. *Nutrients*. (2019) 11:2185. doi: 10.3390/nu11092185
17. Grammatiki M, Rapti E, Karras S, Ajjan RA, Kotsa K. Vitamin D and diabetes mellitus: causal or casual association? *Rev Endocr Metab Disord.* (2017) 18:227–41. doi: 10.1007/s11154-016-9403-y
18. Manson JE, Cook NR, Lee IM, Christen W, Bassuk SS, Mora S, et al. Vitamin D supplements and prevention of cancer and cardiovascular disease. *N Engl J Med.* (2019) 380:33–44. doi: 10.1056/NEJMoa1809944
19. Zittermann A, Pilz S. Vitamin D and cardiovascular disease: an update. *Anticancer Res.* (2019) 39:4627–35. doi: 10.21873/anticanres.13643
20. Skaaby T, Thuesen BH, Linneberg A. Vitamin D, cardiovascular disease and risk factors. *Adv Exp Med Biol.* (2017) 996:221–30. doi: 10.1007/978-3-319-56017-5_18
21. Maddaloni E, Cavallari I, Napoli N, Conte C. Vitamin D and diabetes mellitus. *Front Horm Res.* (2018) 50:161–76. doi: 10.1159/000486083
22. Nielsen NO, Bjerregaard P, Rønn PF, Friis H, Andersen S, Melbye M, et al. Associations between vitamin D status and type 2 diabetes measures among inuit in greenland may be affected by other factors. *PLoS ONE.* (2016) 11:e0152763. doi: 10.1371/journal.pone.0152763
23. Uwitonze AM, Razzaque MS. Role of magnesium in vitamin D activation and function. *J Am Osteopath Assoc.* (2018) 118:181–9. doi: 10.7556/jaoa.2018.037
24. Zittermann A. Magnesium deficit? overlooked cause of low vitamin D status? *BMC Med.* (2013) 11:229. doi: 10.1186/1741-7015-11-229
25. Hardwick LL, Jones MR, Brautbar N, Lee DB. Magnesium absorption: mechanisms and the influence of vitamin D, calcium and phosphate. *J Nutr.* (1991) 121:13–23. doi: 10.1093/jn/121.1.13
26. Gröber U, Schmidt J, Kisters K. Magnesium in prevention and therapy. *Nutrients.* (2015) 7:8199–226. doi: 10.3390/nu7095388
27. Tan CW, Ho LP, Kalimuddin S, Cherng BPZ, Teh YE, Thien SY, et al. Cohort study to evaluate the effect of vitamin D, magnesium, and vitamin B(12) in combination on progression to severe outcomes in older patients with coronavirus (COVID-19). *Nutrition*. (2020) 79–80:111017. doi: 10.1016/j.nut.2020.111017
28. Capozzi A, Scambia G, Lello S. Calcium, vitamin D, vitamin K2, and magnesium supplementation and skeletal health. *Maturitas.* (2020) 140:55–63. doi: 10.1016/j.maturitas.2020.05.020
29. Kostov K. Effects of magnesium deficiency on mechanisms of insulin resistance in Type 2 diabetes: focusing on the processes of insulin secretion and signaling. *Int J Mol Sci.* (2019) 20:1351. doi: 10.3390/ijms20061351
30. Toi PL, Anothaisintawee T, Chaikledkaew U, Briones JR, Reutrakul S, Thakkinstian A. Preventive role of diet interventions and dietary factors in Type 2 diabetes mellitus: an umbrella review. *Nutrients.* (2020) 12:2722. doi: 10.3390/nu12092722
31. Shah IU, Sameen A, Manzoor MF, Ahmed Z, Gao J, Farooq U, et al. Association of dietary calcium, magnesium, and vitamin D with type 2 diabetes among US adults: national health and nutrition examination survey 2007-2014-A

cross-sectional study. *Food Sci Nutr.* (2021) 9:1480–90. doi: 10.1002/fsn3.2118
32. Deng X, Song Y, Manson JE, Signorello LB, Zhang SM, Shrubsole MJ, et al. Magnesium, vitamin D status and mortality: results from US national health and nutrition examination survey (NHANES) 2001 to 2006 and NHANES III. *BMC Med.* (2013) 11:187. doi: 10.1186/1741-7015-11-187
33. Ahluwalia N, Dwyer J, Terry A, Moshfegh A, Johnson C. Update on NHANES dietary data: focus on collection, release, analytical considerations, and uses to inform public policy. *Adv Nutr.* (2016) 7:121–34. doi: 10.3945/an.115.009258
34. Gong R, Luo G, Wang M, Ma L, Sun S, Wei X. Associations between TG/HDL ratio and insulin resistance in the US population: a cross-sectional study. *Endocr Connect.* (2021) 10:1502–12. doi: 10.1530/EC-21-0414
35. Gong R, Xu Z, Wei X. The association between vitamin D3 and diabetes in both hyperuricemia and non-hyperuricemia populations. *Endocrine.* (2021) 74:90–9. doi: 10.1007/s12020-021-02778-y
36. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Weaver, evaluation, treatment, and prevention of vitamin D deficiency: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* (2011) 96:1911–30. doi: 10.1210/jc.2011-0385
37. Zhou M, Pan Y, Jing J, Wang Y, Zhao X, Liu L, et al. Association between β-cell function estimated by HOMA-β and prognosis of non-diabetic patients with ischaemic stroke. *Eur J Neurol.* (2018) 25:549–55. doi: 10.1111/ene.13546
38. Bermúdez-Pirela VJ, Cano C, Medina MT, Souki A, Lemus MA, Leal EM, et al. Metformin plus low-dose glimeperide significantly improves Homeostasis Model Assessment for insulin resistance (HOMA(IR)) and beta-cell function (HOMA(beta-cell)) without hyperinsulinemia in patients with type 2 diabetes mellitus. *Am J Ther.* (2007) 14:194–202. doi: 10.1097/01.pap.0000249909.54047.0e
39. Youk TM, Kang MJ, Song SO, Park EC. Effects of BMI and LDL-cholesterol change pattern on cardiovascular disease in normal adults and diabetics. *BMJ Open Diabetes Res Care*. (2020) 8:e001340. doi: 10.1136/bmjdrc-2020-001340
40. Wu Y, Ding Y, Tanaka Y, Zhang W. Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. *Int J Med Sci.* (2014) 11:1185–200. doi: 10.7150/ijms.10001
41. Kolb H, Martin S. Environmental/lifestyle factors in the pathogenesis and prevention of type 2 diabetes. *BMC Med.* (2017) 15:131. doi: 10.1186/s12916-017-0901-x
42. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat Rev Endocrinol.* (2018) 14:88–98. doi: 10.1038/nrendo.2017.151
43. Wolpin BM, Bao Y, Qian ZR, Wu C, Kraft P, Ogino S, et al. Hyperglycemia, insulin resistance, impaired pancreatic β-cell function, and risk of pancreatic cancer. *J Natl Cancer Inst.* (2013) 105:1027–35. doi: 10.1093/jnci/djt123
44. Lu Y, Wang Y, Sun Y, Li Y, Wang J, Zhao Y, et al. Effects of active vitamin D on insulin resistance and islet β-cell function in non-diabetic chronic kidney disease patients: a randomized controlled study. *Int Urol Nephrol.* (2021). doi: 10.1007/s11255-021-02968-7
45. Mattila C, Knekt P, Männistö S, Rissanen H, Laaksonen MA, Montonen J, et al. Serum 25-hydroxyvitamin D concentration and subsequent risk of type 2 diabetes. *Diabetes Care.* (2007) 30:2569–70. doi: 10.2337/dc07-0292
46. Takaya J, Higashino H, Kobayashi Y. Intracellular magnesium and insulin resistance. *Magnes Res.* (2004) 17:126–36.

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.

frontiers in Endocrinology

OPINION
published: 29 March 2022
doi: 10.3389/fendo.2022.843152

Can Maintaining Optimal Magnesium Balance Reduce the Disease Severity of COVID-19 Patients?

Mark Eskander and Mohammed S. Razzaque *

Department of Pathology, Lake Erie College of Osteopathic Medicine, Erie, PA, United States

Keywords: magnesium, vitamin D, health, disease, hypomagnesemia

The coronavirus disease (COVID-19) caught the world by surprise, claiming millions of lives due to its deadly effects. Ongoing research studies evaluate the measures that can reduce the severity of symptoms in patients infected by the severe acute respiratory syndrome coronavirus 2 (SARS–CoV–2). Magnesium is an essential nutrient that has many studied benefits in humans. This brief commentary aims to describe the potential benefits of magnesium on COVID-19 patients and the reported effects of low versus high magnesium levels in SARS–CoV–2 infected individuals. Also, the potential benefits of vitamin D and how magnesium acts as a cofactor to activate vitamin D functions are elaborated. The results of the existing studies point towards evidence that magnesium may have significant benefits in reducing the severity of COVID-19 symptoms. There is also evidence that magnesium-dependent vitamin D activities may have antiviral effects, thus potentially being able to reduce rates of COVID-19 infection, which is a hypothesis that should be further tested.

The COVID-19 primarily causes respiratory distress but has a broad range of other clinical manifestations and affects various organs and systems in the body. COVID-19 is especially lethal to elderly populations at higher risk (1, 2). As of September 3rd, 2021, the current worldwide death rate of individuals infected by COVID-19 is 4,539,723 (World Health Organization). Many public health measures have already been taken to try to prevent the spread of COVID-19, such as face mask mandates, vaccine inoculation, quarantine, social distancing, limited capacities at public venues, and a shift to online work. However, COVID-19 is still proving to be a health concern as cases of infection continue to rise. The continued infection of individuals by COVID-19 may be due to the high transmissibility of the virus (3–6). It is also proposed that some of the new variants of the virus are even more rapidly transmissible and lethal. Thus, certain preventative measures are essential to find to reduce the severity of symptoms and improve patient outcomes in individuals that have been infected, since the prevention of infection is not always possible.

Basic nutrients that optimize physiologic functions in the body, such as magnesium, may be used as a prophylactic measure that can improve patient outcomes in individuals infected by SARS–CoV–2, and potentially even reduce the intensity of the infection by the virus by potentiating vitamin D functions. In the U.S., more than 50% of people are magnesium deficient (7, 8), and magnesium deficiency is also prevalent in many other countries. The low level of magnesium in the general population may make individuals more vulnerable to viral insults. The high prevalence of magnesium deficiency makes it important to determine how optimal and suboptimal magnesium levels affect COVID-19 patient outcomes, which this paper aims to elaborate on.

OPEN ACCESS

Edited by:
Jeff M. P. Holly,
University of Bristol, United Kingdom

Reviewed by:
Deeptha Sukumar,
Drexel University, United States

***Correspondence:**
Mohammed S. Razzaque
mrazzaque@lecom.edu;
msr.nagasaki@gmail.com

Specialty section:
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *24 December 2021*
Accepted: *02 March 2022*
Published: *29 March 2022*

Citation:
Eskander M and Razzaque MS (2022) Can Maintaining Optimal Magnesium Balance Reduce the Disease Severity of COVID-19 Patients? Front. Endocrinol. 13:843152. doi: 10.3389/fendo.2022.843152

ROLE OF MAGNESIUM IN HEALTH AND DISEASE

Magnesium is an essential nutrient required for many different physiologic functions in the body (9, 10). Magnesium homeostasis is mostly maintained by the cross-talks among intestine, bone, kidneys (**Figure 1**). The recommended daily amount of magnesium intake for 14-18 years is 360 mg (females)/410 mg (males), for 19-30 years is 310 mg (females)/ 400 mg (males), for people 31 years of age and older is 320 mg (females)/420 mg (males). Magnesium is a cofactor for over 600 enzymes in the body with diverse functions that play roles in many different systems (9, 11). One such system is the inflammatory system. Magnesium has been shown to have potent anti-inflammatory effects: low magnesium (0.14 ± 0.02 mmol) levels in rats have been associated with augmented inflammation (12), and higher magnesium levels have been associated with a reduction of C-reactive protein (CRP) levels (13, 14), which is one of the widely used biomarkers to measure inflammation, suggesting that magnesium has the potential to reduce inflammation. This has proven to be true with asthma patients (a disease with lung inflammation); the use of intravenous magnesium sulfate ($MgSO_4$) and high-dose continuous $MgSO_4$ infusion were both shown to reduce the odds of hospitalization in pediatric asthma patients (15, 16). Additionally, the use of nebulized $MgSO_4$ in pediatric asthma patients (17) resulted in a significant reduction in the Yung Asthma Severity Score in pediatric asthma patients compared to pediatric asthma patients treated with a placebo (18). Separate studies showed improved outcomes of asthma patients of all ages when treated with nebulized $MgSO_4$ (15–17). Thus, a wide record of evidence shows the efficacy of different forms of magnesium in reducing the intensity of asthma symptoms in patients of all age groups. This may implicate the clinical benefits of using magnesium in treating lung inflammation of patients with COVID-19. Different forms of magnesium have different levels of bioavailability. Studies show that organic magnesium, such as magnesium citrate, has higher bioavailability than inorganic magnesium, such as magnesium oxide (7, 19).

Another essential role that magnesium plays in the body is its effect on activating vitamin D (11, 20, 21). The enzymes are required to convert the inactive form of vitamin D to the active form of vitamin D where magnesium acts as a cofactor (7, 11) (**Figure 2**). Studies have found the association of vitamin D in improving immune functions (22, 23), cell proliferation and organ regeneration (24), and reducing cardiovascular disease burden (25), as well as the widely accepted role of vitamin D in vascular, oral and bone health along with calcium metabolism (26–30). These health benefits of vitamin D will not be achieved even with adequate vitamin D levels if magnesium is deficient, thus emphasizing the essential role of maintaining optimal magnesium levels to attain desirable benefits of vitamin D. Magnesium deficiency is associated with cardiovascular diseases (31), hypertension (32), osteoporosis (33, 34), and diabetes (35).

VITAMIN D-DEPENDENT EFFECTS OF MAGNESIUM ON COVID-19 OUTCOMES

In searching for the crucial need to reduce the deadly effects of COVID-19, maintaining optimal magnesium levels is an option that should be considered, as it has numerous protective roles in the body that may mitigate the deadly effects of COVID-19 (36). One such role that has been discussed is the activation of vitamin D *via* magnesium, and vitamin D's essential role in improving immune functions (22, 23). Research on vitamin D and the immune system shows that there is a correlation between low vitamin D levels and autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, diabetes mellitus, and

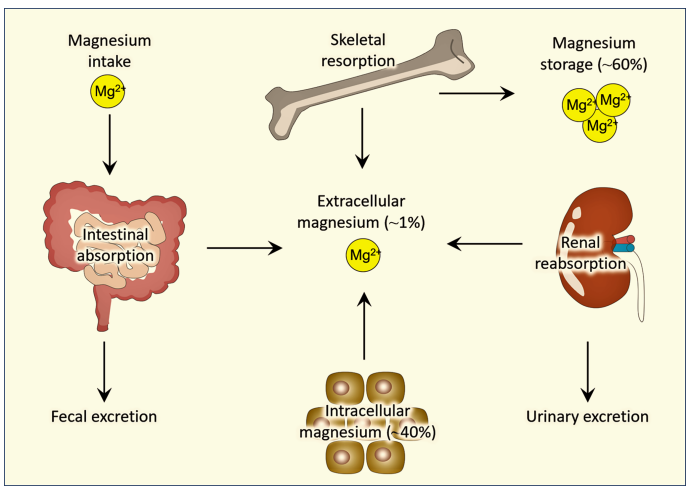


FIGURE 1 | Simplified diagram showing regulation of magnesium homeostasis.

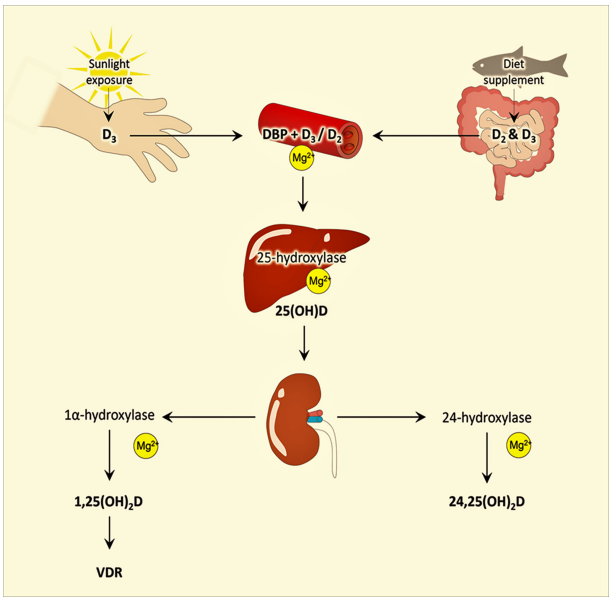


FIGURE 2 | Simplified diagram of the different stages of vitamin D synthesis and role of magnesium in the activation of vitamin D; modified from earlier publications. For simplicity, only the essential steps of vitamin synthesis are included. VDR, vitamin D receptor; DBP, vitamin D binding protein.

inflammatory bowel diseases (37–39). Additionally, several studies have shown that vitamin D has effects in reducing inflammatory cytokines and increasing anti-inflammatory cytokines (40, 41), indicating a potential clinical use of vitamin D in treating diseases that cause excessive inflammation, such as COVID-19. A key mechanism by which vitamin D reduces inflammatory cytokine is by upregulating T regulatory lymphocytes. Magnesium's role in activating vitamin D leads to improved immune function, which can reduce the severity of the cytokine storm in COVID-19 infection (42). The cytokine storm requires a considerable amount of energy and leads to a loss of ATP. Magnesium and phosphate are required for the regeneration of ATP, thus magnesium can help replenish energy stores in the body to restore and boost immune system functions (43).

Recently published articles discussed several possible mechanisms in which vitamin D can help reduce SARS–CoV–2 infections and lessen the mortality of those affected patients (44). One mechanism discussed in which vitamin D can reduce viral infections is by maintaining tight junctions (45, 46), which may reduce viral infections because there is evidence that viral entry into cells may be due to viruses compromising the integrity of tight junctions (47). Another study showed that vitamin D receptor-deficient mice significantly reduced the expression of tight junction proteins specifically in the lungs compared to wild-type mice (48), making the role of vitamin D in reducing SARS–CoV–2 infection relevant since COVID-19 also affects the lungs. The studies show evidence of vitamin D preventing certain viral infections, which is consistent with the hypothesis that vitamin D plays a role in controlling viral infection by maintaining the integrity of tight junctions. One study showed that individuals who were supplemented with 4000 IU/day vitamin D for 10 days were less vulnerable to dengue virus infection than individuals who were supplemented with 1000 IU/day of vitamin D for 10 days (49). Additionally, data shows that vitamin D deficiency is associated with patients who have acquired certain viral infections, including some respiratory tract infections (50, 51).

In a retrospective cohort study conducted in Singapore General Hospital from January 15 to April 15, 2020, patients treated with a combination of vitamin D, magnesium, and vitamin B12 (DMB) showed improved clinical outcomes compared to patients that were not treated with DMB (52). This study highlights the indirect benefit magnesium may have on COVID-19 patients by activating vitamin D. A total of 43 patients all over 50 years of age were analyzed in this study, 17 of which received the DMB treatment, and 26 patients did not receive the treatment. The investigators analyzed patient data to determine if DMB treatment would reduce the need for oxygen therapy and/or intensive care support. The results showed that significantly fewer patients in the DMB group needed oxygen therapy during their treatment compared to the control group (3 of 17 vs 16 of 26, P= 0.006). The data also showed that 1 out of the 17 patients in the DMB group required ICU support compared to 8 out of the 26 patients in the control group (52). This study highlights the importance of magnesium, as in the absence of magnesium, vitamin D will not be activated to play a protective and preventive role against the SARS–CoV–2 virus.

LOW MAGNESIUM AND COVID-19

The role of magnesium in reducing asthma (lung inflammation) symptoms that was previously mentioned is key evidence that shows how magnesium may reduce COVID-19 symptoms. Since previous studies show that magnesium can reduce symptoms of asthma (15–18) and diminish inflammation (12–14), it is plausible that magnesium may help reduce the severity of COVID-19 symptoms by reducing lung inflammation. Additionally, magnesium deficiency has been associated with increased IL-6, a proinflammatory cytokine and a likely target for COVID-19 therapies (53).

Recent research suggests that magnesium does have protective effects against COVID-19 symptoms (**Table 1**). One retrospective cohort study analyzed essential and toxic metals levels in blood samples of 306 COVID-19 patients admitted to Tongji hospital in Wuhan, China (54). The patients were hospitalized for a mean of 30 days and were categorized by severity of disease according to Guidelines of the Diagnosis and Treatment of New Coronavirus Pneumonia published by the National Health Commission of China. The results of the study showed lower magnesium levels in patients with more severe COVID-19 symptoms. The median magnesium level in patients with severe COVID-19 symptoms was 38.33 mg/L, while the median magnesium level in the non-severe symptoms group was 39.46 mg/L, yielding a P value of 0.002 between the two groups.

TABLE 1 | Published studies with magnesium status in patients with COVID-19.

Investigators	Number of patients studied	Results
Zeng et al. (54)	306	COVID-19 patients with lower magnesium levels had more severe symptoms.
Alamdari et al. (55)	396	COVID-19 patients with higher magnesium levels had lower mortality rates.
Gunay et al. (56)	629	COVID-19 patients with lower magnesium levels had a higher degree of myocardial damage.
Zhu et al. (57)	83	Hypomagnesemia was more prevalent in COVID-19 patients who did not survive.
Beigmohammadi et al. (58)	60	Lower magnesium levels in COVID-19 patients correlated to higher disease severity and risk of mortality.
Pulido-Perez et al. (59)	118	Lower magnesium levels were associated with increased mortality.
Quilliot et al. (60)	300	Hypomagnesemia was associated with 61% of all patients in study. Moderate cases were associated with hypomagnesemia; critical cases were associated with higher magnesium levels.
Sharma et al. (61)	193	Severity of the disease was greater in patients with hypermagnesemia.

This study indicates that higher levels of magnesium may be protective against severe COVID-19 symptoms. Another retrospective study analyzed 396 COVID-19 patients who survived and 63 patients who did not survive in order to determine prognostic factors associated with death (55). The study was performed from January 30th to April 5th, 2020, and studied patients admitted to Shahid Modarres Hospital in Tehran, Iran. Among many other factors, blood magnesium levels of each patient upon admission to the hospital were studied. In the group of patients that survived, the average magnesium level was 1.83 ± 0.24 me/L compared to 1.61 ± 0.19 me/L in the group of patients that did not survive, yielding a P-value of < 0.0001 (normal magnesium range is 1.5-2.5 me/L). Thus, this study also indicates that magnesium might have a protective role against COVID-19.

A retrospective cohort study done on 629 COVID-19 patients admitted to the hospital aimed to find a relationship between serum magnesium levels and myocardial damage and prognosis of disease (56). Serum troponin levels above the 99th percentile upper reference limit (24 - 30 pg/mL) was defined as myocardial damage. Prognosis of disease was determined by survival, thus the two groups in this study were the survival group and non-survival group. The median blood magnesium levels of the non-survival group were 1.94 mg/dl and in the survival group it was 2.03 mg/dl (P = 0.027) (normal range of serum magnesium = 1.7 - 2.5 mg/dL). The median troponin levels in the non-survival group were 25.2 pg/ml and in the survival group it was 4.5 pg/ml (P <0.001). The non-survival group's median troponin levels were above the 99th percentile upper reference limit range, thus they were defined as having myocardial damage, while the survival group did not. Therefore, this study uncovered a significant correlation between lower magnesium levels and higher degree of myocardial damage in COVID-19 patients (56).

Another retrospective cohort study aimed to determine the significance of low magnesium levels in COVID-19 patients (57). 83 patients who were hospitalized in the Guanggu Hospital District, Wuhan Third Hospital, China were studied. The serum magnesium levels of the patients were studied, and the patients were separated into different groups based on disease severity (moderate, severe, critical), which was classified according to the fifth edition of China's guidelines. The incidence of hypomagnesemia in each group of severity were as follows: 12.50% in the moderate group, 3.85% in the severe group, and 43.75% in the critical group. The results showed statistical significance for hypomagnesemia being more prevalent in the critical group than in the moderate and severe groups (P < 0.05). The results also showed that hypomagnesemia was more prevalent in non-survivors (40%) than in survivors (17.65%) (P < 0.05).

One cross-sectional study analyzing 60 COVID-19 patients admitted to the Intensive Care Unit (ICU) of Imam Khomeini Hospital in Iran between March and June 2020 focused on the association of serum level of micronutrients with the severity of COVID-19 disease (58). The severity of disease was measured with the Acute Physiology and Chronic Health Evaluation (APACHE) scoring system, where a score of ≥25 was consistent with high risk of mortality. Of the 60 patients studied, there were 20 patients with an APACHE score ≥25 and 40 patients with an APACHE score <25. It was found that levels of serum vitamin D, zinc, and magnesium were each significantly lower in the group with an APACHE score ≥25 (P < 0.001).

A retrospective study on 118 patients studied the relationship of renal function and serum magnesium levels on COVID-19 patients with type 2 diabetes (59). The patients were split into two groups: one group included patients with type 2 diabetes and the other included patients without type 2 diabetes. Renal function was measured by the patient's estimated glomerular filtration rate (eGFR). Patients with type 2 diabetes had a lower eGFR than patients without type 2 diabetes, indicating a reduced renal function in the COVID-19 patients with type 2 diabetes (59.7 ± 32.8 vs. 78.4 ± 33.8 mL/min per 1.73 m^2) (P = 0.008). Additionally, Patients with type 2 diabetes had lower serum magnesium levels than patients without type 2 diabetes (1.9 ± 0.3 vs. 2.1 ± 0.3 mEq/L) (P = 0.012). Statistical analysis of the patient outcomes showed that the type 2 diabetes group was associated with significantly increased risk of death, meaning that lower eGFR and magnesium levels were associated with increased mortality (59).

HIGH MAGNESIUM AND COVID-19

A prospective cohort study analyzed the serum magnesium levels of 300 patients in Nancy Brabois University Hospital between March 1, 2020, and April 29, 2020, and each patient was graded for COVID-19 severity according to WHO guidelines (60). The study showed a high prevalence of low magnesium levels in hospitalized COVID-19 patients; about 61% of the 300 patients in the study presented with hypomagnesemia (<0.75 mmol/L). However, this study revealed that most of the patients with

critical cases had high magnesium levels, and most of the patients with moderate cases had low magnesium levels. The results of this study showed that the prevalence of hypomagnesemia (<0.75 mmol/L) was significantly higher in the group of patients who had a moderate case of COVID-19 (average Mg: 0.73 mmol/L) compared to the group of patients who had a critical case of COVID-19 (average Mg: 0.79 mmol/L) (P<0.001). The group of patients with critical cases of COVID-19 had the highest magnesium levels compared to the moderate and severe groups.

Although this study found that magnesium levels tend to be lower in patients with COVID-19 infection, one study that focused on pregnant women infected with COVID-19 found that pregnant women infected with COVID-19 had higher serum magnesium levels (62). This study was a systematic review of 385 pregnant women from 33 studies with COVID-19. 95.6% of the infected women in this study had a mild case of COVID-19, while 3.6% had a severe case, and 0.8% had a critical case. The study compared certain element levels, including magnesium, in pregnant women with COVID-19 infection and without infection as a control group. The results showed that pregnant women with COVID-19 infection in their first and third trimester had significantly higher magnesium levels than in the control group (first trimester: 1,557 ± 0,211 vs 1,848 ± 0,335, P < 0,0001) (third trimester: 1,947 ± 0,657 vs 2,767 ± 0,394, P < 0,0001). However, there was no correlation between magnesium levels and the severity of disease found in this study. The investigators could not find a definitive reason for this rise in magnesium levels seen in pregnant women infected by COVID-19.

In addition to previous studies mentioned, critical COVID-19 cases were also found to be associated with hypermagnesemia in a retrospective cohort study of 193 COVID-19 patients in a medical center in California conducted from March 13, 2020, to February 2, 2021 (61). The authors of the study hypothesized that hypermagnesemia would be a predictor of mortality and morbidity in COVID-19 patients because hypermagnesemia is associated with mortality in other critical illnesses. Patients were separated according to their magnesium levels upon admission to the hospital: 104 patients were in the hypermagnesemia group (Mg level ≥2.5 mg/dL) and 89 patients were in the normomagnesemia group (Mg level 1.7 - 2.5mg/dL). The outcomes and biomarkers of each group were compared. The severity of disease was greater in the hypermagnesemia group, indicated by 48% of patients with hypermagnesemia being admitted into the ICU versus only 15% of patients in the normomagnesemia group (P<0.001), the average duration of hospital stay was 15.42 days in the hypermagnesemia group compared to 6.7 days in normomagnesemia group (P=0.0001), and of the 35 patients in the study who required a ventilator, 34 of them were in the hypermagnesemia group (P<0.0001). Cough (P=0.005) and dyspnea (P=0.001) were also significantly more prevalent in the hypermagnesemia group compared to the normomagnesemia group. The electrolyte imbalance and mineral ion dysregulation are usually encountered in patients with ventilator (critical stages of the disease process), which is likely to be the consequence of multi-organ dysfunctions.

These results may be due to the adverse effects of hypermagnesemia. Of clinical relevance, hypermagnesemia has been shown to cause adverse cardiovascular, neurological, and respiratory effects (63). Since there are various units for measuring magnesium that are commonly used, errors in administering proper doses of magnesium are a common cause of its overdoses. Two case reports regarding the treatment of two different alcoholic withdrawal patients highlight this issue along with the symptoms associated with magnesium overdose (64). In the course of these patients' treatment, they were ordered to be administered 2 g of magnesium sulfate intravenously. However, patients were given 20 g of magnesium sulfate by mistake leading to magnesium overdose, leading to cardiac arrest in both the patients, followed by successful resuscitation (64). Adverse cardiac effects of magnesium overdose include delays in conduction of electrical impulses in the heart, asystole (cardiac arrest). Other adverse effects of magnesium overdose include apnea, coma, neurologic deterioration, and muscle weakness.

CONCLUSIONS

There is strong evidence of magnesium playing a role in reducing the severity of asthma symptoms (15–18), COVID-19 symptoms (54–59), and inflammation (12–14). Magnesium has been shown to exert anti-inflammatory effects both independently and as a result of activating vitamin D (11–14, 20, 21, 53). The anti-inflammatory effects of magnesium are potentially responsible for reducing symptoms of asthma patients treated with magnesium. Adequate magnesium levels have been associated with lower mortality rates in COVID-19 patients and less severe symptoms (52, 54–59). Natural sources of magnesium (avocado, spinach, almonds, pumpkin seeds, whole grains, black beans, wheat, and oatmeal) and magnesium supplementation are easily accessible and affordable that can be consumed to reduce the disease burden of COVID-19 patients. Magnesium's role in activating vitamin D is essential in that vitamin D has been shown to reduce the rate of viral infection in patients with several respiratory tract infections. Thus, more research should be conducted to determine the magnesium-dependent effects of vitamin D in reducing the risk of infection in patients with COVID-19.

AUTHOR CONTRIBUTIONS

ME collected information and drafted the manuscript. MR conceptualized and reviewed the manuscript. Both authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We want to express our sincere gratitude to Dr. Nuraly Akimbekov, PhD (Al-Farabi Kazakh National University, Kazakhstan) for his help in drawing the illustrations. ME is an Osteopathic Medical Student (OMS II) at the Lake Erie College of Osteopathic Medicine, Erie (USA).

REFERENCES

1. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical Features of Patients Infected With 2019 Novel Coronavirus in Wuhan, China. *Lancet* (2020) 395:497–506. doi: 10.1016/S0140-6736(20)30183-5
2. Shi S, Qin M, Shen B, Cai Y, Liu T, Yang F, et al. Association of Cardiac Injury With Mortality in Hospitalized Patients With COVID-19 in Wuhan, China. *JAMA Cardiol* (2020) 5:802–10. doi: 10.1001/jamacardio.2020.0950
3. Cheng HY, Jian SW, Liu DP, Ng TC, Huang WT, Lin HH. Contact Tracing Assessment of COVID-19 Transmission Dynamics in Taiwan and Risk at Different Exposure Periods Before and After Symptom Onset. *JAMA Intern Med* (2020) 180:1156–63. doi: 10.1001/jamainternmed.2020.2020
4. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, et al. Temporal Dynamics in Viral Shedding and Transmissibility of COVID-19. *Nat Med* (2020) 26:672–5. doi: 10.1038/s41591-020-0869-5
5. Viana R, Moyo S, Amoako DG, Tegally H, Scheepers C, Althaus CL, et al. Rapid Epidemic Expansion of the SARS-Cov-2 Omicron Variant in Southern Africa. *Nature* (2022). doi: 10.1038/s41586-022-04411-y
6. Garrett N, Tapley A, Andriesen J, Seocharan I, Fisher LH, Bunts L, et al. High Rate of Asymptomatic Carriage Associated With Variant Strain Omicron. *medRxiv* (2022). doi: 10.1101/2021.12.20.21268130
7. Razzaque MS. Magnesium: Are We Consuming Enough? *Nutrients* (2018) 10. doi: 10.3390/nu10121863
8. DiNicolantonio JJ, O'Keefe JH, Wilson W. Subclinical Magnesium Deficiency: A Principal Driver of Cardiovascular Disease and a Public Health Crisis. *Open Heart* (2018) 5:e000668. doi: 10.1136/openhrt-2017-000668
9. Long S, Romani AM. Role of Cellular Magnesium in Human Diseases. *Austin J Nutr Food Sci* (2014) 2:1051.
10. Uwitonze AM, Rahman S, Ojeh N, Grant WB, Kaur H, Haq A, et al. Oral Manifestations of Magnesium and Vitamin D Inadequacy. *J Steroid Biochem Mol Biol* (2020) 200:105636. doi: 10.1016/j.jsbmb.2020.105636
11. Uwitonze AM, Razzaque MS. Role of Magnesium in Vitamin D Activation and Function. *J Am Osteopath Assoc* (2018) 118:181–9. doi: 10.7556/jaoa.2018.037
12. Malpuech-Brugère C, Nowacki W, Rock E, Gueux E, Mazur A, Rayssiguier Y. Enhanced Tumor Necrosis Factor-Alpha Production Following Endotoxin Challenge in Rats Is an Early Event During Magnesium Deficiency. *Biochim Biophys Acta* (1999) 1453:35–40. doi: 10.1016/S0925-4439(98)00081-7
13. Dibaba DT, Xun P, He K. Dietary Magnesium Intake is Inversely Associated With Serum C-Reactive Protein Levels: Meta-Analysis and Systematic Review. *Eur J Clin Nutr* (2014) 68:510–6. doi: 10.1038/ejcn.2014.7
14. Mahabir S. Dietary Magnesium and Inflammation. *Eur J Clin Nutr* (2014) 68:970. doi: 10.1038/ejcn.2014.110
15. Rowe BH, Bretzlaff JA, Bourdon C, Bota GW, Camargo CA Jr. Intravenous Magnesium Sulfate Treatment for Acute Asthma in the Emergency Department: A Systematic Review of the Literature. *Ann Emerg Med* (2000) 36:181–90. doi: 10.1067/mem.2000.105659
16. Kew KM, Kirtchuk L, Michell CI. Intravenous Magnesium Sulfate for Treating Adults With Acute Asthma in the Emergency Department. *Cochrane Database Syst Rev* (2014):Cd010909. doi: 10.1002/14651858.CD010909
17. Powell CV, Kolamunnage-Dona R, Lowe J, Boland A, Petrou S, Doull I, et al. Magnesium Trial in Children (MAGNETIC): A Randomised, Placebo-Controlled Trial and Economic Evaluation of Nebulised Magnesium Sulphate in Acute Severe Asthma in Children. *Health Technol Assess* (2013) 17v-vi:1–216. doi: 10.3310/hta17450
18. Blitz M, Blitz S, Hughes R, Diner B, Beasley R, Knopp J, et al. Aerosolized Magnesium Sulfate for Acute Asthma: A Systematic Review. *Chest* (2005) 128:337–44. doi: 10.1378/chest.128.1.337
19. Kappeler D, Heimbeck I, Herpich C, Naue N, Höfler J, Timmer W, et al. Higher Bioavailability of Magnesium Citrate as Compared to Magnesium Oxide Shown by Evaluation of Urinary Excretion and Serum Levels After Single-Dose Administration in a Randomized Cross-Over Study. *BMC Nutr* (2017) 3:7. doi: 10.1186/s40795-016-0121-3
20. Risco F, Traba ML. Influence of Magnesium on the *In Vitro* Synthesis of 24,25-Dihydroxyvitamin D3 and 1 Alpha, 25-Dihydroxyvitamin D3. *Magnes Res* (1992) 5:5–14.
21. Risco F, Traba ML. Possible Involvement of a Magnesium Dependent Mitochondrial Alkaline Phosphatase in the Regulation of the 25-Hydroxyvitamin D3-1 Alpha-And 25-Hydroxyvitamin D3-24r-Hydroxylases in LLC-PK1 Cells. *Magnes Res* (1994) 7:169–78.
22. Ao T, Kikuta J, Ishii M. The Effects of Vitamin D on Immune System and Inflammatory Diseases. *Biomolecules* (2021) 11. doi: 10.3390/biom11111624
23. Maruotti N, Cantatore FP. Vitamin D and the Immune System. *J Rheumatol* (2010) 37:491–5. doi: 10.3899/jrheum.090797
24. Wong MS, Leisegang MS, Kruse C, Vogel J, Schürmann C, Dehne N, et al. Vitamin D Promotes Vascular Regeneration. *Circulation* (2014) 130:976–86. doi: 10.1161/CIRCULATIONAHA.114.010650
25. Kheiri B, Abdalla A, Osman M, Ahmed S, Hassan M, Bachuwa G. Vitamin D Deficiency and Risk of Cardiovascular Diseases: A Narrative Review. *Clin Hypertens* (2018) 24:9. doi: 10.1186/s40885-018-0094-4
26. Wimalawansa SJ, Razzaque MS, Al-Daghri NM. Calcium and Vitamin D in Human Health: Hype or Real? *J Steroid Biochem Mol Biol* (2018) 180:4–14. doi: 10.1016/j.jsbmb.2017.12.009
27. Uwitonze AM, Murererehe J, Ineza MC, Harelimana EI, Nsabimana U, Uwambaye P, et al. Effects of Vitamin D Status on Oral Health. *J Steroid Biochem Mol Biol* (2018) 175:190–4. doi: 10.1016/j.jsbmb.2017.01.020
28. Lanske B, Razzaque MS. Vitamin D and Aging: Old Concepts and New Insights. *J Nutr Biochem* (2007) 18:771–7. doi: 10.1016/j.jnutbio.2007.02.002
29. Razzaque MS. The Dualistic Role of Vitamin D in Vascular Calcifications. *Kidney Int* (2011) 79:708–14. doi: 10.1038/ki.2010.432
30. Brown RB, Haq A, Stanford CF, Razzaque MS. Vitamin D, Phosphate, and Vasculotoxicity. *Can J Physiol Pharmacol* (2015) 93:1077–82. doi: 10.1139/cjpp-2015-0083
31. Qu X, Jin F, Hao Y, Li H, Tang T, Wang H, et al. Magnesium and the Risk of Cardiovascular Events: A Meta-Analysis of Prospective Cohort Studies. *PloS One* (2013) 8:e57720. doi: 10.1371/journal.pone.0057720
32. Altura BM, Altura BT, Gebrewold A, Ising H, Günther T. Magnesium Deficiency and Hypertension: Correlation Between Magnesium-Deficient Diets and Microcirculatory Changes *in Situ*. *Science* (1984) 223:1315–7. doi: 10.1126/science.6701524
33. Groenendijk I, van Delft M, Versloot P, van Loon LJC, de Groot L. Impact of Magnesium on Bone Health in Older Adults: A Systematic Review and Meta-Analysis. *Bone* (2022) 154:116233. doi: 10.1016/j.bone.2021.116233
34. Erem S, Atfi A, Razzaque MS. Anabolic Effects of Vitamin D and Magnesium in Aging Bone. *J Steroid Biochem Mol Biol* (2019) 193:105400. doi: 10.1016/j.jsbmb.2019.105400
35. Zahra H, Berriche O, Mizouri R, Boukhayatia F, Khiari M, Gamoudi A, et al. Plasmatic Magnesium Deficiency in 101 Outpatients Living With Type 2 Diabetes Mellitus. *Clin Pract* (2021) 11:791–800. doi: 10.3390/clinpract11040095
36. Tang CF, Ding H, Jiao RQ, Wu XX, Kong LD. Possibility of Magnesium Supplementation for Supportive Treatment in Patients With COVID-19. *Eur J Pharmacol* (2020) 886:173546. doi: 10.1016/j.ejphar.2020.173546
37. Głąbska D, Kołota A, Lachowicz K, Skolmowska D, Stachoń M, Guzek D. Vitamin D Supplementation and Mental Health in Multiple Sclerosis Patients: A Systematic Review. *Nutrients* (2021) 13. doi: 10.3390/nu13124207
38. Franco AS, Freitas TQ, Bernardo WM, Pereira RMR. Vitamin D Supplementation and Disease Activity in Patients With Immune-Mediated Rheumatic Diseases: A Systematic Review and Meta-Analysis. *Med (Baltimore)* (2017) 96:e7024. doi: 10.1097/MD.0000000000007024
39. Akimbekov NS, Digel I, Sherelkhan DK, Lutfor AB, Razzaque MS. Vitamin D and the Host-Gut Microbiome: A Brief Overview. *Acta Histochem Cytochem* (2020) 53:33–42. doi: 10.1267/ahc.20011
40. Barker T, Martins TB, Hill HR, Kjeldsberg CR, Dixon BM, Schneider ED, et al. Vitamin D Sufficiency Associates With an Increase in Anti-Inflammatory Cytokines After Intense Exercise in Humans. *Cytokine* (2014) 65:134–7. doi: 10.1016/j.cyto.2013.12.004
41. Calton EK, Keane KN, Newsholme P, Soares MJ. The Impact of Vitamin D Levels on Inflammatory Status: A Systematic Review of Immune Cell Studies. *PloS One* (2015) 10:e0141770. doi: 10.1371/journal.pone.0141770
42. DiNicolantonio JJ, O'Keefe JH. Magnesium and Vitamin D Deficiency as a Potential Cause of Immune Dysfunction, Cytokine Storm and Disseminated Intravascular Coagulation in Covid-19 Patients. *Mo Med* (2021) 118:68–73.
43. van Kempen T, Deixler E. SARS-CoV-2: Influence of Phosphate and Magnesium, Moderated by Vitamin D, on Energy (ATP) Metabolism and

on Severity of COVID-19. *Am J Physiol Endocrinol Metab* (2021) 320:E2–e6. doi: 10.1152/ajpendo.00474.2020
44. Grant WB. Vitamin D's Role in Reducing Risk of SARS-CoV-2 and COVID-19 Incidence, Severity, and Death. *Nutrients* (2021) 14. doi: 10.3390/nu14010183
45. Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J, et al. Novel Role of the Vitamin D Receptor in Maintaining the Integrity of the Intestinal Mucosal Barrier. *Am J Physiol Gastrointest Liver Physiol* (2008) 294:G208–216. doi: 10.1152/ajpgi.00398.2007
46. Zhang YG, Wu S, Lu R, Zhou D, Zhou J, Carmeliet G, et al. Tight Junction CLDN2 Gene Is a Direct Target of the Vitamin D Receptor. *Sci Rep* (2015) 5:10642. doi: 10.1038/srep10642
47. Short KR, Kasper J, van der Aa S, Andeweg AC, Zaaraoui-Boutahar F, Goeijenbier M, et al. Influenza Virus Damages the Alveolar Barrier by Disrupting Epithelial Cell Tight Junctions. *Eur Respir J* (2016) 47:954–66. doi: 10.1183/13993003.01282-2015
48. Chen H, Lu R, Zhang YG, Sun J. Vitamin D Receptor Deletion Leads to the Destruction of Tight and Adherens Junctions in Lungs. *Tissue Barriers* (2018) 6:1–13. doi: 10.1080/21688370.2018.1540904
49. Martínez-Moreno J, Hernandez JC, Urcuqui-Inchima S. Effect of High Doses of Vitamin D Supplementation on Dengue Virus Replication, Toll-Like Receptor Expression, and Cytokine Profiles on Dendritic Cells. *Mol Cell Biochem* (2020) 464:169–80. doi: 10.1007/s11010-019-03658-w
50. Ginde AA, Mansbach JM, Camargo CA Jr. Association Between Serum 25-Hydroxyvitamin D Level and Upper Respiratory Tract Infection in the Third National Health and Nutrition Examination Survey. *Arch Intern Med* (2009) 169:384–90. doi: 10.1001/archinternmed.2008.560
51. Siddiqui M, Manansala JS, Abdulrahman HA, Nasrallah GK, Smatti MK, Younes N, et al. Immune Modulatory Effects of Vitamin D on Viral Infections. *Nutrients* (2020) 12. doi: 10.3390/nu12092879
52. Tan CW, Ho LP, Kalimuddin S, Cherng BPZ, Teh YE, Thien SY, et al. Cohort Study to Evaluate the Effect of Vitamin D, Magnesium, and Vitamin B(12) in Combination on Progression to Severe Outcomes in Older Patients With Coronavirus (COVID-19). *Nutrition* (2020) 79-80:111017. doi: 10.1016/j.nut.2020.111017
53. Wallace TC. Combating COVID-19 and Building Immune Resilience: A Potential Role for Magnesium Nutrition? *J Am Coll Nutr* (2020) 39:685–93. doi: 10.1080/07315724.2020.1785971
54. Zeng HL, Yang Q, Yuan P, Wang X, Cheng L. Associations of Essential and Toxic Metals/Metalloids in Whole Blood With Both Disease Severity and Mortality in Patients With COVID-19. *FASEB J* (2021) 35:e21392. doi: 10.1096/fj.202002346RR
55. Alamdari NM, Afaghi S, Rahimi FS, Tarki FE, Tavana S, Zali A, et al. Mortality Risk Factors Among Hospitalized COVID-19 Patients in a Major Referral Center in Iran. *Tohoku J Exp Med* (2020) 252:73–84. doi: 10.1620/tjem.252.73
56. Gunay S, Caliskan S, Sigirli D. Relationship of Magnesemia With Myocardial Damage and Mortality in Patients With COVID-19. *Magnes Res* (2021) 34:93–102. doi: 10.1684/mrh.2021.0485
57. Zhu L, Bao X, Bi J, Lin Y, Shan C, Fan X, et al. Serum Magnesium in Patients With Severe Acute Respiratory Syndrome Coronavirus 2 From Wuhan, China. *Magnes Res* (2021) 34:103–13. doi: 10.1684/mrh.2021.0488
58. Beigmohammadi MT, Bitarafan S, Abdollahi A, Amoozadeh L, Salahshour F, Mahmoodi Ali Abadi M, et al. The Association Between Serum Levels of Micronutrients and the Severity of Disease in Patients With COVID-19. *Nutrition* (2021) 91-92:111400. doi: 10.1016/j.nut.2021.111400
59. Pulido-Perez P, Pondigo-de Los Angeles JA, Hernandez-Hernandez ME, Torres-Rasgado E, Romero JR. Renal Function, Serum Magnesium Levels and Mortality in COVID-19 Patients With Type 2 Diabetes. *Magnes Res* (2021) 34:20–31. doi: 10.1684/mrh.2021.0481
60. Quilliot D, Bonsack O, Jaussaud R, Mazur A. Dysmagnesemia in Covid-19 Cohort Patients: Prevalence and Associated Factors. *Magnes Res* (2020) 33:114–22. doi: 10.1684/mrh.2021.0476
61. Sharma R, Heidari A, Johnson RH, Advani S, Petersen G. Serum Magnesium Levels in Hospitalized Patients With SARS-CoV-2. *J Investig Med* (2021) 70:409–14. doi: 10.2139/ssrn.3811430
62. Anuk AT, Polat N, Akdas S, Erol SA, Tanacan A, Biriken D, et al. the Relation Between Trace Element Status (Zinc, Copper, Magnesium) and Clinical Outcomes in COVID-19 Infection During Pregnancy. *Biol Trace Elem Res* (2021) 199:3608–17. doi: 10.1007/s12011-020-02496-y
63. Cascella M, Vaqar S. Hypermagnesemia. In: *Statpearls*. Treasure Island (FL: StatPearls Publishing Copyright © 2022, StatPearls Publishing LLC. (2022).
64. Vissers RJ, Purssell R. Iatrogenic Magnesium Overdose: Two Case Reports. *J Emerg Med* (1996) 14:187–91. doi: 10.1016/0736-4679(95)02115-9

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.

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 07 June 2022
doi: 10.3389/fnut.2022.878665

Dietary Magnesium Intake Level Modifies the Association Between Vitamin D and Insulin Resistance: A Large Cross-Sectional Analysis of American Adults

Ya Liu[1†], Rongpeng Gong[1†], Haixiu Ma[1,2], Siai Chen[1], Jingwei Sun[1], Jiarui Qi[1], Yidan Pang[1], Juan An[1] and Zhanhai Su[1]*

[1] Department of Basic Medical Sciences, Qinghai University Medical College, Xining, China, [2] Key Laboratory for High Altitude Medicine, Research Center for High Altitude Medicine, Xining, China

OPEN ACCESS

Edited by:
Mohammed S. Razzaque, Lake Erie College of Osteopathic Medicine, United States

Reviewed by:
Anwar Abdullah Borai, King Saud bin Abdulaziz University for Health Sciences, Saudi Arabia
Mostafa Waly, Sultan Qaboos University, Oman

***Correspondence:**
Juan An
anjuan@qhu.edu.cn

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

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

Received: *18 February 2022*
Accepted: *11 April 2022*
Published: *07 June 2022*

Citation:
Liu Y, Gong R, Ma H, Chen S, Sun J, Qi J, Pang Y, An J and Su Z (2022) Dietary Magnesium Intake Level Modifies the Association Between Vitamin D and Insulin Resistance: A Large Cross-Sectional Analysis of American Adults. Front. Nutr. 9:878665. doi: 10.3389/fnut.2022.878665

Background: Previous clinical studies and randomized controlled trials have revealed that low serum vitamin D levels are associated with the risk of developing insulin resistance. Magnesium has been reported to be a protective factor for insulin resistance, and magnesium has been considered an important co-factor for vitamin D activation. However, the effect of dietary magnesium intake on the relationship between vitamin D and the risk of developing insulin resistance has not been comprehensively investigated. Therefore, we designed this cross-sectional analysis to assess whether dietary magnesium intake modifies the association of vitamin D and insulin resistance.

Methods: A total of 4,878 participants (male: 48.2%) from 4 consecutive cycles of the National Health and Nutrition Examination Survey (2007–2014) were included in this study after a rigorous screening process. Participants were stratified by their dietary magnesium intake into low-intake (<267 mg/day) and high-intake (≥267 mg/day) groups. We assessed differences between serum vitamin D levels and the risk of developing insulin resistance (interaction test), using a weighted multivariate logistic regression to analyze differences between participants with low and high magnesium intake levels.

Results: There was a negative association between vitamin D and insulin resistance in the US adult population [OR: 0.93 (0.88–0.98)], $P < 0.001$. Dietary magnesium intake strengthened the association (P for interaction < 0.001). In the low dietary magnesium intake group, vitamin D was negatively associated with the insulin resistance [OR: 0.94 (0.90–0.98)]; in the high dietary magnesium intake group, vitamin D was negatively associated with insulin resistance [OR: 0.92 (0.88–0.96)].

Conclusion: Among adults in the United States, we found an independent association between vitamin D level and insulin resistance, and this association was modified according to different levels of magnesium intake.

Keywords: vitamin D, cross-sectional studies, dietary magnesium intake, insulin resistance, American adults

INTRODUCTION

Insulin resistance (IR) refers to an efficiency decline in the performance of insulin to promote glucose uptake and utilization in tissues or cells (1, 2). It can be caused by a variety of reasons and pushes the body to compensate by producing too much insulin to keep the blood sugar within a normal range. IR is a core component of metabolic syndrome and type 2 diabetes (3–5). It may precede other cardiometabolic risk factors, as mentioned in the study of Gong et al. (6). Yin et al. also conducted a meta-analysis considering IR and the risk of thyroid cancer in 2018 and found that people with IR had a significantly greater chance of developing thyroid cancer (7). IR is also associated with the incidence of colorectal cancer (8, 9) and lung cancer (10, 11). In 2021, the International Diabetes Federation published guidelines stating that 629 million people aged 20–79 years are expected to develop type 2 diabetes by 2045 all over the world, for which one of the main causes is IR (7). In summary, to understand the risk factors underlying the development of health care is significant for the health of the World's people.

Vitamin D (Vit D) is a lipid-soluble vitamin whose main function is to maintain the balance between calcium metabolism and bone formation in the human body. Vit D is activated in the body to acquire the biological activity of a hormone, with 1, 25-bishydroxyvitamin D3 acting as its main active form. According to related studies, Vit D level may be a protective factor against the development of IR (12–15). However, this conclusion is considered controversial at present. A cross-sectional analysis by Schleu et al. found that lower Vit D levels were strongly associated with increased IR in obese women (16). Szymczak-Pajor and Śliwińska (17) suggested that Vit D deficiency is one of the factors that accelerates the development of IR. In contrast, a randomized controlled trial by Gulseth et al. found that Vit D levels and IR were not correlated with one another (18) and similar findings were also reported by Margaret and Lansang (19). The reason for these variations may be that the adjustment strategies were not consistent across studies. Among them, we are particularly concerned about the absence of a certain factor in most of these studies: dietary magnesium intake.

Magnesium is the second most abundant divalent ion in cells following potassium ions, and it has been recognized as a cofactor in > 300 enzymatic reactions. It is essential for the modulation of blood pressure (20), insulin metabolism (21), and other physiological functions. Meanwhile, magnesium is closely related to Vit D synthesis, and previous studies have shown that magnesium is necessary for the movement and activation of Vit D in the blood (22). A randomized clinical trial published in 2018 by Dai et al. showed that magnesium optimizes Vit D status in the body with a bidirectional regulatory effect; in other words, magnesium can be optimized according to the body's original Vit D level so that Vit D levels are maintained in the normal range (23). Further, all enzymes used for the metabolism of Vit D seem to require magnesium, which acts as a co-factor in the enzymatic reactions of the liver and kidneys (24). Magnesium intake alone or its interaction with Vit D intake may contribute to Vit D status (25, 26). The enzymatic activation of 25-hydroxylase in the liver and 1α-hydroxylase in the kidneys is a process that requires magnesium. Magnesium is also needed to deactivate Vit D when levels are too high (22). Previous studies presented that concentrations of cytochrome P450 (CYP) enzymes are modified by magnesium level (27). Cytochrome P450 enzymes include not only the Vit D–activating enzymes [i.e., 25-hydroxylase (e.g., CYP2R1) and 1α-hydroxylase (i.e., CYP27B1)] but also Vit D–deactivating enzymes [i.e., 24-hydroxylase (i.e., CYP24A1 and CYP3A4)]. 25-Hydroxylase synthesizes 25 (OH)D from Vit D3 or Vit D2 in the liver, and then 1α-hydroxylase synthesizes active 1, 25 (OH) 2D from 25 (OH)D in the kidney. 24-Hydroxylase metabolizes both 25 (OH)D and 1, 25 (OH) 2D to inactive forms: 24, 25-dihydroxyVit D and 1, 24, 25-trihydroxyVit D, respectively. Finally, CYP3A4 degrades 24, 25-dihydroxyVit D and 1, 24, 25-trihydroxyVit D (23). Other studies have shown that Vit D is transported through the body in combination with a carrier protein, i.e., Vit D-binding protein, and the activity of this protein is also dependent on magnesium (22). Thus, magnesium is a co-factor for Vit D biosynthesis, transport, and activation.

As we know magnesium is an activator of Vit D and also modulates IR, the question of whether magnesium can influence the link between Vit D and IR deserves consideration. To date, however, there has been little research on this issue. Therefore, we conducted a clinical study of the effect of magnesium intake on the relationship between Vit D and IR. In the present study, we hypothesized that magnesium ingestion could affect the association between Vit D and IR. The aim of this investigation was to explore the effect of magnesium intake on the relationship between Vit D and IR using a nationally representative public database in the United States in an effort to provide some reference for subsequent revelation of its mechanism of action.

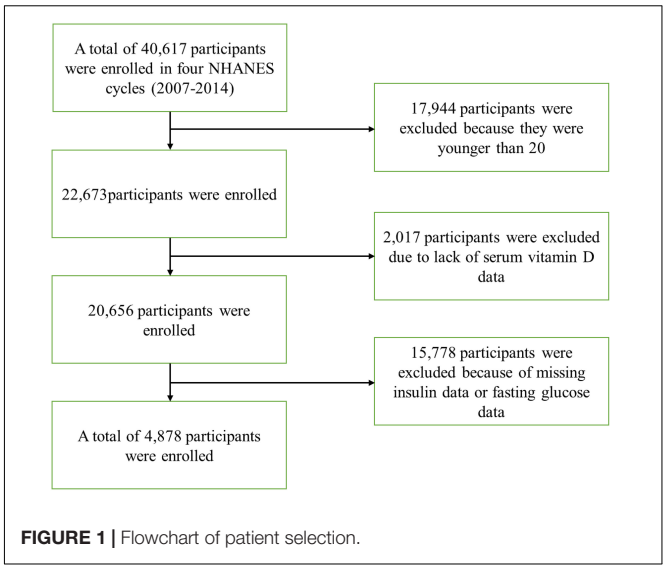


FIGURE 1 | Flowchart of patient selection.

MATERIALS AND METHODS

Data Sources

This study was a large cross-sectional analysis using data from four cycles (2007–2014) of the National Health and

TABLE 1 | Basic information description of participants.

Variables	Total (*n* = 4,878)	Dietary magnesium intake (mg/d)		*P*-value
		<267 mg/d (*n* = 2,436)	≥267 mg/d (*n* = 2,442)	
Age (year), mean ± SD	49.2 ± 17.7	50.3 ± 18.4	48.0 ± 16.9	<0.001
BMI (kg/m^2), mean ± SD	28.9 ± 6.8	29.2 ± 7.0	28.6 ± 6.6	0.004
FPG (mmol/L), mean ± SD	6.0 ± 1.9	6.0 ± 1.9	5.9 ± 1.9	0.406
OGTT (mmol/L), mean ± SD	7.7 ± 4.3	7.9 ± 4.3	7.4 ± 4.2	<0.001
Serum-Vit D (nmol/L), median (IQR)	61.4 (44.2, 79.3)	58.6 (41.1, 77.0)	63.8 (48.0, 81.5)	<0.001
Sex, *n* (%)				<0.001
Male	2,353 (48.2)	947 (38.9)	1,406 (57.6)	
Female	2,525 (51.8)	1,489 (61.1)	1,036 (42.4)	
Race, *n* (%)				<0.001
Mexican–American	716 (14.7)	294 (12.1)	422 (17.3)	
Other Hispanic	477 (9.8)	243 (10)	234 (9.6)	
Non-Hispanic white	2,307 (47.3)	1,139 (46.8)	1,168 (47.8)	
Non-Hispanic black	913 (18.7)	544 (22.3)	369 (15.1)	
Other races	465 (9.5)	216 (8.9)	249 (10.2)	
Obesity, *n* (%)				0.001
No	3,134 (64.2)	1,510 (62)	1,624 (66.5)	
Yes	1,744 (35.8)	926 (38)	818 (33.5)	
Education, *n* (%)				<0.001
Did not graduate from high school	1,228 (25.2)	688 (28.2)	540 (22.1)	
Graduated from high school	1,068 (21.9)	600 (24.6)	468 (19.2)	
College education or above	2,582 (52.9)	1,148 (47.1)	1,434 (58.7)	
Activity, *n* (%)				0.772
Vigorous work activity	899 (18.4)	464 (19)	435 (17.8)	
Moderate work activity	1,032 (21.2)	505 (20.7)	527 (21.6)	
Walk or bicycle	682 (14.0)	343 (14.1)	339 (13.9)	
Vigorous recreational activities	330 (6.8)	168 (6.9)	162 (6.6)	
Moderate recreational activities	1,935 (39.7)	956 (39.2)	979 (40.1)	
Diabetes, *n* (%)				<0.001
No	3,905 (80.1)	1,892 (77.7)	2,013 (82.4)	
Yes	973 (19.9)	544 (22.3)	429 (17.6)	
Season of examination, *n* (%)				0.327
Winter	2,304 (47.2)	1,133 (46.5)	1,171 (48)	
Summer	2,574 (52.8)	1,303 (53.5)	1,271 (52)	
Dietary factors				
Energy (kcal)	2106.1 ± 10.3	1582.5 ± 6.7	2628.4 ± 15.1	<0.001
Protein (gm)	81.9 ± 42.9	59.1 ± 24.8	104.6 ± 45.0	<0.001
Fiber (gm)	16.7 ± 10.3	10.7 ± 5.1	22.7 ± 10.7	<0.001
Calcium (mg)	920.7 ± 603.4	639.1 ± 342.2	1201.6 ± 672.7	<0.001

BMI, Body Mass Index; FPG, Fasting plasma glucose; OGGT, Oral Glucose Tolerance Test.

Nutrition Examination Survey (NHANES).[1] The NHANES project is a research project of U.S. citizens that uses a multi-stage stratified probability design with a collection sample representative of the overall sample of non-institutionalized U.S. citizens. These data include demographic data, food data, physical measurements, laboratory data, and questionnaire data. All NHANES-based studies are approved by the National Health Statistics Research Ethics Review Board. Ethical approval and more detailed information can be found on the NHANES Ethics Review Committee website[2] (28).

Study Design and Participant Population

This study was designed as a cross-sectional analysis. The target independent variable was the serum Vit D level recorded at the time that participants were tested. The dependent variable was whether the participant was diagnosed with IR. Grouping was done by median magnesium intake, with participants added to

[1]https://www.cdc.gov/nchs/nhanes/

[2]https://www.cdc.gov/nchs/nhanes/irba98.htm

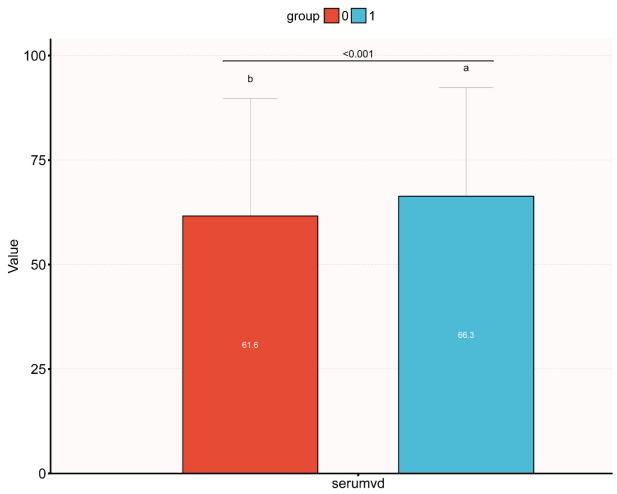


FIGURE 2 | Bar figure of the differences between different vitamin D levels in the high and low dietary magnesium intake groups. Median vitamin D levels were significantly higher in the high magnesium intake group than in the low dietary magnesium intake group (0: low dietary magnesium intake group, 1: high dietary magnesium intake group, 66.3 vs. 61.6 nmol/L, $p < 0.001$).

a low-intake (<267 mg/d) group (n = 2,436) or a high-intake (≥267 mg/d) group (n = 2,442).

A total of 40,617 participants completed interviews and examinations at the Mobile Examination Center (MEC) from 2007 to 2014. Participants with any of the following conditions were excluded from the current study: (1) age below 20 years (n = 17,944); (2) no serum Vit D testing (n = 2,017); and (3) missing insulin data or fasting glucose data (n = 15,778). Finally, a total of 4,878 participants were enrolled.

Data Collection

All study data were collected by trained professionals and included demographics (age, sex, race, education, etc.), anthropometric measurements (height, waist circumference, weight, body mass index [BMI], etc.), health-related behaviors (smoking and exercise), and biochemical tests [fasting plasma glucose, oral glucose tolerance test (OGTT), etc.]. All information was collected and blood samples were drawn in an MEC; basic information was collated immediately and serum samples were managed in scientific storage, then sent to the Laboratory Sciences Division of the National Center for Environmental Health, the Centers for Disease Control and Prevention (CDC), and designated authorized institutions for analysis.

Measurement of Magnesium Intake

The magnesium intake protocol used in this study was the consensus reached during the regular NHANES workshops for expert assessment of the protocol (29). The 24-h food-recall method has previously been used to determine dietary intake in large cross-sectional studies. In this study, data on the first 24 h of magnesium dietary intake were collected through a dietary-recall interview at the MEC. Daily magnesium intake was classified as high or low intake based on the median value (267 mg/day).

Measurement of Vit D

Immediately after serum was collected at the MEC, it was stored frozen at -30°C and subsequently shipped uniformly to the CDC Environmental Health Laboratory in Atlanta, Georgia, for Vit D measurement. Vit D levels were defined as the sum of Vit D3 and Vit D2 concentrations. Laboratory analysis was performed by ultra-high performance liquid chromatography–tandem mass-spectrometry (30).

Identification of Insulin Resistance

In previous research, the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index was recognized as a good indicator of IR (31). The HOMA-IR index is calculated as fasting glucose - insulin (μU/mL) × fasting glucose (mmol/L)/22.5. In a study involving IR in American adults, a HOMA-IR value of ≥ 2.73 was considered indicative of IR. Therefore, in the present study, IR was defined by a HOMA-IR value of ≥ 2.73 (32).

Definitions of Other Variables

To confirm diabetes, the measured fasting glucose level was multiplied by 0.056 (rounded to three decimal places) to convert the unit from mg/dL to mmol/L. Diabetes was diagnosed when the following conditions were met: fasting glucose level of ≥ 7.0 mmol/L, OGTT result of ≥ 11.1 mmol/L, physician diagnosis, self-reported diagnosis, or taking diabetes medication (33).

Participants who fit into any of the following race categories were included: Mexican–American, other Hispanic, non-Hispanic white, non-Hispanic black, or another race. Their education was divided into three categories: high school graduates, college graduates or higher. Smoking levels included current, former and never smokers. Those who had smoked ≥ 100 cigarettes or more in the past and reported smoking on a few days or every day at the time of the interview were considered current smokers, those who had smoked < 100 cigarettes in the past but were not currently smoking were considered ex-smokers, and those who had smoked < 100 cigarettes in the past were considered non-smokers. BMI was calculated using height and weight values. Weight was measured by the researchers using an electronic sports measurement device (Seca GmbH, Hamburg, Germany), which is accurate in millimeters. Body weight was measured by researchers using a digital scale (Toledo Scale; Mettler-Toledo, LLC, Columbus, OH, United States), and, after measurement, pounds were converted to kilograms. The formula used for BMI was: BMI = weight (kg)/height (m^2). Finally, dietary data were obtained from a dietary retrospective interview set up to collect dietary information for the previous 24 h, including total dietary energy, Vit D, calcium, magnesium, protein, and fiber intakes.

Statistical Methods

NHANES selects 5,000 people each year from a sampling frame of 15 different locations in all U.S. counties. Thus, its data have broad U.S. population band variability. To prevent bias and inaccurate estimation of results due to over-sampling of minority groups, we used one of the weight values officially recommended

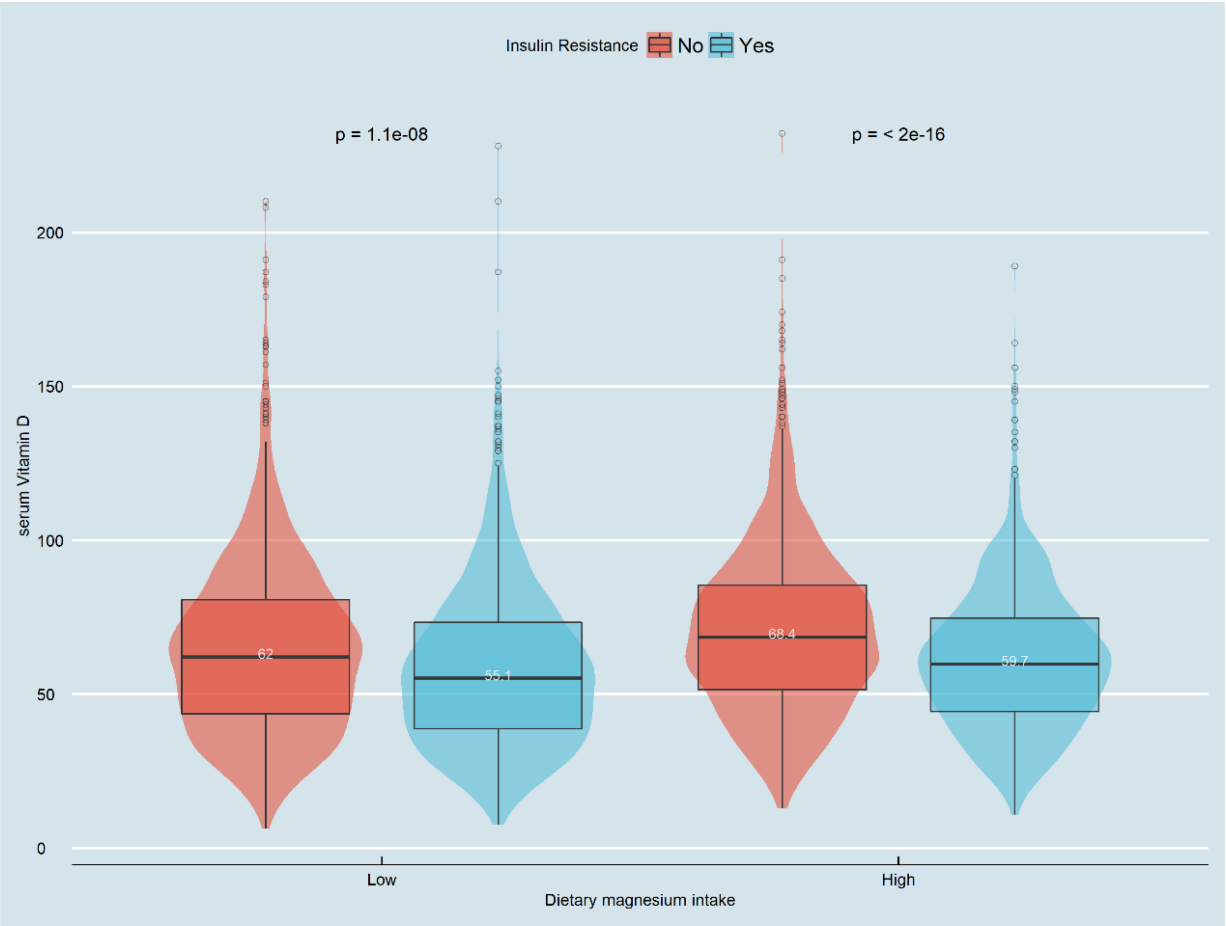


FIGURE 3 | Violin chart of distribution of serum vitamin D in patients with IR group by magnesium intake. In the low-magnesium group, serum vitamin D levels in those with insulin resistance were significantly lower than those without insulin resistance (55.1 vs. 62.0 nmol/L, $P < 0.001$). In the high-magnesium group, serum vitamin D levels in those with insulin resistance were significantly lower than those without insulin resistance (59.7 vs. 68.4 nmol/L, $p < 0.001$).

by NHANES, which means that all our subsequent studies were analyzed based on weighted models.

All data were analyzed using R version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria), with continuous variables represented by detailed sample descriptions with a mean confidence interval (CI) of 95%. Categorical variables were represented by counts and weighted percentages. Skewed distributions were based on median and Q1–Q3 values. Normal distributions were described by median and standard deviation values. Continuous variables were compared between groups using Student's *t*-test or the Mann–Whitney *U*-test based on the normality of the distribution, and comparisons were made using Fisher's exact probability method. Covariates were selected based on potential confounders that may be associated with Vit D and IR. Gender, age, race, smoking, BMI, obesity, dietary intake, physical activity, and education were selected as covariates based on a combination of previous literature, international standards, and relevant clinical experience. Multiple interpolation was used to fill in the missing covariates with the aim of maximizing statistical power and minimizing bias. In addition, sensitivity analyses were conducted to see if the resulting complete data differed significantly from the original data. These evaluations showed that the data after multiple interpolation did not differ significantly from the original data and were not statistically significant ($P > 0.05$). Therefore, all results of our multivariate analysis were based on the dataset developed after multiple interpolation according to Rubin's criterion.

Three multivariate logistic regression models were developed to analyze the relationship between Vit D and IR in the sample at different magnesium intakes, and smooth fitted curves were constructed. $P < 0.05$ (two-sided) was considered to be statistically significant. We also developed three linear regression models to analyze the relationship between Vit D and the HOMA-IR index at different magnesium intake levels. A sensitivity analysis was completed to ensure the robustness of the analysis. We transformed Vit D into a categorical variable and calculated *P*-values for trends. The aim was to test the possibility of observing the presence of non-linearity between Vit D and IR when Vit D level was used as a categorical variable (34).

TABLE 2 | Association of covariates and IR.

Variable	OR (95%CI)	*P*-value
Age	1.01 (1.01∼1.01)	<0.001
sex, *n* (%)		
Male	1	
Female	0.8 (0.71∼0.89)	<0.001
Race/ethnicity, *n* (%)		
Mexican–American	1	
Other Hispanic	0.71 (0.56∼0.9)	0.004
Non-Hispanic white	0.59 (0.5∼0.7)	<0.001
Non-Hispanic black	0.72 (0.59∼0.88)	0.001
Other races	0.43 (0.34∼0.55)	<0.001
Obesity, *n* (%)		
No	1	
Yes	6.73 (5.9∼7.67)	<0.001
Education level, *n* (%)		
Did not graduate from high school	1	
Graduated from high school	0.83 (0.7∼0.98)	0.026
College education or above	0.61 (0.54∼0.7)	<0.001
Smoking status, *n* (%)		
Current smoker	1	
Former smoker	0.95 (0.81∼1.13)	0.588
Never smoker	0.97 (0.84∼1.12)	0.669
Physical activity, *n* (%)		
Vigorous work activity	1	
Moderate work activity	0.97 (0.81∼1.16)	0.721
Walk or bicycle	1.13 (0.92∼1.38)	0.236
Vigorous recreational activities	0.93 (0.72∼1.2)	0.585
Moderate recreational activities	0.98 (0.83∼1.15)	0.774
Season of examination, *n* (%)		
Winter	1	
Summer	0.91 (0.81∼1.02)	0.099
Serum-Vit D	0.99 (0.99∼0.99)	<0.001
Mg-intake	0.99 (0.99∼0.99)	<0.001

Data presented are ORs and 95% CIs.

RESULTS

Basic Information of the Study Population

In the present study, a total of 4,878 participants from four NHANES cycles (2007–2014) were included (**Figure 1**). The basic information of the study population is detailed in **Table 1**. Grouping was performed based on magnesium intake, using a cutoff of 267 mg/d.

The mean age of all participants was 49.2 ± 17.7 years. In the low-intake group, the mean age was 50.3 ± 18.4 years, while, in the high-intake group, the mean age was 48.0 ± 16.9 years, and the difference in age between the two groups was statistically significant ($P < 0.001$). The proportion of obese participants in the low-intake group increased (35.8 → 38%) and the proportion of the same in the high-intake group decreased (35.8 → 33.5%) over time, respectively. In the low-intake group, BMI, fasting plasma glucose, and OGTT levels were higher than those in the high-intake group. In contrast, Vit D levels were significantly higher in the high-intake group than the low-intake group.

Bar Figure Analysis

Figure 2 displays the difference in Vit D level between high and low Mg intake groups (61.6 vs. 66.3 nmol/L, $P < 0.001$). Meanwhile, we observed that the Vit D level differed among the IR-positive and IR-negative groups ($P < 0.001$), as shown in **Figure 3**. The IR-positive group exhibited significantly lower Vit D levels than the IR-negative group (high Mg intake group: 59.7 *vs.* 68.4 nmol/L, $P < 0.001$; low Mg intake group: 55.1 vs. 62.0 nmol/L, $P < 0.001$).

Univariate Analysis of Which Variables Might Be Associated With Insulin Resistance

Univariate logistics regression was used to detect which factors were associated with the occurrence of insulin resistance. As shown in **Table 2**, women had a lower probability of developing insulin resistance relative to men [OR: 0.8 (0.71–0.89)]. Compared to Mexican–Americans, other Hispanics [OR: 0.71 (0.56–0.9)], non-Hispanic whites [OR: 0.59 (0.5–0.7)] non-Hispanic blacks [OR: 0.72 (0.59–0.88)] and other races [0.43 (0.34–0.55)] had a lower probability of insulin resistance. age. factors such as obesity and IR were positively associated. In contrast, factors such as educational level, physical activity, Vit D and dietary magnesium intake were negatively associated with insulin resistance.

Multivariable Logistics Regression Analysis of Vit D and Insulin Resistance

In the present study, three logistic regression models were constructed to analyze the independent association between Vit D level and IR and determine whether this association was influenced by different levels of magnesium intake. The model-based effect ratios (odds ratio [OR]) and 95% CIs shown in **Table 3** indicate that each single-unit increase in Vit D was associated with a corresponding decrease in the probability of IR occurring. For example, in the unadjusted model, the total effect value was 0.99. Each single-unit increase in Vit D meant a 1% reduction in IR (OR 0.99; 95% CI 0.99–0.99). In the high-intake group, the effect ratio (OR) and 95% CI were 0.98 (0.99–0.99), respectively, while in the low-intake group, the effect-value ratio (OR) and 95% CI were 0.99 (0.99–0.99). In model 2, which was adjusted for sociodemographic data only, the overall effect-value ratio (OR) and 95% CI were 0.98 (0.97–0.99); in the high-intake group, the effect-value ratio (OR) and 95% CI were 0.96 (0.93–0.99); and in the low-intake group, the effect-value ratio (OR) and 95% CI were 0.98 (0.97–0.99), respectively. In the fully adjusted model 3, the overall effect-value ratio (OR) and 95% CI were 0.93 (0.88–0.98); in the high-intake group, the effect-value ratio (OR) and 95% CI were 0.92 (0.88–0.96); and in the low-intake group, the effect-value ratio (OR) and 95% CI were 0.93 (0.88–0.98), respectively. The above results suggest an independent association between Vit D level and IR and

TABLE 3 | Interactive effect of vitamin D and dietary magnesium intake on IR (All participants).

	Model 1			Model 2			Model 3		
Variable	**OR (95%CI)**	***P*-value**	***P* for interaction**	**OR (95%CI)**	***P*-value**	***P* for interaction**	**OR (95%CI)**	***P*-value**	***P* for interaction**
Vit D	0.99 (0.99∼0.99)	<0.001		0.98 (0.97∼0.99)	<0.001		0.93 (0.88∼0.98)	<0.001	
Magnesium intake group									
<267 mg/day Vit D	0.99 (0.99∼0.99)	<0.001	<0.001	0.98 (0.97∼0.99)	<0.001	<0.001	0.94 (0.90∼0.98)	<0.001	<0.001
≥267 mg/day Vit D	0.98 (0.97∼0.99)	<0.001		0.96 (0.93∼0.99)	<0.001		0.92 (0.88∼0.96)	<0.001	

Model 1: non-adjusted. Model 2: adjusted age, sex, race. Model 3: adjusted age, sex, race, obesity, education level, physical activity, smoking status, the season of examination, and dietary calcium intake.

TABLE 4 | Interactive effect of vitamin D and dietary magnesium intake on HOMA-IR (All participants).

	Model 1			Model 2			Model 3		
Variable	**β (95%CI)**	***P*-value**	***P* for interaction**	**β (95%CI)**	***P*-value**	***P* for interaction**	**β (95%CI)**	***P*-value**	***P* for interaction**
Vit D	−0.02 (−0.02 to −0.01)	<0.001		−0.03 (−0.04 to −0.02)	<0.001		−0.04 (−0.06 to −0.02)	<0.001	
Magnesium intake group									
<267 mg/day Vit D	−0.02 (−0.02 to −0.01)	<0.001	<0.001	−0.03 (−0.04 to −0.02)	<0.001	<0.001	−0.04 (−0.06 to −0.02)	<0.001	<0.001
≥267 mg/day Vit D	−0.03 (−0.04 to −0.02)	<0.001		−0.04 (−0.05 to −0.03)	<0.001		−0.05 (−0.06 to −0.03)	<0.001	

Model 1: non-adjusted. Model 2: adjusted age, sex, race. Model 3: adjusted age, sex, race, obesity, education level, physical activity, smoking status, the season of examination, and dietary calcium intake.

confirm that this association was influenced by different levels of magnesium intake.

Three linear regression models were constructed to analyze the independent association between the Vit D level and HOMA-IR index and determine whether this association was influenced by different levels of magnesium intake. The model-based effect value β and 95% CI shown in **Table 4** indicate that the Vit D level and HOMA-IR index were independently correlated and influenced by different levels of magnesium intake after adjusting for covariates according to the full model (Model 3). The overall effect value β and 95% CI were −0.04 (−0.06 to −0.02), respectively. Additionally, in the high-intake group, the effect β and 95% CI were −0.05 (−0.06 to −0.03), while in the low-intake group, the effect values β and 95% CI were −0.04 (−0.06 to −0.02).

Linear Association Between Vit D and Insulin Resistance

We analyzed whether there was a linear relationship between Vit D and IR at different levels of magnesium intake. **Figures 4A1,A2** show the association between Vit D and IR after logistic regression without adjusting for latent variables. **Figures 4B1,B2** show the association between Vit D and IR after logistic regression adjusted for model 2. **Figures 4C1,C2** show the association between Vit D and IR after logistic regression adjusted for the full model (model 3). In summary, the associations between Vit D level and the risk of IR occurrence were linear at different levels of magnesium intake.

DISCUSSION

In recent decades, as the global economy has grown rapidly, the number of patients with type 2 diabetes has shown a significant increase in both developing and developed countries (35). Not only does this place a great burden on the world's medical resources, its complications also greatly plague patients physically and psychologically. IR is one of the main causes of type 2 diabetes, and about 90% of cases are caused by IR. Aside from the rising incidence of diabetes, there are far more people suffering from IR. How to effectively prevent IR in advance before the progression to diabetes has also become a matter of great concern to researchers.

In this study, multiple linear regression models were developed to investigate whether there was a significant independent association between Vit D and the HOMA-IR index and to analyze whether the independent association between Vit D and the HOMA-IR index was affected by different dietary magnesium intake levels. In addition, multiple logistic regression models were developed to analyze the independent association between Vit D and IR and to assess whether the independent association between Vit D and IR was affected

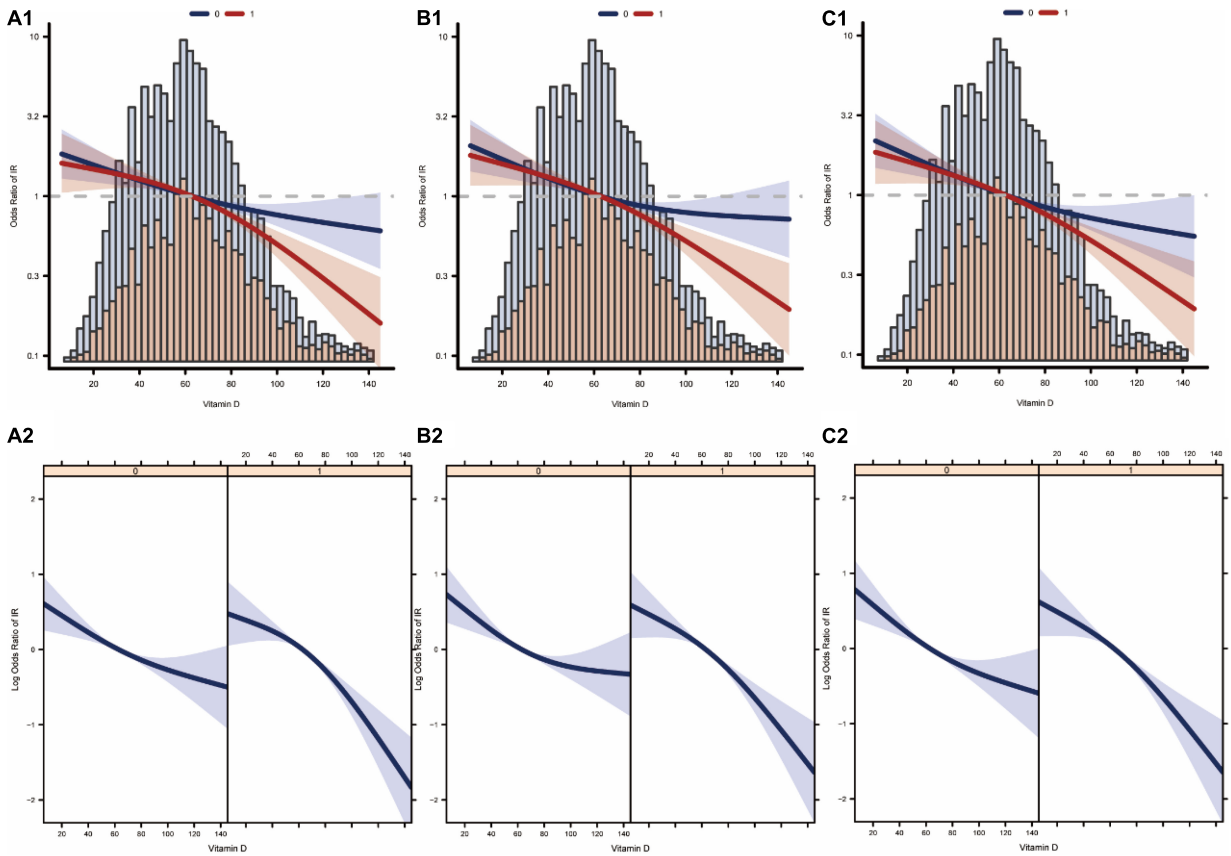


FIGURE 4 | Curve fitting of vitamin D levels and the risk of developing insulin resistance. **(A1,A2)** Are association between vitamin D and insulin resistance at different levels of dietary magnesium intake after adjustment by model 1. **(B1,B2)** Are association between vitamin D and insulin resistance at different levels of dietary magnesium intake after adjustment by model 2. **(C1,C2)** Are association between vitamin D and insulin resistance at different levels of dietary magnesium intake after adjustment by model 3 (0: low dietary magnesium intake group, 1: high dietary magnesium intake group).

at different dietary magnesium intake levels. After excluding the potential confounding effects, the results showed that Vit D levels were independently correlated with the HOMA-IR index and IR in the multivariate regression analysis, and this association seemed to be influenced by different levels of magnesium intake. Smoothed fitted curves also showed a linear relationship between Vit D level and HOMA-IR index at different levels of magnesium intake. The probability of the occurrence of IR decreased alongside increasing Vit D levels. As shown in **Figure 4**, the association between serum vitamin D levels and insulin resistance was stronger in the high-magnesium diet group. As vitamin D levels increased, the incidence of insulin resistance decreased more significantly. This trend is evident in all models.

It has been reported that reduced plasma Vit D levels may produce excessive white adipose tissue, leading to IR and dyslipidemia (36). In a cross-sectional analysis conducted by Bilge et al. (37) in 2015 on a Turkish population of 39 individuals with normal weights and 66 individuals categorized as obese, Vit D was found to be negatively associated with the modified HOMA-IR index after adjusting for laboratory indicators, physical measurements, and other factors Research conducted by Wang et al. (38) also found that Vit D deficiency may lead to increased activity of the nuclear factor kappa-light-chain-enhancer of activated B-cells pathway, which promotes inflammation and leads to IR. These studies also support our results.

Magnesium is the second most abundant intracellular divalent cation after potassium ion, and it has been recognized as a cofactor in > 300 enzymatic reactions, with it being particularly essential for adenosine triphosphate metabolism (39). Low magnesium (2+) levels lead to defective tyrosine kinase activity, and insulin acts on receptors that are later damaged, altering cellular glucose transport and reducing cellular glucose utilization, thereby promoting peripheral IR in type 2 diabetes (21, 40). In addition, our finding is also consistent with another cross-sectional analysis conducted in the NHANES that magnesium intakes interact with serum Vit D levels in relation to type 2 diabetes (36). Thus, magnesium supplementation has the potential to increase Vit D activity such that it increases Vit D's protection of pancreatic β-cells. A randomized clinical trial published in 2018 by Dai et al. showed that magnesium optimizes Vit D status in the body with a bidirectional regulatory effect; in other words, magnesium can be optimized according to the

body's original Vit D level so that Vit D levels are maintained in the normal range (23). Further, all enzymes used for the metabolism of Vit D seem to require magnesium, which acts as a co-factor in the enzymatic reactions of the liver and kidneys (24). Magnesium intake alone or its interaction with Vit D intake may contribute to Vit D status (25, 26). The enzymatic activation of 25-hydroxylase in the liver and 1α-hydroxylase in the kidneys is a process that requires magnesium.

A recent randomized trial found that magnesium treatment greatly reduced imidazole propionate, a microbial metabolite of histidine, compared to the placebo group. Imidazole propionate induces IR, and levels of imidazole propionate were higher in patients with prediabetes and type 2 diabetes. In addition to imipramine propionate, the same randomized trial found that magnesium treatment increased circulating levels of propionic acid and reduced levels of glutamate, two microbial metabolites. In fact, propionic acid and glutamate were associated with a reduced and increased risk of type 2 diabetes, respectively, and were inversely and positively associated with IR. In conclusion, the possible mechanism is that high magnesium intake increases Vit D synthesis on the one hand and improves microbial production of the three amino acid metabolites on the other hand, which in turn reduces IR and the risk of type 2 diabetes (41).

Although the current study is a cross-sectional analysis, our finding is consistent with that in a 2013 prospective cohort study in which the inverse associations between serum Vit D concentrations and risk of mortality due to cardiovascular disease only appeared in those with higher intakes of magnesium, but not in those with lower intakes of magnesium (25).

The present study has some limitations. First, because the NHANES database does not include some specific groups, such as pregnant women and children, it is uncertain whether the results of this study are applicable to these groups. We will analyze these groups in forthcoming studies, therefore this limitation will be addressed in the future. Second, the present study was a cross-sectional investigation, and no causal relationship could be drawn between Vit D levels and the HOMA-IR index as well as IR; thus, further cohort studies are needed to analyze this causal relationship. Finally, our dietary data were obtained from self-reported 24-h dietary-recall interviews and is therefore inevitably subject to some degrees of recall and self-report bias. However, the level of this influence is low and not sufficient to affect our results. This is because NHANES uses professional staff for data collection and a multistage stratified probability design approach for subject selection to decrease such bias. However, the present study also has certain advantages over other studies. In this study, a more comprehensive and more representative sample of participants was selected, with a unique representation of the entire U.S. population. In this study, the missing data were processed using statistical methods that are currently recognized by experts as more scientific (multiple interpolation) to maximize the statistical efficacy of the results as well as minimize the bias. Finally, smoothed fitted curves for Vit D and IR with different dietary magnesium intake levels were plotted to make the results more intuitive.

CONCLUSION

In this study, Vit D levels were found to be independently associated with the HOMA-IR index and IR among American adults after adjusting for potential confounders, and magnesium intake strengthened this association. The results of this study provide new clinical insights. However, because this was a cross-sectional analysis that could not determine the role of magnesium in the association of Vit D levels with IR and HOMA-IR index, more randomized controlled studies or cohort studies are required to provide evidence in the future.

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.

AUTHOR CONTRIBUTIONS

YL and RG conceived the idea and wrote the manuscript. HM, SC, JS, JQ, and YP collected, read the literature, and revised the article. JA and ZS read through and corrected the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Key R&D and Transformation Program of Qinghai Province-Special Project of Science and Technology Assistance (No. 2021-QY-213), the CAS (Light of the West China) Program (No. 2019-33), the Open Project of State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University (No. 2019-ZZ-07), and the Scientific Research Project of Research Team of Medical College of Qinghai University (No. 2020-KYT-2).

REFERENCES

1. Krentz AJ. Insulin resistance. *BMJ.* (1996) 313:1385–9. doi: 10.1136/bmj.313.7069.1385
2. Lebovitz HE. Insulin resistance: definition and consequences. *Exp Clin Endocrinol Diabetes.* (2001) 109(Suppl. 2):S135–48. doi: 10.1055/s-2001-18576
3. Brown AE, Walker M. Genetics of insulin resistance and the metabolic syndrome. *Curr Cardiol Rep.* (2016) 18:75.
4. Isenovic ER, Gluvic Z, Zaric B, Resanovic I, Obradovic M, Mitrovic A, et al. Link between metabolic syndrome and insulin resistance. *Curr Vasc Pharmacol.* (2017) 15:30–9. doi: 10.2174/1570161114666161007164510
5. Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, et al. Pathophysiology of type 2 diabetes. *Int J Mol Sci.* (2020) 21:6275.

6. Gong R, Xu Z, Wei X. The association between vitamin D3 and diabetes in both hyperuricemia and non-hyperuricemia populations. *Endocrine.* (2021) 74:90–9. doi: 10.1007/s12020-021-02778-y
7. Yin DT, He H, Yu K, Xie J, Lei M, Ma R, et al. The association between thyroid cancer and insulin resistance, metabolic syndrome and its components: a systematic review and meta-analysis. *Int J Surg.* (2018) 57:66–75. doi: 10.1016/j.ijsu.2018.07.013
8. Cirillo F, Catellani C, Sartori C, Lazzeroni P, Amarri S, Street ME. Obesity, insulin resistance, and colorectal cancer: could miRNA dysregulation play a role? *Int J Mol Sci.* (2019) 20:2922. doi: 10.3390/ijms20122922
9. Farahani H, Mahmoudi T, Asadi A, Nobakht H, Dabiri R, Hamta A. Insulin resistance and colorectal cancer risk: the role of elevated plasma resistin levels. *J Gastrointest Cancer.* (2020) 51:478–83. doi: 10.1007/s12029-019-00260-7
10. McNabney SM, Henagan TM. Short chain fatty acids in the colon and peripheral tissues: a focus on butyrate, colon cancer, obesity and insulin resistance. *Nutrients.* (2017) 9:1348. doi: 10.3390/nu9121348
11. Petridou ET, Sergentanis TN, Antonopoulos CN, Dessypris N, Matsoukis IL, Aronis K, et al. Insulin resistance: an independent risk factor for lung cancer? *Metabolism.* (2011) 60:1100–6. doi: 10.1016/j.metabol.2010.12.002
12. Hahn S, Haselhorst U, Tan S, Quadbeck B, Schmidt M, Roesler S, et al. Low serum 25-hydroxyvitamin D concentrations are associated with insulin resistance and obesity in women with polycystic ovary syndrome. *Exp Clin Endocrinol Diabetes.* (2006) 114:577–83. doi: 10.1055/s-2006-948308
13. Kamiński M, Uruska A, Rogowicz-Frontczak A, Lipski D, Niedźwiecki P, Różańska O, et al. Insulin resistance in adults with type 1 diabetes is associated with lower vitamin D serum concentration. *J Exp Clin Endocrinol Diabetes.* (2019) 129:396–402. doi: 10.1055/a-0895-5166
14. Peivasteh S, Daneshi-Maskooni M, Vafa M, Nourbakhsh M, Janani L, Maddah M, et al. Vitamin D supplementation improves SIRT1, Irisin, and glucose indices in overweight or obese type 2 diabetic patients: a double-blind randomized placebo-controlled clinical trial. *BMC Fam Pract.* (2020) 21:26. doi: 10.1186/s12875-020-1096-3
15. Contreras-Bolivar V, García-Fontana B, García-Fontana C, Muñoz-Torres M. Mechanisms involved in the relationship between vitamin D and insulin resistance: impact on clinical practice. *Nutrients.* (2021) 13:1986. doi: 10.3390/nu12071986
16. Schleu MF, Barreto-Duarte B, Arriaga MB, Araujo-Pereira M, Ladeia AM, Andrade BB, et al. Lower levels of vitamin D are associated with an increase in insulin resistance in obese Brazilian women. *Nutrients.* (2021) 13:2979. doi: 10.3390/nu13092979
17. Szymczak-Pajor I, Śliwińska A. Analysis of association between vitamin D deficiency and insulin resistance. *Nutrients.* (2019) 11:794. doi: 10.3390/nu11040794
18. Gulseth HL, Wium C, Angel K, Eriksen EF, Birkeland KI. Effects of vitamin D supplementation on insulin sensitivity and insulin secretion in subjects with type 2 diabetes and vitamin D deficiency: a randomized controlled trial. *Diabetes Care.* (2017) 40:872–8. doi: 10.2337/dc16-2302
19. Margaret C, Lansang MC. Effect of treating vitamin D deficiency in uncontrolled type 2 diabetes: a randomized, placebo-controlled study. *Am J Ther.* (2013) 20:638–53. doi: 10.1097/MJT.0000000000000738
20. Houston M. The role of magnesium in hypertension and cardiovascular disease. *J Clin Hypertens (Greenwich).* (2011) 13:843–7. doi: 10.1111/j.1751-7176.2011.00538.x
21. Takaya J, Higashino H, Kobayashi Y. Intracellular magnesium and insulin resistance. *Magnes Res.* (2004) 17:126–36.
22. Uwitonze AM, Razzaque MS. Role of magnesium in vitamin D activation and function. *J Am Osteopath Assoc.* (2018) 118:181–9.
23. Dai Q, Zhu X, Manson JE, Song Y, Li X, Franke AA, et al. Magnesium status and supplementation influence vitamin D status and metabolism: results from a randomized trial. *Am J Clin Nutr.* (2018) 108:1249–58. doi: 10.1093/ajcn/nqy274
24. Marie UA, Razzaque MS. Role of magnesium in vitamin D activation and function. *J Am Osteopath Assoc.* (2018) 118:181–9.
25. Deng X, Song Y, Manson JE, Signorello LB, Zhang SM, Shrubsole MJ, et al. Magnesium, vitamin D status and mortality: results from US national health and nutrition examination survey (NHANES) 2001 to 2006 and NHANES III. *BMC Med.* (2013) 11:187. doi: 10.1186/1741-7015-11-187
26. Rosanoff A, Dai Q, Shapses SA. Essential nutrient interactions: does low or suboptimal magnesium status interact with vitamin D and/or calcium status? *Adv Nutr.* (2016) 7:25–43. doi: 10.3945/an.115.008631
27. Campbell TC, Hayes JR. Role of nutrition in the drug-metabolizing enzyme system. *Pharmacol Rev.* (1974) 26:171–97.
28. Fain JA. NHANES. *Diabetes Educ.* (2017) 43:151.
29. Ahluwalia N, Dwyer J, Terry A, Moshfegh A, Johnson C. Update on NHANES dietary data: focus on collection, release, analytical considerations, and uses to inform public policy. *Adv Nutr.* (2016) 7:121–34. doi: 10.3945/an.115.009258
30. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* (2011) 96:1911–30. doi: 10.1210/jc.2011-0385
31. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* (1985) 28:412–9. doi: 10.1007/BF00280883
32. Sumner AE, Cowie CC. Ethnic differences in the ability of triglyceride levels to identify insulin resistance. *Atherosclerosis.* (2008) 196:696–703. doi: 10.1016/j.atherosclerosis.2006.12.018
33. Harreiter J, Roden M. [Diabetes mellitus-definition, classification, diagnosis, screening and prevention (Update 2019)]. *Wien Klin Wochenschr.* (2019) 131(Suppl. 1):6–15. doi: 10.1007/s00508-019-1450-4
34. Gong R, Liu Y, Luo G, Liu W, Jin Z, Xu Z, et al. Associations of TG/HDL ratio with the risk of prediabetes and diabetes in Chinese adults: a Chinese population cohort study based on open data. *Int J Endocrinol.* (2021) 2021:9949579. doi: 10.1155/2021/9949579
35. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat Rev Endocrinol.* (2018) 14:88–98. doi: 10.1038/nrendo.2017.151
36. Guareschi ZM, Valcanaia AC, Ceglarek VM, Hotz P, Amaral BK, de Souza DW, et al. The effect of chronic oral vitamin D supplementation on adiposity and insulin secretion in hypothalamic obese rats. *Br J Nutr.* (2019) 121:1334–44. doi: 10.1017/S0007114519000667
37. Bilge U, Nalacak M, Ünlüoglu I, Ipek M, Çeler Ö, Akalin A. Relationship between 1,25-dihydroxy vitamin D levels and homeostatic model assessment insulin resistance values in obese subjects. *Niger J Clin Pract.* (1900) 18:377–80. doi: 10.4103/1119-3077.151757
38. Wang W, Zhang J, Wang H, Wang X, Liu S. Vitamin D deficiency enhances insulin resistance by promoting inflammation in type 2 diabetes. *Int J Clin Exp Pathol.* (2019) 12:1859–67.
39. Gröber U, Schmidt J, Kisters K. Magnesium in prevention and therapy. *Nutrients.* (2015) 7:8199–226. doi: 10.3390/nu7095388
40. Kostov K. Effects of magnesium deficiency on mechanisms of insulin resistance in type 2 diabetes: focusing on the processes of insulin secretion and signaling. *Int J Mol Sci.* (2016) 17:1182. doi: 10.3390/ijms20061351
41. Fan L, Yu D, Zhu X, Huang X, Murff HJ, Azcarate-Peril MA, et al. Magnesium and imidazole propionate. *Clin Nutr ESPEN.* (2021) 41:436–8. doi: 10.1016/j.clnesp.2020.12.011

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.

frontiers | Frontiers in Endocrinology

TYPE Mini Review
PUBLISHED 15 July 2022
DOI 10.3389/fendo.2022.967774

OPEN ACCESS

EDITED BY
Mohammed S. Razzaque,
Lake Erie College of Osteopathic Medicine, United States

REVIEWED BY
Ryan Ross,
Rush University Medical Center, United States
Hirotaka Komaba,
Tokai University, Japan
Ronald Brown,
University of Waterloo, Canada
Shuanhu Zhou,
Brigham and Women's Hospital and Harvard Medical School, United States

*CORRESPONDENCE
Toshimi Michigami
michigami@wch.opho.jp

SPECIALTY SECTION
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 13 June 2022
ACCEPTED 29 June 2022
PUBLISHED 15 July 2022

CITATION
Michigami T (2022) Roles of osteocytes in phosphate metabolism. *Front. Endocrinol.* 13:967774. doi: 10.3389/fendo.2022.967774

Roles of osteocytes in phosphate metabolism

Toshimi Michigami*

Department of Bone and Mineral Research, Research Institute, Osaka Women's and Children's Hospital, Osaka Prefectural Hospital Organization, Izumi, Japan

Osteocytes are dendritic cells in the mineralized bone matrix that descend from osteoblasts. They play critical roles in controlling bone mass through the production of sclerostin, an inhibitor of bone formation, and receptor activator of nuclear factor κ B ligand, an inducer of osteoblastic bone resorption. Osteocytes also govern phosphate homeostasis through the production of fibroblast growth factor 23 (FGF23), which lowers serum phosphate levels by increasing renal phosphate excretion and reducing the synthesis of 1,25-dihydroxyvitamin D (1,25$(OH)_2$D), an active metabolite of vitamin D. The production of FGF23 in osteocytes is regulated by various local and systemic factors. *Phosphate-regulating gene homologous to endopeptidase on X chromosome* (*PHEX*), *dentin matrix protein 1* (*DMP1*), and *family with sequence similarity 20, member C* function as local negative regulators of FGF23 production in osteocytes, and their inactivation causes the overproduction of FGF23 and hypophosphatemia. Sclerostin has been suggested to regulate the production of FGF23, which may link the two functions of osteocytes, namely, the control of bone mass and regulation of phosphate homeostasis. Systemic regulators of FGF23 production include 1,25$(OH)_2$D, phosphate, parathyroid hormone, insulin, iron, and inflammation. Therefore, the regulation of FGF23 in osteocytes is complex and multifactorial. Recent mouse studies have suggested that decreases in serum phosphate levels from youth to adulthood are caused by growth-related increases in FGF23 production by osteocytes, which are associated with the down-regulation of *Phex* and *Dmp1*.

KEYWORDS

phosphate, osteoblast, osteocyte, fibroblast growth factor 23, regulation

Introduction

Osteocytes, which are terminally differentiated cells of the osteoblast lineage, are dendritic cells embedded within the mineralized bone matrix (1–3). Although osteocytes are the most abundant among all cells in bone, their location and inaccessibility has

delayed our understanding of their function at the molecular level. In the past few decades, mounting evidence has indicated that osteocytes play important roles in bone homeostasis. They produce sclerostin, a secreted potent suppressor of bone formation (4, 5). Furthermore, critical roles for osteocyte-derived receptor activator of nuclear factor κ B ligand (RANKL) in the control of postnatal bone resorption have been demonstrated in mouse models in which its expression was specifically deleted from osteocytes (6).

Phosphate is an essential nutrient that mediates the majority of biological processes (7). Fibroblast growth factor 23 (FGF23), which functions as a central regulator of phosphate metabolism in mammals, is mainly produced by osteocytes (8). In addition to FGF23, several other molecules responsible for phosphate homeostasis are highly expressed in osteocytes, which include *phosphate-regulating gene homologous to endopeptidase on X chromosome* (*PHEX*), *dentin matrix protein 1* (*DMP1*), and *family with sequence similarity 20, member C* (*FAM20C*), the genes responsible for hereditary hypophosphatemia (8–12). Current concepts on the molecular mechanisms by which osteocytes regulate phosphate metabolism are discussed herein.

Osteocyte differentiation from osteoblasts

Osteocytes account for 90-95% of all bone cells in adult bone and have the longest lifespan (1–3). In the process of osteocytogenesis, a subpopulation of matrix-producing osteoblasts on the bone surface become embedded within the matrix proteins they produce and differentiate into osteocytes with a decrease in the production of the bone matrix, marked changes in morphology, and the expression of genes that constitute the signature of osteocytes (1–3). Approximately 5 to 20% of osteoblasts mature into osteocytes, while the remainder die by apoptosis or become bone lining cells (3). During the maturation of osteoblasts into osteocytes, cell morphology changes to a stellate shape with long processes. Osteocytes reside in lacunae within the mineralized bone matrix, and interconnect with each other and with osteoblasts on the bone surface by their long cytoplasmic processes running through canaliculi. As osteoblasts differentiate into osteocytes, they acquire the expression of molecules that regulate bone homeostasis and phosphate metabolism (1–3).

Control of bone mass by osteocytes

Osteocytes embedded in the bone matrix sense mechanical signals and regulate bone formation and resorption. A previous study reported that the genetic ablation of osteocytes in mice led to osteoporotic bone loss and the suppression of mechanically-induced new bone formation (13). Sclerostin encoded by the *SOST* gene is secreted by osteocytes and suppresses bone formation (5). The inactivation and reduced expression of *SOST* in humans have been shown to be responsible for rare bone sclerosing diseases, such as sclerosteosis 1 and van Buchem diseases (14). Sclerostin binds to low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6 to inhibit Wnt/ß-catenin signaling (15). Wnt/ß-catenin signaling plays a critical role in controlling bone mass by promoting the commitment of mesenchymal progenitor cells into osteoblasts as well as the proliferation and differentiation of osteoblasts. Furthermore, Wnt/ß-catenin signaling has been shown to inhibit the differentiation and activation of osteoclasts (16, 17). Mechanical signaling was found to suppress the expression of sclerostin in osteocytes, which promoted bone formation by enhancing Wnt/β-catenin signaling (4). The mechanosensor channel Piezo1 has recently been suggested to be involved in the suppression of *Sost* expression by mechanical force (18). The bone anabolic effects of parathyroid hormone (PTH) are also mediated by the down-regulation of *Sost* (19).

Osteocytes also regulate osteoclastic bone resorption by producing RANKL. Mice with the conditional deletion of RANKL from osteocytes and some mature osteoblasts exhibited markedly impaired osteoclastic bone resorption after birth, leading to the osteopetrotic phenotype (6). Therefore, osteocytes play critical roles in bone homeostasis by controlling the formation and resorption of bone in postnatal life.

Production and effects of FGF23

As osteoblasts mature into osteocytes, they acquire the expression of various molecules involved in phosphate homeostasis, which include the genes responsible for hereditary hypophosphatemic diseases (1–3, 8). The high expression of these molecules indicates that osteocytes play essential roles in the regulation of phosphate metabolism as well as the control of bone mass.

FGF23, the key regulator in phosphate metabolism, consists of 251 amino acids including an amino-terminal signal sequence of 24 amino acids (20). It is mainly produced by osteocytes and exerts its effects on distant target organs, such as the kidneys. Its endocrine function is suggested to be conferred by its low binding affinity to heparin/heparan sulfate (21). In the kidneys, the main target for FGF23, it increases phosphate excretion by reducing the expression of type IIa and IIc sodium/phosphate (Na^+/Pi) co-transporters (designated as NaPi-IIa and NaPi-IIc, respectively) (20). In addition, FGF23 reduces the production of 1,25-dihydroxyvitamin D [1,25 $(OH)_2D$], an active metabolite of vitamin D, by suppressing the expression of 25-hydroxyvitamin D 1α-hydroxylase and

inducing that of 24-hydroxylase, which leads to the decreased absorption of phosphate in the intestines (20). At physiological concentrations, FGF23 requires a single-pass transmembrane protein, αKlotho as a co-receptor for its signal transduction through the FGF receptor (FGFR) (22, 23). FGF23 is inactivated by proteolytic cleavage between Arg^{179} and Ser^{180}. This cleavage is prevented by the *O*-glycosylation of FGF23 at Thr^{178}, a process that is mediated by UDP-*N*-acetyl-α-D-galacosamine: polypeptide *N*-acetylgalactosaminyltransferase 3 (GalNAc-T3) (24).

Figure 1 summarizes the roles of osteocytes in the control of bone mass and the regulation of phosphate metabolism.

Local regulators of FGF23 production in osteocytes

PHEX, *DMP1*, and *FAM20C* are highly expressed in osteocytes (8). Since inactivating variants of these genes cause the overproduction of FGF23 in osteocytes (25), these molecules are considered to function as local negative regulators of FGF23 production in osteocytes.

The *PHEX* gene is responsible for X-linked hypophosphatemic rickets (XLH), the most common form of hereditary hypophosphatemia (9). Although the PHEX protein is suggested to function as a cell surface-bound, Zinc-dependent protease based on its structure (26), its physiological substrates remain elusive, and FGF23 does not serve as its substrate (27). *DMP1* encodes an extracellular matrix protein belonging to the small integrin-binding ligand, N-linked glycoproteins (SIBLINGs) family, and its inactivating variants cause autosomal recessive hypophosphatemic rickets type 1 (ARHR1) (10, 11).

Studies using *Phex*-deficient hypophosphatemic *Hyp* mice and *Dmp1*-null mice have suggested that enhanced FGFR signaling in osteocytes contributes to the increased production of FGF23 in XLH and ARHR1 (8, 28, 29). In *Hyp* mice, the osteocytic expression of *Fgf1*, *Fgf2*, *Fgfr1–3*, and *Egr-1*, which is a target gene of activated FGFR signaling, was found to be markedly up-regulated (8), and the osteocyte-specific deletion of *Fgfr1* partially restored the overproduction of FGF23 and attenuated hypophosphatemia and mineralization defects (29). In a culture of bone marrow stromal cells isolated from *Dmp1*-null mice, the inhibition of FGFR signaling using SU5402 prevented increases in FGF23 expression levels (28). Furthermore, mice with the transgenic overexpression of high-molecular-weight isoforms of FGF2 in osteoblast lineage cells exhibited elevated FGF23 levels and hypophosphatemic rickets (30). In humans, osteoglophonic dysplasia caused by activating variants in FGFR1 is often associated with hypophosphatemia due to elevated FGF23 levels (31). These findings support activated FGFR signaling in osteocytes increasing the production of FGF23.

The *FAM20C* gene encodes a secreted kinase that phosphorylates a broad range of substrates, including FGF23 and proteins of the SIBLINGs family, such as DMP1 (32, 33). Inactivating variants of *FAM20C* cause Raine syndrome (RNS), an autosomal recessive disease characterized by neonatal osteosclerotic bone dysplasia of an aggressive onset and poor prognosis. Patients with mild RNS may survive and manifest hypophosphatemic rickets due to elevated FGF23 levels as well as dental anomalies (12, 34). FAM20C has been shown to directly phosphorylate FGF23 on Ser^{180}, which inhibits the *O*-glycosylation of FGF23 by GalNAc-T3. Therefore, the inactivation of FAM20C may increase the protein levels of intact FGF23 by reducing its cleavage (33).

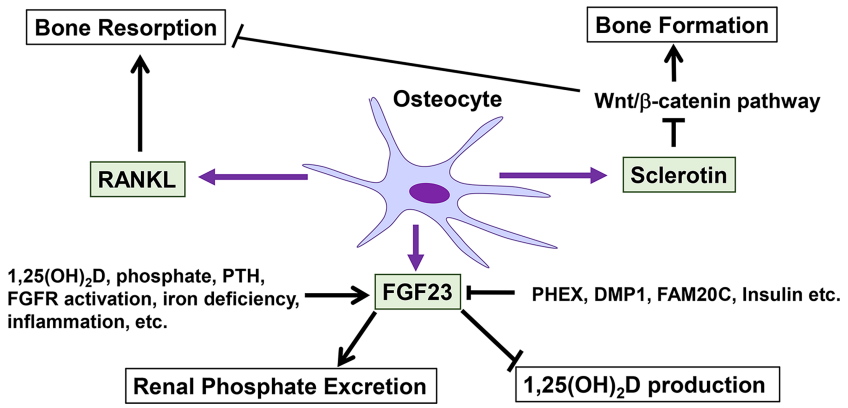


FIGURE 1
Roles of osteocytes in the control of bone mass and the regulation of phosphate metabolism. Osteocytes regulate bone formation and resorption through the production of sclerostin and RANKL. Sclerostin suppresses bone formation by inhibiting Wnt/β-catenin signaling. FGF23 produced by osteocytes plays central roles in phosphate metabolism by increasing renal phosphate excretion and decreasing the production of $1,25(OH)_2D$. The production of FGF23 in osteocytes is influenced by various positive and negative regulators.

Sclerostin may also function as a local regulator of FGF23. A recent study demonstrated that a treatment with an anti-sclerostin antibody reduced serum levels of intact FGF23 and increased serum phosphate levels in wild-type and *Phex*-deficient *Hyp* mice (35). While the serum intact FGF23 levels were reduced by anti-sclerostin antibody, the levels determined by C-terminal assay was found to be unchanged. Since the C-terminal FGF23 assay detects both the intact and cleaved C-terminal fragments of FGF23, the circulating C-terminal fragments of FGF23 were likely to be increased after the treatment with anti-sclerostin antibody (35). Considering a previous report demonstrating that the C-terminal fragments of FGF23 may antagonize the action of biologically active intact FGF23 (36), the elevation in serum phosphate levels following the treatment with anti-sclerostin antibody might be mediated by both the reduction in intact FGF23 levels and the increase in the C-terminal fragments. Although the regulation of FGF23 by sclerostin may be indirect and mediated by the control of bone turnover, a cell study using the osteocytic cell line IDG-SW3 suggested the direct stimulating effects of sclerostin on the synthesis of FGF23 (37). The regulation of FGF23 by sclerostin is interesting because it suggests a connection between the two important functions of osteocytes: the control of bone mass and the regulation of phosphate metabolism.

A clinical study previously demonstrated that the intravenous administration of pamidronate to patients with osteogenesis imperfecta rapidly decreased serum intact FGF23 levels (38), which suggested that bone turnover influences serum FGF23 levels. This concept was supported by a mouse study in which interleukin-1 (IL-1)-induced local bone resorption caused elevations in serum intact FGF23 levels without increasing its mRNA levels, and this elevation in FGF23 was prevented by a pre-treatment with a bisphosphonate pamidronate (39). Similarly, in nephrectomized rats with a high bone turnover renal osteodystrophy, a treatment with a bisphosphonate risedronate suppressed the elevation of serum intact FGF23 levels (40). These findings indicate that the release of FGF23 produced by osteocytes into the circulation is accelerated in association with bone resorption.

Systemic regulators of FGF23 production in osteocytes

In addition to the local regulators described above, FGF23 production by osteocytes is influenced by various systemic factors, some of which are described herein. Among the systemic regulators of FGF23 production, $1,25(OH)_2D$ appears to be a principal regulator and increases the expression of FGF23 in osteoblast lineage cells through the vitamin D receptor (VDR)-mediated transactivation of its gene (41, 42). The importance of $1,25(OH)_2D$ in the regulation of FGF23 is supported by clinical observations showing that patients with vitamin D deficiency have low levels of serum intact FGF23 (43).

PTH also stimulates the production of FGF23, as suggested by the elevated serum levels of FGF23 in patients and mouse models of hyperparathyroidism (44, 45). Elevated serum levels of intact FGF23 were also reported in patients with Jansen type metaphyseal chondrodysplasia, a skeletal dysplasia caused by activating variants in PTH receptor 1 (PTH1R) (46). The importance of PTH signaling in osteocytes for the regulation of FGF23 production has been shown in mouse studies demonstrating that the constitutive activation of PTH1R in osteocytes using a *Dmp1* promoter increased the production of FGF23 (47).

Phosphate itself also regulates the production of FGF23 in osteocytes. Previous studies reported that dietary phosphate loading increased serum intact FGF23 levels in both humans and mice (48, 49). We recently showed that a 72-hour treatment of primary mouse osteocytes with high phosphate increased FGF23 production *in vitro*, and this increase occurred at the protein level rather than at the mRNA level (50). Furthermore, a treatment of osteoblast lineage cells with high phosphate up-regulated the expression of the *Galnt3* gene, which prevented the cleavage-mediated inactivation of FGF23 (51).

Recent studies demonstrated that insulin signaling suppressed the osteocytic production of FGF23 through the activation of the AKT pathway. In clinical settings, negative correlations were reported between increases in plasma insulin levels after oral glucose loading and plasma intact FGF23 levels (52). A treatment of the cultured osteoblastic cell line UMR106 with insulin and insulin-like growth factor 1 (IGF-1) also suppressed the production of FGF23 through the activation of the AKT pathway and the inhibition of forkhead box protein 1 (FOXO1) (52). We recently reported that the osteocyte-specific deletion of phosphatase and tensin homolog deleted from chromosome 10 (PTEN), the molecule that antagonizes the insulin-induced activation of AKT, resulted in a decrease in the production of FGF23 in osteocytes, a reduction in renal phosphate excretion, and the attenuation of hyperphosphatemia (53). The knockdown of PTEN expression in UMR1-6 cells decreased the expression of *Fgf23*, which was partially restored by a treatment with rapamycin, suggesting the involvement of AKT/mechanistic target of rapamycin complex 1 (mTORC1) (53). These findings suggest that the insulin- and IGF1-induced activation of AKT in osteocytes inhibit the production of FGF23 through the FOXO1 and mTORC1 pathways.

Iron deficiency increases the production of FGF23 through the hypoxia-inducible factor 1α (HIF1α)-mediated transactivation of the gene (54, 55). The activation of the HIF pathway promotes the production of the hematopoietic hormone erythropoietin, which has been shown to increase the production of FGF23 (56).

Increased serum levels of intact FGF23 may be observed in patients with inflammatory diseases (57), and various

proinflammatory cytokines, such as tumor necrosis factor α, IL-1β, and IL-6, have been reported to increase the expression of *Fgf23* (58). Several mechanisms, including the activation of the HIF pathway, the involvement of NF-κB and signal transducer and activator of transcription 3, and lipocalin 2-mediated induction, have been suggested to contribute to inflammation-associated increases in the production of FGF23 (59–62).

Many other factors have also been suggested to regulate the production of FGF23, and there are several excellent review articles on this topic (57, 58). Therefore, the regulation of FGF23 is multifactorial and complex, and has not yet been elucidated in detail.

Growth-related changes in osteocytes and alterations in phosphate metabolism

Serum levels of phosphate are higher in children than in adults (63), which may be due to the high need for phosphate for the growth of the skeleton and soft tissues. However, the mechanisms underlying growth-related changes in phosphate metabolism remain unclear. Since osteocytes play central roles in phosphate homeostasis, we recently investigated the relationship between growth-related skeletal changes and alterations in phosphate metabolism from youth to adulthood using young (4-week-old) and adult (12-week-old) mice (50). Although serum phosphate levels were lower in young mice, serum intact FGF23 levels and the osteocytic production of FGF23 increased from youth to adulthood and were associated with the enhancement of the FGF23-mediated-bone-kidney axis (50). An analysis of osteocytes isolated from young and adult mice revealed that the mRNA and protein levels of Dmp1 and mRNA levels of Phex declined from youth to adulthood. Since they function in the negative regulation of FGF23 production, the down-regulation of Dmp1 and Phex may be one of the mechanisms contributing to growth-related increases in the production of FGF23 and decreases in serum phosphate levels (50). In isolated osteoblasts and osteocytes, gene responses to elevated extracellular phosphate levels were also markedly altered from youth to adulthood (50). These findings provide evidence for the critical roles of osteocytes in growth-related alterations in phosphate metabolism (**Figure 2**).

Phosphate sensing in osteocytes

To maintain phosphate homeostasis, organisms need to sense environmental and internal levels of phosphate and adapt to changes. Although the molecular mechanisms for phosphate sensing have been extensively investigated in unicellular organisms, such as bacteria and yeast, as well as in plants (64–66), the mechanisms by which mammals sense phosphate levels in individual cells or the whole body currently remain unknown. Previous studies, including ours, demonstrated that an elevation in extracellular phosphate directly exerted its effects on various cell types, including bone cells, through the activation of signaling pathways, such as the FGFR and Raf/MEK/ERK pathways (7, 67–70). The responsiveness of cells to elevated extracellular phosphate

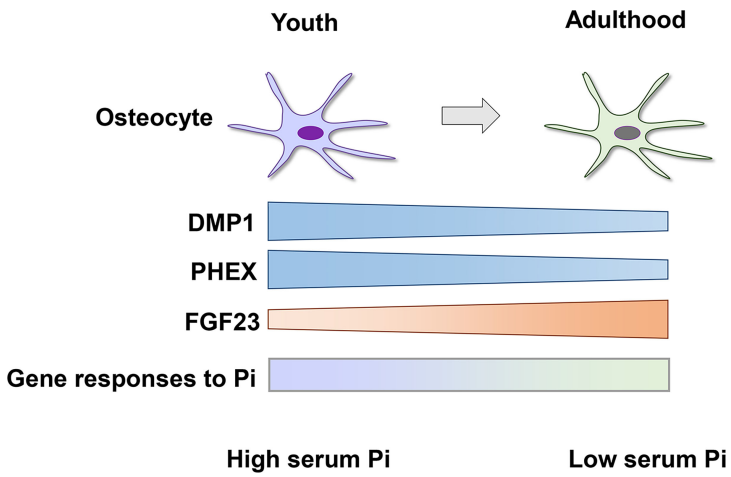


FIGURE 2
Growth-related changes in osteocytes cause a decrease in serum phosphate levels from youth to adulthood. The osteocytic expression of DMP1 and PHEX declines from youth to adulthood, which leads to an increase in the production of FGF23 and decrease in serum phosphate (Pi) levels. Gene responses of osteocytes to extracellular Pi are distinct between youth and adulthood (depicted as a shaded bar), suggesting growth-related differences in the sensitivity to the altered availability of Pi.

levels indicates that phosphate availability is detectable at the individual cell level. Since osteocytes play a central role in phosphate homeostasis, they may sense phosphate availability in the whole body. This concept appears to be supported by the close relationship between growth-related changes in osteocytic gene expression and their responses to phosphate and alterations in phosphate metabolism from youth to adulthood (50). A previous study reported that phosphate loading in mice up-regulated the skeletal expression of *Galnt3* by activating FGFR and increased the production of FGF23, suggesting the involvement of FGFR in phosphate sensing in mammals (51).

The parathyroid glands also respond to altered levels of extracellular phosphate. The secretion of PTH is stimulated by phosphate, and a recent study has suggested that this process is mediated by a direct action of phosphate on the calcium-sensing receptor (71). Thus, calcium-sensing receptor may function as a phosphate sensor in the parathyroids.

Conclusion

Osteocytes embedded in the mineralized bone matrix play central roles in the regulation of phosphate metabolism as well as in the control of bone mass. They control bone formation and resorption by producing sclerostin and RANKL, respectively. FGF23 produced mainly by osteocytes functions as a key regulator of phosphate homeostasis, and it increases renal phosphate excretion and decreases the synthesis of 1,25(OH)$_2$D. The production of FGF23 in osteocytes is influenced by multiple local and systemic regulators, and some of the local regulators, such as PHEX, DMP1, and FAM20C, were found to be responsible for hereditary hypophosphatemic diseases associated with the overproduction of FGF23. Serum phosphate levels are higher in children to meet the high needs for phosphate during growth. Mouse studies have suggested that decreases in serum phosphate levels from youth to adulthood are associated with growth-related increases in the production of FGF23 in osteocytes, which may be attributed to the down-regulation of PHEX and DMP1. Since osteocytes govern phosphate homeostasis, they may be responsible for sensing phosphate availability in the whole body. Although the mechanisms by which mammals sense phosphate levels remain largely unknown, FGFR appears to be involved in the process. Further clarification of the mechanisms by which osteocytes sense phosphate availability and regulate the production of FGF23 will contribute to a more detailed understanding of the pathogenesis of conditions with abnormal phosphate metabolism and the development of effective treatments.

Author contributions

TM developed the concept and prepared the manuscript.

Funding

Preparation of the manuscript was supported by a grant from Japan Society for the Promotion of Science (JSPS KAKENHI Grant Number 21K07835) to TM.

Conflict of interest

The 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

References

1. Bonewald LF. The amazing osteocyte. *J Bone Miner Res* (2011) 26(2):229–38. doi: 10.1002/jbmr.320

2. Dallas SL, Prideaux M, Bonewald LF. The osteocyte: an endocrine cell and more. *Endocr Rev* (2013) 34(5):658–90. doi: 10.1210/er.2012-1026

3. Delgado-Calle J, Bellido T. The osteocyte as a signaling cell. *Physiol Rev* (2022) 102(1):379–410. doi: 10.1152/physrev.00043.2020

4. Robling AG, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, et al. Mechanical stimulation of bone *in vivo* reduces osteocyte expression of sost/sclerostin. *J Biol Chem* (2008) 283(9):5866–75. doi: 10.1074/jbc.M705092200

5. Delgado-Calle J, Sato AY, Bellido T. Role and mechanism of action of sclerostin in bone. *Bone* (2017) 96:29–37. doi: 10.1016/j.bone.2016.10.007

6. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med* (2011) 17(10):1231–4. doi: 10.1038/nm.2452

7. Michigami T, Kawai M, Yamazaki M, Ozono K. Phosphate as a signaling molecule and its sensing mechanism. *Physiol Rev* (2018) 98(4):2317–48. doi: 10.1152/physrev.00022.2017

8. Miyagawa K, Yamazaki M, Kawai M, Nishino J, Koshimizu T, Ohata Y, et al. Dysregulated gene expression in the primary osteoblasts and osteocytes isolated from hypophosphatemic hyp mice. *PloS One* (2014) 9(4):e93840. doi: 10.1371/journal.pone.0093840

9. Francis SH F, Korn B, Reinhardt R, de Jong P, Poustka A, Lehrach H, et al. A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. the HYP consortium. *Nat Genet* (1995) 11 (2):130–6. doi: 10.1038/ng1095-130

10. Lorenz-Depiereux B, Bastepe M, Benet-Pages A, Amyere M, Wagenstaller J, Muller-Barth U, et al. DMP1 mutations in autosomal recessive hypophosphatemia

implicate a bone matrix protein in the regulation of phosphate homeostasis. *Nat Genet* (2006) 38(11):1248–50. doi: 10.1038/ng1868

11. Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B, et al. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet* (2006) 38(11):1310–5. doi: 10.1038/ng1905

12. Rafaelsen SH, Raeder H, Fagerheim AK, Knappskog P, Carpenter TO, Johansson S, et al. Exome sequencing reveals FAM20c mutations associated with fibroblast growth factor 23-related hypophosphatemia, dental anomalies, and ectopic calcification. *J Bone Miner Res* (2013) 28(6):1378–85. doi: 10.1002/jbmr.1850

13. Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, et al. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab* (2007) 5(6):464–75. doi: 10.1016/j.cmet.2007.05.001

14. Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, et al. Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *Am J Hum Genet* (2001) 68(3):577–89. doi: 10.1086/318811

15. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, et al. Sclerostin binds to LRP5/6 and antagonizes canonical wnt signaling. *J Biol Chem* (2005) 280 (20):19883–7. doi: 10.1074/jbc.M413274200

16. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med* (2013) 19(2):179–92. doi: 10.1038/nm.3074

17. Kubota T, Michigami T, Ozono K. Wnt signaling in bone metabolism. *J Bone Miner Metab* (2009) 27(3):265–71. doi: 10.1007/s00774-009-0064-8

18. Sasaki F, Hayashi M, Mouri Y, Nakamura S, Adachi T, Nakashima T. Mechanotransduction *via* the Piezo1-akt pathway underlies sost suppression in osteocytes. *Biochem Biophys Res Commun* (2020) 521(3):806–13. doi: 10.1016/j.bbrc.2019.10.174

19. Leupin O, Kramer I, Collette NM, Loots GG, Natt F, Kneissel M, et al. Control of the SOST bone enhancer by PTH using MEF2 transcription factors. *J Bone Miner Res* (2007) 22(12):1957–67. doi: 10.1359/jbmr.070804

20. Shimada T, Mizutani S, Muto T, Yoneya T, Hino R, Takeda S, et al. Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia. *Proc Natl Acad Sci U S A* (2001) 98(11):6500–5. doi: 10.1073/pnas.101545198

21. Goetz R, Beenken A, Ibrahimi OA, Kalinina J, Olsen SK, Eliseenkova AV, et al. Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. *Mol Cell Biol* (2007) 27(9):3417–28. doi: 10.1128/MCB.02249-06

22. Kurosu H, Ogawa Y, Miyoshi M, Yamamoto M, Nandi A, Rosenblatt KP, et al. Regulation of fibroblast growth factor-23 signaling by klotho. *J Biol Chem* (2006) 281(10):6120–3. doi: 10.1074/jbc.C500457200

23. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, et al. Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* (2006) 444(7120):770–4. doi: 10.1038/nature05315

24. Kato K, Jeanneau C, Tarp MA, Benet-Pages A, Lorenz-Depiereux B, Bennett EP, et al. Polypeptide GalNAc-transferase T3 and familial tumoral calcinosis. secretion of fibroblast growth factor 23 requires O-glycosylation. *J Biol Chem* (2006) 281(27):18370–7. doi: 10.1074/jbc.M602469200

25. Fukumoto S. FGF23-related hypophosphatemic rickets/osteomalacia: diagnosis and new treatment. *J Mol Endocrinol* (2021) 66(2):R57–65. doi: 10.1530/JME-20-0089

26. Drezner MK. PHEX gene and hypophosphatemia. *Kidney Int* (2000) 57 (1):9–18. doi: 10.1046/j.1523-1755.2000.00807.x

27. Benet-Pages A, Lorenz-Depiereux B, Zischka H, White KE, Econs MJ, Strom TM. FGF23 is processed by proprotein convertases but not by PHEX. *Bone* (2004) 35(2):455–62. doi: 10.1016/j.bone.2004.04.002

28. Martin A, Liu S, David V, Li H, Karydis A, Feng JQ, et al. Bone proteins PHEX and DMP1 regulate fibroblastic growth factor Fgf23 expression in osteocytes through a common pathway involving FGF receptor (FGFR) signaling. *FASEB J* (2011) 25(8):2551–62. doi: 10.1096/fj.10-177816

29. Xiao Z, Huang J, Cao L, Liang Y, Han X, Quarles LD. Osteocyte-specific deletion of Fgfr1 suppresses FGF23. *PloS One* (2014) 9(8):e104154. doi: 10.1371/journal.pone.0104154

30. Xiao L, Naganawa T, Lorenzo J, Carpenter TO, Coffin JD, Hurley MM. Nuclear isoforms of fibroblast growth factor 2 are novel inducers of hypophosphatemia *via* modulation of FGF23 and KLOTHO. *J Biol Chem* (2010) 285(4):2834–46. doi: 10.1074/jbc.M109.030577

31. White KE, Cabral JM, Davis SI, Fishburn T, Evans WE, Ichikawa S, et al. Mutations that cause osteoglophonic dysplasia define novel roles for FGFR1 in bone elongation. *Am J Hum Genet* (2005) 76(2):361–7. doi: 10.1086/427956

32. Tagliabracci VS, Engel JL, Wen J, Wiley SE, Worby CA, Kinch LN, et al. Secreted kinase phosphorylates extracellular proteins that regulate biomineralization. *Science* (2012) 336(6085):1150–3. doi: 10.1126/science.1217817

33. Tagliabracci VS, Engel JL, Wiley SE, Xiao J, Gonzalez DJ, Nidumanda Appaiah H, et al. Dynamic regulation of FGF23 by Fam20C phosphorylation, GalNAc-T3 glycosylation, and furin proteolysis. *Proc Natl Acad Sci U S A* (2014) 111(15):5520–5. doi: 10.1073/pnas.1402218111

34. Takeyari S, Yamamoto T, Kinoshita Y, Fukumoto S, Glorieux FH, Michigami T, et al. Hypophosphatemic osteomalacia and bone sclerosis caused by a novel homozygous mutation of the FAM20C gene in an elderly man with a mild variant of raine syndrome. *Bone* (2014) 67:56–62. doi: 10.1016/j.bone.2014.06.026

35. Carpenter KA, Ross RD. Sclerostin antibody treatment increases bone mass and normalizes circulating phosphate levels in growing hyp mice. *J Bone Miner Res* (2020) 35(3):596–607. doi: 10.1002/jbmr.3923

36. Goetz R, Nakada Y, Hu MC, Kurosu H, Wang L, Nakatani T, et al. Isolated c-terminal tail of FGF23 alleviates hypophosphatemia by inhibiting FGF23-FGFR-Klotho complex formation. *Proc Natl Acad Sci U S A* (2010) 107(1):407–12. doi: 10.1073/pnas.0902006107

37. Ito N, Prideaux M, Wijenayaka AR, Yang D, Ormsby RT, Bonewald LF, et al. Sclerostin directly stimulates osteocyte synthesis of fibroblast growth factor-23. *Calcif Tissue Int* (2021) 109(1):66–76. doi: 10.1007/s00223-021-00823-6

38. Kitaoka T, Namba N, Miura K, Kubota T, Ohata Y, Fujiwara M, et al. Decrease in serum FGF23 levels after intravenous infusion of pamidronate in patients with osteogenesis imperfecta. *J Bone Miner Metab* (2011) 29(5):598–605. doi: 10.1007/s00774-011-0262-z

39. Yamazaki M, Kawai M, Miyagawa K, Ohata Y, Tachikawa K, Kinoshita S, et al. Interleukin-1-induced acute bone resorption facilitates the secretion of fibroblast growth factor 23 into the circulation. *J Bone Miner Metab* (2015) 33 (3):342–54. doi: 10.1007/s00774-014-0598-2

40. Ishida H, Komaba H, Hamano N, Yamato H, Sawada K, Wada T, et al. Skeletal and mineral metabolic effects of risedronate in a rat model of high-turnover renal osteodystrophy. *J Bone Miner Metab* (2020) 38(4):501–10. doi: 10.1007/s00774-020-01095-0

41. Liu S, Tang W, Zhou J, Stubbs JR, Luo Q, Pi M, et al. Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin d. *J Am Soc Nephrol* (2006) 17(5):1305–15. doi: 10.1681/ASN.2005111185

42. Haussler MR, Livingston S, Sabir ZL, Haussler CA, Jurutka PW. Vitamin d receptor mediates a myriad of biological actions dependent on its 1,25-dihydroxyvitamin d ligand: Distinct regulatory themes revealed by induction of klotho and fibroblast growth factor-23. *JBMR Plus* (2021) 5(1):e10432. doi: 10.1002/jbm4.10432

43. Endo I, Fukumoto S, Ozono K, Namba N, Tanaka H, Inoue D, et al. Clinical usefulness of measurement of fibroblast growth factor 23 (FGF23) in hypophosphatemic patients: proposal of diagnostic criteria using FGF23 measurement. *Bone* (2008) 42(6):1235–9. doi: 10.1016/j.bone.2008.02.014

44. Kobayashi K, Imanishi Y, Miyauchi A, Onoda N, Kawata T, Tahara H, et al. Regulation of plasma fibroblast growth factor 23 by calcium in primary hyperparathyroidism. *Eur J Endocrinol* (2006) 154(1):93–9. doi: 10.1530/eje.1.02053

45. Kawata T, Imanishi Y, Kobayashi K, Miki T, Arnold A, Inaba M, et al. Parathyroid hormone regulates fibroblast growth factor-23 in a mouse model of primary hyperparathyroidism. *J Am Soc Nephrol* (2007) 18(10):2683–8. doi: 10.1681/ASN.2006070783

46. Brown WW, Juppner H, Langman CB, Price H, Farrow EG, White KE, et al. Hypophosphatemia with elevations in serum fibroblast growth factor 23 in a child with jansen's metaphyseal chondrodysplasia. *J Clin Endocrinol Metab* (2009) 94 (1):17–20. doi: 10.1210/jc.2008-0220

47. Rhee Y, Bivi N, Farrow E, Lezcano V, Plotkin LI, White KE, et al. Parathyroid hormone receptor signaling in osteocytes increases the expression of fibroblast growth factor-23 *in vitro* and *in vivo*. *Bone* (2011) 49(4):636–43. doi: 10.1016/j.bone.2011.06.025

48. Ferrari SL, Bonjour JP, Rizzoli R. Fibroblast growth factor-23 relationship to dietary phosphate and renal phosphate handling in healthy young men. *J Clin Endocrinol Metab* (2005) 90(3):1519–24. doi: 10.1210/jc.2004-1039

49. Perwad F, Azam N, Zhang MY, Yamashita T, Tenenhouse HS, Portale AA. Dietary and serum phosphorus regulate fibroblast growth factor 23 expression and 1,25-dihydroxyvitamin d metabolism in mice. *Endocrinology* (2005) 146(12):5358–64. doi: 10.1210/en.2005-0777

50. Michigami T, Tachikawa K, Yamazaki M, Nakanishi T, Kawai M, Ozono K. Growth-related skeletal changes and alterations in phosphate metabolism. *Bone* (2022) 161:116430. doi: 10.1016/j.bone.2022.116430

51. Takashi Y, Kosako H, Sawatsubashi S, Kinoshita Y, Ito N, Tsoumpra MK, et al. Activation of unliganded FGF receptor by extracellular phosphate potentiates proteolytic protection of FGF23 by its O-glycosylation. *Proc Natl Acad Sci USA* (2019) 116(23):11418–27. doi: 10.1073/pnas.1815166116

52. Bar L, Feger M, Fajol A, Klotz LO, Zeng S, Lang F, et al. Insulin suppresses the production of fibroblast growth factor 23 (FGF23). *Proc Natl Acad Sci USA* (2018) 115(22):5804–9. doi: 10.1073/pnas.1800160115

53. Kawai M, Kinoshita S, Ozono K, Michigami T. Lack of PTEN in osteocytes increases circulating phosphate concentrations by decreasing intact fibroblast growth factor 23 levels. *Sci Rep* (2020) 10(1):21501. doi: 10.1038/s41598-020-78692-6

54. Farrow EG, Yu X, Summers LJ, Davis SI, Fleet JC, Allen MR, et al. Iron deficiency drives an autosomal dominant hypophosphatemic rickets (ADHR) phenotype in fibroblast growth factor-23 (Fgf23) knock-in mice. *Proc Natl Acad Sci USA* (2011) 108(46):E1146–55. doi: 10.1073/pnas.1110905108

55. Wheeler JA, Clinkenbeard EL. Regulation of fibroblast growth factor 23 by iron, EPO, and HIF. *Curr Mol Biol Rep* (2019) 5(1):8–17. doi: 10.1007/s40610-019-0110-9

56. Daryadel A, Bettoni C, Haider T, Imenez Silva PH, Schnitzbauer U, Pastor-Arroyo EM, et al. Erythropoietin stimulates fibroblast growth factor 23 (FGF23) in mice and men. *Pflugers Arch* (2018) 470(10):1569–82. doi: 10.1007/s00424-018-2171-7

57. Rausch S, Foller M. The regulation of FGF23 under physiological and pathophysiological conditions. *Pflugers Arch* (2022) 474(3):281–92. doi: 10.1007/s00424-022-02668-w

58. Simic P, Babitt JL. Regulation of FGF23: Beyond bone. *Curr Osteoporos Rep* (2021) 19(6):563–73. doi: 10.1007/s11914-021-00703-w

59. Zhang B, Yan J, Umbach AT, Fakhri H, Fajol A, Schmidt S, et al. NFkappaB-sensitive Orai1 expression in the regulation of FGF23 release. *J Mol Med (Berl)* (2016) 94(5):557–66. doi: 10.1007/s00109-015-1370-3

60. Durlacher-Betzer K, Hassan A, Levi R, Axelrod J, Silver J, Naveh-Many T. Interleukin-6 contributes to the increase in fibroblast growth factor 23 expression in acute and chronic kidney disease. *Kidney Int* (2018) 94(2):315–25. doi: 10.1016/j.kint.2018.02.026

61. Courbon G, Francis C, Gerber C, Neuburg S, Wang X, Lynch E, et al. Lipocalin 2 stimulates bone fibroblast growth factor 23 production in chronic kidney disease. *Bone Res* (2021) 9(1):35. doi: 10.1038/s41413-021-00154-0

62. David V, Francis C, Babitt JL. Ironing out the cross talk between FGF23 and inflammation. *Am J Physiol Renal Physiol* (2017) 312(1):F1–8. doi: 10.1152/ajprenal.00359.2016

63. Yamazaki Y, Imura A, Urakawa I, Shimada T, Murakami J, Aono Y, et al. Establishment of sandwich ELISA for soluble alpha-klotho measurement: Age-dependent change of soluble alpha-klotho levels in healthy subjects. *Biochem Biophys Res Commun* (2010) 398(3):513–8. doi: 10.1016/j.bbrc.2010.06.110

64. Silver J, Dranitzki-Elhalel M. Sensing phosphate across the kingdoms. *Curr Opin Nephrol Hypertens* (2003) 12(4):357–61. doi: 10.1097/00041552-200307000-00003

65. Mouillon JM, Persson BL. New aspects on phosphate sensing and signalling in saccharomyces cerevisiae. *FEMS Yeast Res* (2006) 6(2):171–6. doi: 10.1111/j.1567-1364.2006.00036.x

66. Chiou TJ, Lin SI. Signaling network in sensing phosphate availability in plants. *Annu Rev Plant Biol* (2011) 62:185–206. doi: 10.1146/annurev-arplant-042110-103849

67. Yamazaki M, Ozono K, Okada T, Tachikawa K, Kondou H, Ohata Y, et al. Both FGF23 and extracellular phosphate activate Raf/MEK/ERK pathway *via* FGF receptors in HEK293 cells. *J Cell Biochem* (2010) 111(5):1210–21. doi: 10.1002/jcb.22842

68. Kimata M, Michigami T, Tachikawa K, Okada T, Koshimizu T, Yamazaki M, et al. Signaling of extracellular inorganic phosphate up-regulates cyclin D1 expression in proliferating chondrocytes *via* the Na+/Pi cotransporter pit-1 and Raf/MEK/ERK pathway. *Bone* (2010) 47(5):938–47. doi: 10.1016/j.bone.2010.08.006

69. Nishino J, Yamazaki M, Kawai M, Tachikawa K, Yamamoto K, Miyagawa K, et al. Extracellular phosphate induces the expression of dentin matrix protein 1 through the FGF receptor in osteoblasts. *J Cell Biochem* (2017) 118(5):1151–63. doi: 10.1002/jcb.25742

70. Beck GR Jr., Knecht N. Osteopontin regulation by inorganic phosphate is ERK1/2-, protein kinase c-, and proteasome-dependent. *J Biol Chem* (2003) 278(43):41921–9. doi: 10.1074/jbc.M304470200

71. Centeno PP, Herberger A, Mun HC, Tu C, Nemeth EF, Chang W, et al. Phosphate acts directly on the calcium-sensing receptor to stimulate parathyroid hormone secretion. *Nat Commun* (2019) 10(1):4693. doi: 10.1038/s41467-019-12399-9

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 09 August 2022
DOI 10.3389/fendo.2022.937380

OPEN ACCESS

EDITED BY
Mohammed S. Razzaque,
Lake Erie College of Osteopathic Medicine, United States

REVIEWED BY
Evelyn Frias-Toral,
Catholic University of Santiago de Guayaquil, Ecuador
Hua Yue,
Shanghai Jiao Tong University, China
Chihua Li,
Columbia University Irving Medical Center, United States

*CORRESPONDENCE
Li-hao Sun
slh10945@rjh.com.cn
Bei Tao
tb11454@rjh.com.cn
Jian-min Liu
ljm10586@rjh.com.cn

†Present Address:
Ling-ying Ma,
Department of Rheumatology, Zhongshan Hospital, Fu-dan University, Shanghai, China

SPECIALTY SECTION
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

RECEIVED 06 May 2022
ACCEPTED 18 July 2022
PUBLISHED 09 August 2022

CITATION
Yang Y-y, Zhang D, Ma L-y, Hou Y-f, Bi Y-f, Xu Y, Xu M, Zhao H-y, Sun L-h, Tao B and Liu J-m (2022) Association of famine exposure and the serum calcium level in healthy Chinese adults.
Front. Endocrinol. 13:937380.
doi: 10.3389/fendo.2022.937380

Association of famine exposure and the serum calcium level in healthy Chinese adults

Yu-ying Yang[1,2], Deng Zhang[1,2], Ling-ying Ma[1,2†], Yan-fang Hou[1,2], Yu-fang Bi[1,2], Yu Xu[1,2], Min Xu[1,2], Hong-yan Zhao[1,2], Li-hao Sun[1,2*], Bei Tao[1,2*] and Jian-min Liu[1,2*]

[1] Department of Endocrine and Metabolic Diseases, Shanghai Institute of Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2]Shanghai National Clinical Research Center for Metabolic Diseases, Key Laboratory for Endocrine and Metabolic Diseases of the National Health Commission of the PR China, Shanghai National Center for Translational Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Objective: Famine exposure and higher serum calcium levels are related with increased risk of many disorders, including Alzheimer's disease, atherosclerosis, diabetes, and osteoporosis. Whether famine exposure has any effect on serum calcium level is unclear. Besides, the normal reference range of serum calcium is variable among different populations. Our aims are 1) determining the reference interval of calcium in Chinese adults; 2) exploring its relationship with famine exposure.

Methods: Data in this study was from a cross-sectional study of the epidemiologic investigation carried out during March-August 2010 in Jiading district, Shanghai, China. Nine thousand and two hundred eleven participants with estimated glomerular filtration rate (eGFR) ≥60ml/min/1.73m^2 were involved to calculate reference interval of total calcium from 10569 participants aged 40 years or older. The analysis of famine exposure was conducted in 9315 participants with complete serum biochemical data and birth year information.

Results: After rejecting outliers, the 95% reference interval of total serum calcium was 2.122~2.518 mmol/L. The equation of albumin-adjusted calcium was: Total calcium + 0.019* (49-Albumin), with a 95% reference interval of 2.151~2.500 mmol/L. Compared to the age-balanced control group, there was an increased risk of being at the upper quartile of total serum calcium (OR=1.350, 95%CI=1.199-1.521) and albumin-adjusted calcium (OR=1.381, 95%CI=1.234-1.544) in subjects experienced famine exposure in childhood. Females were more vulnerable to this impact (OR= 1.621, 95%CI= 1.396-1.883 for total serum calcium; OR=1.722, 95%CI= 1.497-1.980 for albumin-adjusted calcium).

Conclusions: Famine exposure is an important environmental factor associated with the changes in circulating calcium concentrations, the newly

established serum calcium normal range and albumin-adjusted calcium equation, together with the history of childhood famine exposure, might be useful in identifying subjects with abnormal calcium homeostasis and related diseases, especially in females.

KEYWORDS

famine, serum calcium, albumin-adjusted calcium, reference interval, hypercalcemia

Introduction

Famine experience, especially during early life, has gathered increasing attention worldwide. Studies from the Dutch famine as well as the Great China's Famine showed that experiencing food shortage during early life is associated with a higher risk of osteoporosis, vertebral fracture, type 2 diabetes, obesity, coronary artery disease, cognition decline, and schizophrenia (1–10). However, the underlying mechanism, especially the common causes or factors responsible for or related to these varieties of diseases, is poorly understood.

During the past two decades, mounts of evidence have revealed the interaction between skeleton metabolism and the functionalities of organs and systems (11–14). It is reported that bone resorption, with its consequence of motivating skeletal calcium into circulation, is one of the major mediating factors for such a connection (15–17). In fact, among those diseases related to famine exposure, many of them are also associated with higher serum calcium levels (18–23). For example, individuals having higher serum calcium, although still in the normal range, are at higher risk of intracranial atherosclerosis (18) and presence of calcified coronary atherosclerotic plaque (24), cognition decline, and clinical progression of Alzheimer's disease (20), prevalence of adult overweight or obesity (25), incident type 2 diabetes (21), and lower bone mineral densities (BMDs) (22, 23). These studies indicated that elevated serum calcium level is not only an indicator but also the causal factor of the pathological processes. Thus, it makes establishing an adequate reference interval of serum calcium and finding its influencing factors essential to distinguish these pathological conditions at an early stage.

Serum calcium is closely regulated within an exquisitely narrow range. However, variation exists among different ethnics. A study regarding US civilian population showed that Mexican-Americans have lower serum calcium levels than Hispanics, while non-Hispanic blacks have higher serum calcium concentrations than non-Hispanic whites (26). On the other hand, in disease conditions like chronic kidney disease (CKD), black patients had lower serum calcium concentrations compared with white patients (27). Likely, the equation used to calculate albumin-adjusted calcium varies among different countries and regions (28, 29), and the use of population-specific equations improved the diagnostic accuracy of the adjusted calcium than the commonly used equation described by Payne et al. in 1973 (30, 31). These findings indicate that serum calcium levels may be influenced by ethnicity and it is necessary to determine adequate reference intervals as well as albumin-adjusted calcium equation regarding specific races, geographic regions and populations.

Serum calcium is regulated mainly by three systems: intestinal resorption, kidney reabsorption, and bone resorption. Low serum calcium or some pathological conditions can trigger a series of pathophysiological processes to increase calcium absorption by the intestines and reabsorption in the kidney (32, 33). More importantly, the release of calcium from the skeleton through bone resorption contributed significantly to an elevation of serum calcium level (34). In addition, recent studies reported that nutrition status may influence serum calcium level (35, 36) and famine exposure is also associated with metabolic bone abnormalities (7, 37). Thus, it is of interest and necessary to investigate whether serum calcium levels are affected by famine exposure.

In this study, we aimed to establish a reference interval of serum calcium level and equation for albumin-adjusted calcium in Chinese adults; and to explore the relationship between serum calcium level and famine exposure.

Materials and methods

Participants

Data in this study were from a cross-sectional study of the epidemiologic investigation carried out during March-August 2010 in Jiading district, Shanghai, China (38). The study population was sampled using cluster sampling method. Ten thousand and five hundred sixty-nine men and women aged 40 years or older were invited by telephone or door-to-door visit to participate in this study. Among them, 10375 (98.2%) agreed to participate. 9211 participants with an estimated glomerular filtration rate (eGFR) ≥60ml/min/1.73m^2 were involved to calculate reference intervals of total calcium.

8172 participants with normal hepatic and renal function (20≤albumin<55g/L, alanine transaminase (ALT)< 41U/L, alkaline phosphatase alkaline phosphatase (ALP) < 130U/L, blood urea nitrogen (BUN) <15mmol/L, creatinine (Cr) < 200umol/L) were included to calculate albumin-adjusted calcium equation according to the previously described protocol (39). And 9315 participants with complete serum biochemical parameters data and birth year information were included for the analysis of the association between famine exposure and serum calcium levels (Figure 1).

The study protocol was approved by the Institutional Review Board of the Rui-jin Hospital, Shanghai Jiao Tong University School of Medicine, and informed consent was obtained from all participants.

Data collection

Anthropometric measurements were performed by the trained staff according to the standardized protocol. Height was measured to the nearest 0.1 cm, and weight was recorded to the nearest 0.1 kg with light clothing and no shoes. Body mass index (BMI) was calculated as body weight in kilograms divided by height squared in meters.

Venous blood samples were collected after an overnight fast. Serum Ca, P, ALT, AST, γGT, ALP, albumin, uric acid (UA), BUN, and Cr were measured using the autoanalyzer (Modular E170; Roche). CKD-EPI Creatinine Equation (2009) was used to calculate eGFR.

Definition of famine exposure

The Great China's Famine occurred from 1959 to 1962. According to the birth year, participants were divided into four groups: non-exposed (born after 1 Jan 1963), fetal exposure (born between 1 Jan 1959 to 31 Dec 1962), childhood exposure (born between 1 Jan 1949 to 31 Dec 1958), adolescent exposure (born between 1 Jan 1941 to 31 Dec 1948), and adulthood exposure (born before 31 Dec 1940).

Because there are no overlaps in the birth years among the 5 famine exposed groups, to limit the effect of age difference on the results, we combined the non-exposed (post-famine) group and adolescent exposure (pre-famine) group as the age-balanced control group of childhood exposure group (mean age: 55.94 vs 56.48 years) (40–42).

Statistical analysis

To establish the total calcium reference interval, first, we excluded outliers that lay more than 3 quartiles above or below the interquartile range. The 95% reference interval was defined by mean ± 1.96*SD with 95% confidence intervals (95% CI).

The albumin-adjusted calcium equation was derived from the linear regression analysis using serum albumin as the dependent variable and serum calcium as the independent variable.

All continuous variables were presented as medians (interquartile ranges), and categorical variables were presented as proportions. Odds ratio (ORs) and 95% CIs were calculated using a multivariable-adjusted logistic regression model to analyze the association of famine exposure with serum total calcium and albumin-adjusted calcium. Mann–Whitney U-test was performed to compare serum total calcium levels and albumin-adjusted calcium between genders. A p-value less than 0.05 was considered statistically significant. The statistical analysis was performed using SPSS 23.0 (SPSS, Inc.)

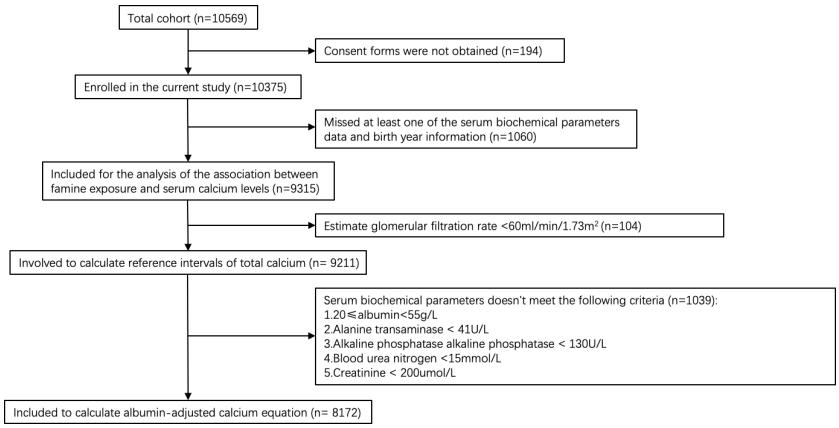


FIGURE 1
Flow chart of the participants selection of this study.

Results

Reference interval for total calcium

Clinical characteristics of participants with consent forms and complete serum biochemical parameters were shown in Table 1. A total of 9211 participants with eGFR≥60ml/min/1.73m^2 were involved to calculate reference intervals of total calcium. After rejecting outliers (n=1), the calculated results were shown in Table 2. The mean calcium level in the total cohort was 2.320mmol/L and females had higher mean total serum calcium levels than males (2.325 mmol/L vs 2.313 mmol/L, P=0.000).

Albumin-adjusted calcium equation

To derive the albumin-adjusted calcium, first, we established the albumin-adjusted calcium equation based on 8172 participants selected according to the previously protocol (39). The y-intercept was 1.389 and the slope was 0.019 (Figure 2) which made the equation:

Total calcium (mmol/L) = 0.019*albumin(g/L) + 1.389.

It is established that albumin-adjusted calcium=total calcium-(slope*albumin) + (mean normal total calcium-intercept calcium) (39), which is:

Albumin-adjusted calcium = Total calcium + 0.019 × (albumin) (mean total calcium–1.389)

Albumin-adjusted calcium = Total calcium + 0.019 × (albumin) (2.320- 1.389)

Albumin-adjusted calcium = Total calcium + 0.019 × (albumin) 0.931

Albumin-adjusted calcium = Total calcium + 0.019 × (49-Albumin)

Reference interval for albumin-adjusted calcium

We calculated the albumin-adjusted calcium according to the above equation for 9211 participants with eGFR≥60ml/min/1.73m^2. After rejecting outliers (11 outliers, 0.12%) by the method mentioned above, 9200 participants were included for the calculation of the reference interval of albumin-adjusted calcium, and the results were shown in Table 2. The mean albumin-adjusted calcium level in the total cohort was 2.325mmol/L and females had higher mean albumin-adjusted serum calcium levels than males (2.332 mmol/L vs 2.314 mmol/L, P=0.000).

Clinical characteristics of participants according to famine exposure

We further studied whether exposure to famine at different stages of life had any effect on the serum calcium level in adulthood. The characteristics of 9315 participants were listed

TABLE 1 Clinical characteristics of participants according to famine exposure.

		Famine exposure			
	Non-exposed	Fetal	Childhood	Adolescent	Adult
n	1455	1097	3592	1911	1260
Age (year)	44.05 (42-46)	49.72 (49-51)	56.48 (54-59)	65.00 (63-67)	74.88 (72-77)
Male (%)	563 (38.69%)	408 (37.19%)	1253 (34.88%)	784 (41.03%)	553 (43.89%)
BMI (kg/m2)	24.53 (22.17-26.51)	25.02 (22.64-27.22)	25.20 (23.08-27.19)	25.39 (23.17-27.47)	24.93 (22.56-27.2)
Ca (mmol/L)	2.30 (2.24-2.36)	2.31 (2.25-2.38)	2.33 (2.27-2.4)	2.32 (2.25-2.38)	2.31 (2.25-2.38)
ALB-adjusted Ca (mmol/L)	2.30 (2.25-2.35)	2.32 (2.26-2.37)	2.33 (2.27-2.39)	2.33 (2.27-2.38)	2.34 (2.28-2.39)
P (mmol/L)	1.23 (1.09-1.36)	1.24 (1.09-1.39)	1.26 (1.13-1.4)	1.26 (1.11-1.41)	1.24 (1.11-1.38)
ALB (g/L)	48.90 (47.30-50.80)	48.86 (47.30-50.50)	49.02 (47.60-50.60)	48.53 (47.00-50.10)	47.52 (45.80-49.38)
ALT (IU/L)	23.65 (12.10-26.10)	21.97 (13.20-25.80)	22.55 (14.30-25.80)	21.76 (14.20-24.60)	19.59 (13.03-22.58)
AST (IU/L)	21.98 (16.30-23.20)	22.15 (17.30-23.95)	23.36 (18.50-25.40)	24.11 (19.10-26.30)	24.82 (19.63-27.20)
γGT (IU/L)	29.62 (13.00-31.00)	32.13 (14.00-35.00)	31.24 (15.00-33.00)	32.43 (16.00-36.00)	34.81 (16.00-38.00)
ALP (IU/L)	68.99 (56.00-79.00)	75.03 (60.00-86.00)	82.54 (67.00-95.00)	83.71 (69.00-96.00)	85.28 (68.00-99.00)
UA (umol/L)	279.82 (208.80-338.80)	286.17 (225.45-337.80)	298.60 (235.65-350.68)	311.23 (244.80-366.50)	322.89 (257.65-381.25)
BUN (mmol/L)	4.84 (4.00-5.50)	5.14 (4.20-5.90)	5.34 (4.40-6.10)	5.56 (4.60-6.30)	5.78 (4.60-6.60)
Cr (umol/L)	59.28 (49.40-67.50)	59.87 (50.60-66.90)	60.39 (51.40-67.40)	64.22 (53.50-71.10)	68.30 (56.03-77.20)
eGFR (ml/min/1.73m2)	110.29 (107.11-115.09)	105.52 (102.79-109.86)	99.64 (96.76-104.55)	91.71 (88.83-97.60)	82.70 (77.97-90.32)

Data were present as median (interquartile range) or proportions.
BMI, body mass index; ALB, albumin; ALT, alanine transaminase; AST, aspartate transaminase; γGT, Gamma Glutamyl Transpeptidase; ALP, alkaline phosphatase; UA, serum uric acid; BUN, blood urea nitrogen; Cr, serum creatin; eGFR, estimated glomerular filtration rate.

TABLE 2 Calculated reference intervals of serum total calcium and albumin-adjusted calcium.

	The lower limit of 95% reference interval (mmol/L) (95% confidence intervals)	The upper limit of 95% reference interval (mmol/L) (95% confidence intervals)
Serum total calcium (mmol/L)		
Total cohort	2.122 (2.120-2.124)	2.518 (2.516-2.520)
Male	2.113 (2.110-2.117)	2.512 (2.509-2.515)
Female	2.128 (2.125-2.130)	2.521 (2.519-2.524)
Albumin-adjusted calcium (mmol/L)		
Total cohort	2.151 (2.149-2.153)	2.500 (2.498-2.501)
Male	2.141 (2.138-2.144)	2.488 (2.485-2.491)
Female	2.159 (2.157-2.161)	2.505 (2.503-2.508)

according to different life stages of exposure to famine. As shown in Table 1, participants who experienced famine had higher total calcium levels as well as albumin-adjusted calcium levels compared to non-exposed participants. Those exposed in childhood had the highest total calcium level and those exposed in adulthood had the highest albumin-adjusted calcium level.

The association between famine exposure and serum calcium levels

To further evaluate the association between famine exposure and calcium level. We assorted the participant according to the quartiles of total calcium and analyzed the relationship between famine exposure and being at the upper quartile of total calcium. The ORs (95% CI) of being at the upper quartile of total calcium levels were 1.28(1.06-1.55), 1.67(1.44-1.94), 1.25(1.05-1.48), and 1.27(1.05-1.52) for fetal, childhood, adolescent and adulthood exposure to famine, respectively. After adjusting for age, sex, eGFR, and albumin, only participants who experienced famine in childhood had a significantly higher risk (OR=1.87, 95%CI=1.31-2.67) of being at the upper quartile of serum calcium (Table 3).

It was reported that females are more vulnerable to famine-associated metabolic dysregulation (43–46). We wondered whether there was bias in the influence of famine exposure on serum calcium levels between males and females. As demonstrated in Table 3, after multivariable adjustment, female participants, not males, who experienced famine in childhood were more likely to have higher calcium levels. Similar results were derived in the analysis of famine exposure and being upper quartile of albumin-adjusted calcium. Female participants who experienced famine in both fetal and childhood were more likely to have higher albumin-adjusted calcium levels after multivariable adjustment (Table 4).

To control the effect of age on the outcomes of famine, we compared the childhood exposure group with the age-balanced

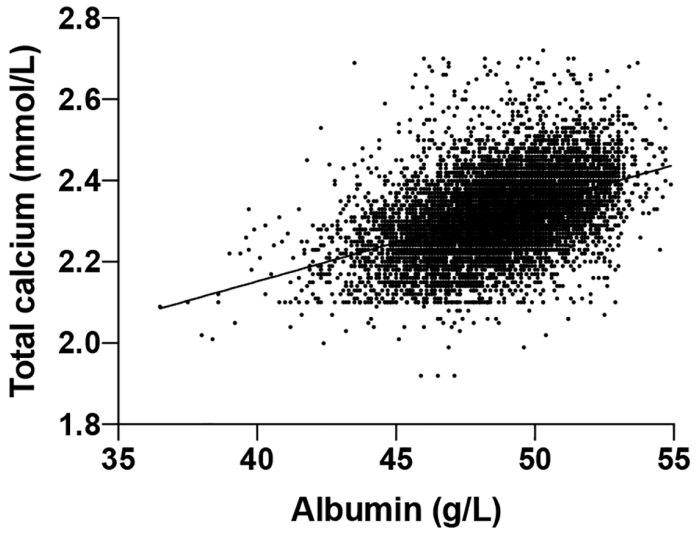


FIGURE 2
Scatter plot of total calcium (y-axis) and albumin (x-axis).

TABLE 3 | ORs (95% CI) for being at the upper quartile of serum total calcium of exposure to famine at different life stages.

	Famine exposure				
Non-exposed		**Fetal**	**Childhood**	**Adolescent**	**Adult**
Whole cohort					
Case/total	279/1455	256/1097	1021/3592	436/1911	291/1260
Model 1	1.00(ref)	**1.28(1.06-1.55)**	**1.67(1.44-1.94)**	**1.25(1.05-1.48)**	**1.27(1.05-1.52)**
Model 2	1.00(ref)	1.33(0.95-1.86)	**1.99(1.44-2.76)**	1.72(0.82-3.63)	**4.295(1.72-10.75)**
Model 3	1.00(ref)	1.21(0.84-1.74)	**1.87(1.31-2.67)**	1.30(0.59-2.88)	2.08(0.76-5.67)
Male					
Case/total	151/563	87/408	274/1253	153/784	108/553
Model 1	1.00(ref)	0.739(0.55-1.00)	**0.764(0.61-0.96)**	**0.662(0.51-0.86)**	**0.662(0.50-0.88)**
Model 2	1.00(ref)	0.879(0.54-1.44)	1.046(0.61-1.80)	1.068(0.33-3.41)	3.744(0.91-15.49)
Model 3	1.00(ref)	0.86 (0.50-1.46)	0.855(0.50-1.46)	0.855(0.25-2.97)	2.305(0.49-10.84)
Female					
Case/total	128/892	169/689	747/2339	283/1127	183/707
Model 1	1.00(ref)	**1.94(1.50-2.50)**	**2.801(2.28-3.44)**	**2.001(1.59-2.52)**	**2.085(1.62-2.68)**
Model 2	1.00(ref)	**1.854(1.17-2.95)**	**3.238(2.13-4.92)**	**2.991(1.12-8.02)**	**5.348(1.58-18.06)**
Model 3	1.00(ref)	1.623(0.99-2.67)	**2.915(1.85-4.60)**	1.974(0.69-5.62)	2.197(0.58-8.27)

Model 1: unadjusted.
Model 2: adjusted for age and sex.
Model 3: adjusted for age, sex, eGFR, and ALB.
Bold values are ORs reached statistical significance.

control group. The results showed that exposure to famine in childhood was related to higher total serum calcium (OR=1.350, 95%CI=1.199-1.521) and albumin-adjusted calcium (OR=1.381, 95%CI=1.234-1.544) in their adulthood. Subgroup analysis showed that this effect only existed in females (Tables 5, 6).

Discussion

In this study, we provided the reference interval of serum calcium and established a new albumin-adjusted calcium equation for Chinese adults, and further found that

TABLE 4 ORs (95% CI) for being at the upper quartile of serum albumin-adjusted calcium of exposed to famine at different life stages.

	Famine exposure				
Non-exposed		**Fetal**	**Childhood**	**Adolescent**	**Adult**
Whole cohort					
Case/total	234/1455	248/1097	984/3592	486/1911	376/1260
Model 1	1.00(ref)	**1.52(1.25-1.86)**	**1.97(1.68-2.31)**	**1.78(1.50-2.12)**	**2.22(1.85-2.67)**
Model 2	1.00(ref)	1.23(0.87-1.75)	**2.13(1.52-2.99)**	1.24(0.59-2.63)	1.99(0.84-4.73)
Model 3	1.00(ref)	1.24(0.87-1.76)	**2.15(1.53-3.02)**	1.28(0.60-2.72)	2.00(0.84-4.78)
Male					
Case/total	119/563	69/408	237/1253	161/784	137/553
Model 1	1.00(ref)	0.76(0.55-1.06)	0.87(0.68-1.11)	0.96(0.74-1.26)	1.23(0.93-1.63)
Model 2	1.00(ref)	0.61(0.35-1.03)	0.94(0.53-1.69)	0.68(0.21-2.27)	1.28(0.33-4.88)
Model 3	1.00(ref)	0.64(0.37-1.10)	0.98(0.55-1.75)	0.73(0.22-2.44)	1.20(0.31-4.62)
Female					
Case/total	115/892	179/689	747/2339	325/1127	239/707
Model 1	1.00(ref)	**2.37(1.83-3.07)**	**3.17(2.56-3.93)**	**2.74(2.17-3.46)**	**3.45(2.69-4.43)**
Model 2	1.00(ref)	**2.06(1.28-3.32)**	**3.59(2.35-5.49)**	2.17(0.82-5.75)	2.88(0.92-9.04)
Model 3	1.00(ref)	**2.05(1.27-3.30)**	**3.59(2.34-5.50)**	2.19(0.82-5.82)	3.11(0.98-9.89)

Model 1: unadjusted.
Model 2: adjusted for age and sex.
Model 3: adjusted for age, sex and eGFR.
Bold values are ORs reached statistical significance.

participants who experienced famine in early life (fetal and childhood) have higher total and albumin-adjusted serum calcium levels, especially in females.

In a most recent study, more than 170 thousand European residents were investigated to derive reference interval of serum calcium (29). In our study, we also used a large population cohort to derive the Chinses-specific reference intervals of both total and albumin-adjusted serum calcium. The intervals were relatively narrow in our cohort, indicating that the cohort we used had relatively adequate homogeneity and representativeness. In addition, due to the variation among populations and methodology used for the measurement of total calcium and albumin levels, the use of a locally derived albumin-adjusted calcium equation is recommended by the Association for Clinical Biochemistry and Laboratory Medicine (ACB) (28). Our study provided a new equation for calculating albumin-adjusted calcium, which derived a higher value than commonly used equation (adjusted-calcium(mmol/L) = total calcium(mmol/L) + 0.02(40-albumin)) and the equation reported by the European study (adjusted-calcium(mmol/L) = total calcium (mmol/L) + 0.0177(45.2-albumin)) (29). It is noteworthy that compared to the European study, the average total and albumin-adjusted calcium concentrations were lower in our cohort. It is reported that the corrected serum calcium level of African-Americans is higher than Caucasians and Hispanics (47). More evidence is needed to confirm that the difference of circulating calcium between Asians and Europeans is caused by race.

In subgroup analysis, we found that the mean total and albumin-adjusted calcium concentrations were higher in females, which is in line with the European study. The reason behind such a phenomenon is multi-faceted: females go through a rapid estrogen decline during menopause, which augments the bone resorption rate. Thus, postmenopausal women have higher serum calcium than premenopausal women (48). In the meantime, males undergo a relatively moderate transition and serum calcium falls with aging, thus, in the aged population, females have higher serum calcium level than males (48). In our study, almost 80% of the participants were over 50 years old and this may be responsible for the higher serum calcium level in females of the total cohort. When we analyzed the participants under 50 years old, the results showed that serum calcium level was higher in males (data not shown), which is also in line with the previous reports (48, 49). All these findings suggest that it might be necessary to use country-specific or ethnic specific and even gender-specific reference intervals of serum calcium as well as albumin-adjusted calcium equation.

Despite serum calcium concentration being strictly regulated within an exquisitely narrow range, our study found that it is influenced by famine exposure during early life. Malnutrition is a predominant result caused by edible food deprivation during famine exposure (50). A study reported that thin children (16% of BMI lower than normal control) have a higher level of bone resorption marker C-terminal telopeptide of collagen type I (CTX) than normal-weight peers (51). Similar results are found in anorexia nervosa (AN) patients. In AN, although serum calcium concentration remains in the normal range, urinary calcium excretion is elevated while intestinal calcium absorption is unchanged (35, 36), indicating the loss of calcium from the skeleton due to increased bone resorption. Thus, increased serum calcium in individuals exposed to famine may be caused by enhanced bone resorption triggered by a nutrition deficiency.

TABLE 5 The risk of being at the upper quartile of serum total calcium in later life following exposure to famine during childhood using age-balanced control group.

	Age-balanced control	Childhood exposure
Whole cohort		
Case/total	715/3366	1021/3592
Model 1	1.00(ref)	**1.472(1.319-1.643)**
Model 2	1.00(ref)	**1.454(1.302-1.623)**
Model 3	1.00(ref)	**1.350(1.199-1.521)**
Male		
Case/total	304/1347	274/1253
Model 1	1.00(ref)	0.960(0.798-1.155)
Model 2	1.00(ref)	0.976(0.811-1.176)
Model 3	1.00(ref)	1.008(0.825-1.231)
Female		
Case/total	411/2019	747/2339
Model 1	1.00(ref)	**1.836(1.598-2.109)**
Model 2	1.00(ref)	**1.842(1.601-2.118)**
Model 3s	1.00(ref)	**1.621(1.396-1.883)**

Model 1: unadjusted.
Model 2: adjusted for age and sex.
Model 3: adjusted for age, sex, eGFR, and ALB.
Bold values are ORs reached statistical significance.

TABLE 6 The risk of being at the upper quartile of serum albumin-adjusted calcium in later life following exposure to famine during childhood using age-balanced control group.

	Age-balanced control	Childhood exposure
Whole cohort		
Case/total	720/3366	984/3592
Model 1	1.00(ref)	**1.387(1.242-1.548)**
Model 2	1.00(ref)	**1.367(1.223-1.527)**
Model 3	1.00(ref)	**1.381(1.234-1.544)**
Male		
Case/total	280/1347	237/1253
Model 1	1.00(ref)	0.889(0.733-1.078)
Model 2	1.00(ref)	0.890(0.733-1.079)
Model 3	1.00(ref)	0.912(0.751-1.108)
Female		
Case/total	440/2019	747/2339
Model 1	1.00(ref)	**1.684(1.469-1.931)**
Model 2	1.00(ref)	**1.718(1.495-1.974)**
Model 3	1.00(ref)	**1.722(1.497-1.980)**

Model 1: unadjusted.
Model 2: adjusted for age and sex.
Model 3: adjusted for age, sex and eGFR.
Bold values are ORs reached statistical significance.

Measuring serum total as well as albumin-adjusted calcium level and establishing their normal reference range is not only indispensable for diagnosing diseases with overt disturbed calcium metabolism such as hyperparathyroidism and hypoparathyroidism but its variations, even within the normal range, are also associated with other extra-skeleton disorders. It was reported that subjects in the upper three quartiles of corrected serum calcium concentration had a significantly increased risk for intracranial atherosclerosis compared with the lowest quartile (18). The prevalence of overweight/obesity almost doubled in the upper total serum calcium level quartile compared to the lowest quartile (25). In two longitudinal studies, participants who had higher serum calcium were 1.6-2.3 times risky to develop diabetes and AD during 2-8 years of follow-up (20, 21). More importantly, the causal effect of high calcium level on lower BMDs at lumber-spine and whole-body is confirmed by two recent Mendelian-randomization (MR) studies, this effect is even independent of the most three important calcium-modulating hormones: parathyroid hormone (PTH), vitamin D, and phosphate concentrations. Similarly, clinical trials demonstrated that high-dose (10000IU daily) vitamin D supplementation (with 9% of study participants experiencing hypercalcemia at the end of the trial) leads to accelerated bone loss compared to low-dose(400IU daily and none of hypercalcemia) (52, 53). The results derived from our study that early-life famine exposure has an impact on serum calcium concentrations in adulthood emphasized that for those with famine exposure in early life, it is necessary to evaluate their serum calcium level. This might be important to screen subjects at high risk of the above-mentioned calcium-related and famine-related diseases. In addition, whether there is a need to re-evaluate the reference interval of serum calcium and albumin-adjusted calcium equation when the nutrition status is greatly changed (improved) in the future is another interesting topic.

In subgroup analysis, we found that after multivariable adjustment, the relationship between famine and serum calcium only exists in female participants. One explanation is that during evolution, mammalian females have been exposed to more severe selection pressure than males during food shortages (54). Besides, in Chinese traditional culture, parents were intended to provide better nutrition to boys than girls when facing food shortages (55), thus there might be a severity difference in famine exposure between females and males.

The mechanism underlying elevated serum calcium and famine in early life is unclear; it might be related to enhanced bone resorption during famine exposure. In rodents, food restriction reduces cortical bone mass and cortical thickness while trabecular percent bone volume (BV/TV) was significantly lower in the food restriction group (56–58). Moreover, there is an increase in osteoclasts number and bone resorption in caloric restriction mice (59, 60), which is in line with the previous hypothesis that bone resorption activity was enhanced during famine exposure. Further studies revealed that serum leptin, which inhibits osteoclast generation (61), is decreased in food restriction mice (57, 59, 60), which may lead to the activation of osteoclastogenesis. On the other hand, dietary energy restriction elevates glucocorticoid hormone levels (62), and methylprednisolone treatment will increase osteoclast activity (63). However, more evidence is needed to support the hypothesis that famine exposure may result in increased bone resorptive activity and thus higher serum calcium levels.

There are some limitations in this study. Firstly, serum concentrations of PTH and 25OHD were not measured, which have critical roles in maintaining calcium homeostasis. However, vitamin D metabolism has been shown to behave normally in malnourished children (64) and serum calcium doesn't relate to PTH levels in AN patients (35). It indicates that there are other mechanisms to regulate serum calcium in undernutrition conditions. Secondly, the serum concentrations of bone resorption and bone formation markers were not evaluated in our study. Thirdly, the severity and precise duration of famine exposure, confounding including place of birth and residence, and familial socioeconomic status (SES) at the time of the famine are unknown, thus the potential dose-response relationship between famine and serum calcium has not been studied in our research. A recent review addressed some recommendations that might help improve future Chinese famine studies (65). Besides, further studies regarding the mediation effect of calcium in famine-related health outcomes are needed.

Conclusion

Our results suggest famine exposure is an important environmental factor responsible for the changes in circulating calcium concentrations, the newly established normal range of serum calcium and albumin adjusted calcium equation, together with the history of famine exposure in childhood, might be helpful in early identifying subjects with abnormal calcium homeostasis and related diseases, especially in females.

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 Institutional Review Board of the Rui-jin Hospital, Shanghai Jiao Tong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

J-mL, L-hS, and BT designed the study, Y-yY analyzed the data and write the manuscript. DZ and Y-fH verified the underlying data reported in the manuscript. L-yM, Y-fB, YX, and MX collect the data. J-mL interpreted the results and revised the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

This research did not recieve any specific grant form any funding agency in the public, commercial or not-for-frofit sector.

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.

The reviewer HY declared a shared affiliation with the author(s) to the handling editor at the time of review.

Publisher's note

References

1. Susser ES, Lin SP. Schizophrenia after prenatal exposure to the Dutch hunger winter of 1944-1945. *Arch Gen Psychiatry* (1992) 49:983–8. doi: 10.1001/archpsyc.1992.01820120071010

2. Ravelli AC, van der Meulen JH, Osmond C, Barker DJ, Bleker OP. Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* (1999) 70:811–6. doi: 10.1093/ajcn/70.5.811

3. Kahn HS, Graff M, Stein AD, Lumey LH. A fingerprint marker from early gestation associated with diabetes in middle age: the Dutch hunger winter families study. *Int J Epidemiol* (2009) 38:101–9. doi: 10.1093/ije/dyn158

4. van Abeelen AFM, Elias SG, Bossuyt PMM, Grobbee DE, van der Schouw YT, Roseboom TJ, et al. Famine exposure in the young and the risk of type 2 diabetes in adulthood. *Diabetes* (2012) 61:2255–60. doi: 10.2337/db11-1559

5. Hoek HW, Susser E, Buck KA, Lumey LH, Lin SP, Gorman JM. Schizoid personality disorder after prenatal exposure to famine. *Am J Psychiatry* (1996) 153:1637–9. doi: 10.1176/ajp.153.12.1637

6. Roseboom TJ. Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. *Heart* (2000) 84:595–8. doi: 10.1136/heart.84.6.595

7. Zong L, Cai L, Liang J, Lin W, Yao J, Huang H, et al. Exposure to famine in early life and the risk of osteoporosis in adulthood: A prospective study. *Endocr Pract* (2019) 25:299–305. doi: 10.4158/EP-2018-0419

8. Lu J, Li M, Xu Y, Bi Y, Qin Y, Li Q, et al. Early life famine exposure, ideal cardiovascular health metrics, and risk of incident diabetes: Findings from the 4C study. *Diabetes Care* (2020) 43:1902–9. doi: 10.2337/dc19-2325

9. Rong H, Lai X, Mahmoudi E, Fang H. Early-life exposure to the Chinese famine and risk of cognitive decline. *J Clin Med* (2019) 8:484. doi: 10.3390/jcm8040484

10. Xu H, Zhang Z, Li L, Liu J. Early life exposure to china's 1959-61 famine and midlife cognition. *Int J Epidemiol* (2018) 47:109–20. doi: 10.1093/ije/dyx222

11. Shen Y, Chen L, Zhou J, Wang C, Gao F, Zhu W, et al. Low total osteocalcin levels are associated with all-cause and cardiovascular mortality among patients with type 2 diabetes: A real-world study. *Cardiovasc Diabetol* (2022) 21:98. doi: 10.1186/s12933-022-01539-z

12. Yadav VK, Berger JM, Singh P, Nagarajan P, Karsenty G. Embryonic osteocalcin signaling determines lifelong adrenal steroidogenesis and homeostasis in the mouse. *J Clin Invest* (2022) 132(4):e153752. doi: 10.1172/JCI153752

13. Hou Yf, Shan C, Zhuang Sy, Zhuang Qq, Ghosh A, Zhu Kc, et al. Gut microbiota-derived propionate mediates the neuroprotective effect of osteocalcin in a mouse model of parkinson's disease. *Microbiome* (2021) 9:1–18. doi: 10.1186/s40168-020-00988-6

14. Ye X, Yu R, Jiang F, Hou X, Wei L, Bao Y, et al. Osteocalcin and risks of incident diabetes and diabetic kidney disease: A 4.6-year prospective cohort study. *Diabetes Care* (2022) 45:830–6. doi: 10.2337/dc21-2113

15. Xuan Y, Sun LH, Liu DM, Zhao L, Tao B, Wang WQ, et al. Positive association between serum levels of bone resorption marker CTX and HbA1c in women with normal glucose tolerance. *J Clin Endocrinol Metab* (2015) 100:274–81. doi: 10.1210/jc.2014-2583

16. Liu TT, Liu DM, Xuan Y, Zhao L, Sun LH, Zhao DD, et al. The association between the baseline bone resorption marker CTX and incident dysglycemia after 4 years. *Bone Res* (2017) 5:1–7. doi: 10.1038/boneres.2017.20

17. Lacombe J, Karsenty G, Ferron M. *In vivo* analysis of the contribution of bone resorption to the control of glucose metabolism in mice. *Mol Metab* (2013) 2:498–504. doi: 10.1016/j.molmet.2013.08.004

18. Kang K. Serum calcium and phosphate concentrations and intracranial atherosclerosis. *Atherosclerosis* (2014) 232:249–53. doi: 10.1016/j.atherosclerosis.2013.11.030

19. Yuan S, Yu L, Gou W, Wang L, Sun J, Li D, et al. Health effects of high serum calcium levels: Updated phenome-wide mendelian randomisation investigation and review of mendelian randomisation studies. *EBioMedicine* (2022) 76:103865. doi: 10.1016/j.ebiom.2022.103865

20. Ma LZ, Wang ZX, Wang ZT, Hou XH, Shen XN, Ou YN, et al. Serum calcium predicts cognitive decline and clinical progression of alzheimer's disease. *Neurotox Res* (2021) 39:609–17. doi: 10.1007/s12640-020-00312-y

21. Rooney MR, Pankow JS, Sibley SD, Selvin E, Reis JP, Michos ED, et al. Serum calcium and incident type 2 diabetes: The atherosclerosis risk in communities (ARIC) study. *Am J Clin Nutr* (2016) 104:1023–9. doi: 10.3945/ajcn.115.130021

22. Pan K, Tu R, Yao X, Zhu Z. Associations between serum calcium, 25(OH)D level and bone mineral density in adolescents. *Adv Rheumatol (London England)* (2021) 61:16. doi: 10.1186/s42358-021-00174-8

23. Qu Z, Yang F, Yan Y, Hong J, Wang W, Li S, et al. Relationship between serum nutritional factors and bone mineral density: A mendelian randomization study. *J Clin Endocrinol Metab* (2021) 106:e2434–43. doi: 10.1210/clinem/dgab085

24. Zhu H, Wang H, Jia Y, Cheng L, Cheng X. Increased serum calcium levels are associated with carotid atherosclerotic plaque in normocalcaemic individuals with type 2 diabetes. *Ther Adv Endocrinol Metab* (2021) 12:2042018821995369. doi: 10.1177/2042018821995369

25. Ren XH, Yao YS, He LP, Jin YL, Chang WW, Li J, et al. Overweight and obesity associated with increased total serum calcium level: Comparison of cross-sectional data in the health screening for teaching faculty. *Biol Trace Elem Res* (2013) 156:74–8. doi: 10.1007/s12011-013-9856-8

26. Ngo Nkondjock VR, Wabo TMC, Kosgey JC, Zhang Y, Amporfro DA, Adnan H, et al. Insulin resistance, serum calcium and hypertension: A cross-sectional study of a multiracial population, and a similarity assessment of results from a single-race population's study. *Diabetes Metab Syndr Obes Targets Ther* (2021) 14:3361–73. doi: 10.2147/DMSO.S259409

27. Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *J Am Soc Nephrol* (2004) 15:2208–18. doi: 10.1097/01.ASN.0000133041.27682.A2

28. Mohd Ariffin ZA, Jamaluddin FA. Albumin adjusted calcium: Study in a tertiary care hospital. *Malays J Pathol* (2020) 42:395–400.

29. Schini M, Hannan FM, Walsh JS, Eastell R. Reference interval for albumin-adjusted calcium based on a large UK population. *Clin Endocrinol (Oxf)* (2021) 94:34–9. doi: 10.1111/cen.14326

30. Jassam N, Hayden K, Dearman R, Allgar V, Barth JH. Prospective study comparing the outcome of a population-specific adjusted calcium equation to ionized calcium. *Ann Clin Biochem* (2020) 57:316–24. doi: 10.1177/0004563220926542

31. Payne RB, Little AJ, Williams RB, Milner JR. Interpretation of serum calcium in patients with abnormal serum proteins. *Br Med J* (1973) 4:643–6. doi: 10.1136/bmj.4.5893.643

32. Fleet JC. The role of vitamin d in the endocrinology controlling calcium homeostasis. *Mol Cell Endocrinol* (2017) 453:36–45. doi: 10.1016/j.mce.2017.04.008

33. Khundmiri SJ, Murray RD, Lederer E. PTH and vitamin d. *Compr Physiol* (2016) 6:561–601. doi: 10.1002/cphy.c140071

34. Hurwitz S. Homeostatic control of plasma calcium concentration. *Crit Rev Biochem Mol Biol* (1996) 31:41–100. doi: 10.3109/10409239609110575

35. Lenherr-Taube N, Trajcevski K, Sochett E, Katzman DK. Low PTH levels in adolescents with anorexia nervosa. *Front Pediatr* (2020) 8:99. doi: 10.3389/fped.2020.00099

36. Abrams SA, Silber TJ, Esteban NV, Vieira NE, Stuff JE, Meyers R, et al. Mineral balance and bone turnover in adolescents with anorexia nervosa. *J Pediatr* (1993) 123:326–31. doi: 10.1016/S0022-3476(05)81714-7

37. Kin CFW, Shan WSY, Shun LJC, Chung LP, Jean W. Experience of famine and bone health in post-menopausal women. *Int J Epidemiol* (2007) 36:1143–50. doi: 10.1093/ije/dym149

38. Liu JM, Ma LY, Bi YF, Xu Y, Huang Y, Xu M, et al. A population-based study examining calcaneus quantitative ultrasound and its optimal cut-points to discriminate osteoporotic fractures among 9352 Chinese women and men. *J Clin Endocrinol Metab* (2012) 97:800–9. doi: 10.1210/jc.2011-1654

39. Barth JH, Fiddy JB, Payne RB. Adjustment of serum total calcium for albumin concentration: Effects of non-linearity and of regression differences between laboratories. *Ann Clin Biochem Int J Lab Med* (1996) 33:55–8. doi: 10.1177/000456329603300108

40. Li C, Lumey LH. Exposure to the Chinese famine of 1959-61 in early life and long-term health conditions: A systematic review and meta-analysis. *Int J Epidemiol* (2017) 46:1157–70. doi: 10.1093/ije/dyx013

41. Wang Z, Zou Z, Yang Z, Dong Y, Song J, Dong B, et al. The association between fetal-stage exposure to the China famine and risk of diabetes mellitus in adulthood: Results from the China health and retirement longitudinal study. *BMC Public Health* (2018) 18:1205. doi: 10.1186/s12889-018-6134-x

42. Li J, Yang Q, An R, Sesso HD, Zhong VW, Chan KHK, et al. Famine and trajectories of body mass index, waist circumference, and blood pressure in two generations: Results from the CHNS from 1993-2015. *Hypertens* (2022) 79:518–31. doi: 10.1161/HYPERTENSIONAHA.121.18022

43. Li C, Tobi EW, Heijmans BT, Lumey LH. The effect of the Chinese famine on type 2 diabetes mellitus epidemics. *Nat Rev Endocrinol* (2019) 15:313–4. doi: 10.1038/s41574-019-0195-5

44. Liu H, Chen X, Shi T, Qu G, Zhao T, Xuan K, et al. Association of famine exposure with the risk of type 2 diabetes: A meta-analysis. *Clin Nutr* (2020) 39:1717–23. doi: 10.1016/j.clnu.2019.08.002

45. Qi H, Hu C, Wang S, Zhang Y, Du R, Zhang J, et al. Early life famine exposure, adulthood obesity patterns and the risk of nonalcoholic fatty liver disease. *Liver Int* (2020) 40:2694–705. doi: 10.1111/liv.14572

46. Shi Z, Ji L, Ma RCW, Zimmet P. Early life exposure to 1959-1961 Chinese famine exacerbates association between diabetes and cardiovascular disease. *J Diabetes* (2020) 12:134–41. doi: 10.1111/1753-0407.12975

47. Rubin MR, Rundek T, McMahon DJ, Lee H-S, Sacco RL, Silverberg SJ. Carotid artery plaque thickness is associated with increased serum calcium levels: The northern Manhattan study. *Atherosclerosis* (2007) 194:426–32. doi: 10.1016/j.atherosclerosis.2006.08.027

48. Jorde R, Sundsfjord J, Bønaa KH. Determinants of serum calcium in men and women. *Tromsø study Eur J Epidemiol* (2001) 17:1117–23. doi: 10.1023/A:1021272831251

49. Goldstein DA. (1990).

50. Guven C, Hoang T, Rahman MH, Ulubaşoğlu MA. Long-term effects of malnutrition on early-life famine survivors and their offspring: New evidence from the great Vietnam famine 1944-45. *Health Econ* (2021) 30:1600–27. doi: 10.1002/hec.4274

51. Ambroszkiewicz J, Gajewska J, Rowicka G, Klemarczyk W, Chelchowska M. Assessment of biochemical bone turnover markers and bone mineral density in thin and normal-weight children. *Cartilage* (2018) 9:255–62. doi: 10.1177/1947603516686145

52. Burt LA, Billington EO, Rose MS, Raymond DA, Hanley DA, Boyd SK. Effect of high-dose vitamin d supplementation on volumetric bone density and bone strength: A randomized clinical trial. *JAMA - J Am Med Assoc* (2019) 322:736–45. doi: 10.1001/jama.2019.11889

53. Burt LA, Billington EO, Rose MS, Kremer R, Hanley DA, Boyd SK. Adverse effects of high-dose vitamin d supplementation on volumetric bone density are greater in females than males. *J Bone Miner Res* (2020) 35:2404–14. doi: 10.1002/jbmr.4152

54. Hoyenga KB, Hoyenga KT. Gender and energy balance: Sex differences in adaptations for feast and famine. *Physiol Behav* (1982) 28:545–63. doi: 10.1016/0031-9384(82)90153-6

55. Zhou C, Wang XL, Zhou XD, Hesketh T. Son preference and sex-selective abortion in China: Informing policy options. *Int J Public Health* (2012) 57:459–65. doi: 10.1007/s00038-011-0267-3

56. Ito E, Sato Y, Kobayashi T, Nakamura S, Kaneko Y, Soma T, et al. Food restriction reduces cortical bone mass and serum insulin-like growth factor-1 levels and promotes uterine atrophy in mice. *Biochem Biophys Res Commun* (2021) 534:165–71. doi: 10.1016/j.bbrc.2020.11.122

57. Pando R, Masarwi M, Shtaif B, Idelevich A, Monsonego-Ornan E, Shahar R, et al. Bone quality is affected by food restriction and by nutrition-induced catch-up growth. *J Endocrinol* (2014) 223:227–39. doi: 10.1530/JOE-14-0486

58. Ferguson VL, Greenberg AR, Bateman TA, Ayers RA, Simske SJ. The effects of age and dietary restriction without nutritional supplementation on whole bone structural properties in C57BL/6J mice. *BioMed Sci Instrum* (1999) 35:85–91.

59. Hamrick MW, Ding K-H, Ponnala S, Ferrari SL, Isales CM. Caloric restriction decreases cortical bone mass but spares trabecular bone in the mouse skeleton: Implications for the regulation of bone mass by body weight. *J Bone Miner Res* (2008) 23:870–8. doi: 10.1359/jbmr.080213

60. Devlin MJ, Cloutier AM, Thomas NA, Panus DA, Lotinun S, Pinz I, et al. Caloric restriction leads to high marrow adiposity and low bone mass in growing mice. *J Bone Miner Res* (2010) 25:2078–88. doi: 10.1002/jbmr.82

61. Holloway WR, Collier FM, Aitken CJ, Myers DE, Hodge JM, Malakellis M, et al. Leptin inhibits osteoclast generation. *J Bone Miner Res* (2002) 17:200–9. doi: 10.1359/jbmr.2002.17.2.200

62. Yaktine AL, Vaughn R, Blackwood D, Duysen E, Birt DF. Dietary energy restriction in the SENCAR mouse: Elevation of glucocorticoid hormone levels but no change in distribution of glucocorticoid receptor in epidermal cells. *Mol Carcinog* (1998) 21:62–9. doi: 10.1002/(SICI)1098-2744(199801)21:1<62::AID-MC8>3.0.CO;2-R

63. Bellini G, Torella M, Manzo I, Tortora C, Luongo L, Punzo F, et al. PKCβII-mediated cross-talk of TRPV1/CB2 modulates the glucocorticoid-induced osteoclast overactivity. *Pharmacol Res* (2017) 115:267–74. doi: 10.1016/j.phrs.2016.11.039

64. Raghuramulu N, Reddy V. Studies on vitamin d metabolism in malnourished children. *Br J Nutr* (1982) 47:231–4. doi: 10.1079/BJN19820030

65. Li C, Lumey L. Early life exposure to the Chinese famine of 1959-61 and type 2 diabetes in adulthood: A systematic review and meta-analysis. *Nutrients* (2022) 14:2855. doi: 10.3390/nu14142855

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 11 August 2022
DOI 10.3389/fendo.2022.985297

OPEN ACCESS

EDITED BY
Mohammed S. Razzaque,
Lake Erie College of Osteopathic Medicine, United States

REVIEWED BY
Ali Tatlıcı,
Selçuk University, Turkey
Hrvoje Karninčić,
University of Split, Croatia

*CORRESPONDENCE
Konstantin Gurevich
kgurevich@mail.ru

SPECIALTY SECTION
This article was submitted to Translational Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 03 July 2022
ACCEPTED 22 July 2022
PUBLISHED 11 August 2022

CITATION
Zaborova V, Zolnikov O, Dzhakhaya N, Bueverova E, Sedova A, Kurbatova A, Putilo V, Yakovleva M, Shantyr I, Kastyro I, Ozimek M, Korolev D, Krikheli N, Gurevich K and Heinrich KM (2022) The study of the relevance of macro- and microelements in the hair of young wrestlers depending on the style of wrestling.
Front. Endocrinol. 13:985297.
doi: 10.3389/fendo.2022.985297

The study of the relevance of macro- and microelements in the hair of young wrestlers depending on the style of wrestling

Victoria Zaborova[1,2], Oxana Zolnikov[3], Natiya Dzhakhaya[3], Elena Bueverova[3], Alla Sedova[3], Anastasia Kurbatova[3], Victor Putilo[3], Maria Yakovleva[4], Igor Shantyr[4], Igor Kastyro[5], Mariusz Ozimek[6], Dmitry Korolev[7], Natella Krikheli[7], Konstantin Gurevich[8,9]* and Katie M. Heinrich[10]

[1]Institute of Clinical Medicine, I.M. Sechenov First Moscow State Medical University, Moscow, Russia, [2]Moscow Institute of Physics and Technology, Dolgoprudny, Russia, [3]I.M. Sechenov First Moscow State Medical University, Moscow, Russia, [4]Nikiforov Russian Center of Emergency and Radiation Medicine, Ministry of Emergency Situations, St. Petersburg, Russia, [5]Peoples' Friendship University of Russia, Moscow, Russia, [6]Institute of Sport, Department of Track and Field's Sports, University of Physical Education, Krakow, Poland, [7]Moscow State University of Medicine and Dentistry, Moscow, Russia, [8]UNESCO Chair, Moscow State University of Medicine and Dentistry, Moscow, Russia, [9]Research Institute of Health Organization and Medical Management of the Moscow City Health Department, Moscow, Russia, [10]Department of Kinesiology, College of Health and Human Sciences, Kansas State University, Manhattan, KS, United States

While participating in an intensive training process, the athlete's body requires not only energy, but also specific macro- and microelements. The purpose of this study was to show the meaning of monitoring the level of mineral trace elements in athletes-wrestlers during physical activity. As an experimental group, 66 male wrestlers aged 18-20 years with at least 3 years of intensive wrestling experience were examined. The control group consisted of 92 young cadets of military school aged 18-20 years, who had previous sports training, but were not engaged in wrestling. To determine the quantitative content of trace elements, the hair was cut from the back of the head for the entire length in an amount of at least 0.1 g. an examined using the mass spectrometer ICP-MS Agilent 7900. Strong positive correlations were found for sodium with potassium and rubidium, magnesium with calcium, potassium with rubidium, and rubidium with caesium among wrestlers. Wrestlers were found to have higher levels of a number of macro- and microelements, including toxic ones.

KEYWORDS

trace elements, hair, athletes, sodium, magnesium, calcium, rubidium, potassium

Introduction

When undergoing intensive physical training, an athlete's body requires not only energy, but also a specific balance of macro- and microelements. Minerals that enter the human body with water and food are used for metabolic needs and act as cofactors of key vitamins and enzymes, among others. Due to diverse functions of minerals, young athletes often track their presence, with one such method using a hair sample (1). As well, different sport specializations have demonstrated differences in the balance of macro- and microelements. Thus, for male athletes with repetitive aerobic movements, deficiencies of calcium (Ca), cobalt (Co), copper (Cu), magnesium (Mg), phosphorus (P), zinc (Zn), iodine (I), potassium (K), and nitrogen (N) are described (2). In gymnasts, an increase in selenium (Se), Zn, and Cu has been described (3). The development of microelement imbalance reduces training process efficiency and sports performance and increases the recovery time after playing sports (4).

Differences in microelement imbalance between well-trained athletes and those with low physical activity have been established, such that the athletes had lower levels of toxic microelements including cadmium (Cd) and lead (Pb) (5). Physical training "until failure" also leads to specific changes in the balance of microelements (6). Also important is the duration of training in the development of microelement imbalance in athletes (7, 8). The role of iron (Fe) in the ability of athletes to adequately respond to the training process is actively discussed. Iron deficiency is accompanied by reduced adjustment to physical load, such as lower aerobic power (9, 10). At iron deficiency the serum ferritin level (i.e., total body iron stores) changes (11) and can result in microcytosis (12).

The role of gender in the development of iron deficiency among female athletes has been thoroughly studied in relation to the menstrual cycle (13). Women in sports are more likely to develop iron deficiency than those who do not (14, 15). Different types of sporting activities have been related to significant differences in iron levels which have an impact on women's chances of developing an iron deficiency thought to be due to low dietary iron consumption and iron loss through menstruation and hormonal response to training (16, 17).

Since intensive exercise is accompanied by the formation of active forms of oxygen, Se is of particular importance to athletes. In particular, the activity of the enzyme glutathione peroxidase, a highly effective antioxidant enzyme, depends largely on the presence of Se. These Se properties can potentially be applied to improve sporting performance and recovery after training (18). The use of Se additives can increase athletic performance as well as protect muscle tissue from oxidative processes (19, 20). It is shown that correction of microelements deficiency in athletes increases their endurance and sportsmanship (21). Using an variable microelement dosing mode (e.g., oral, intravenous, transdermal) is essential in order to achieve an optimal result (22). However, the first step is to determine key levels of microelements among specific populations. To date, limited research has examined this for young wrestlers.

A recent systematic review notes that micronutrient deficiencies/imbalances can play a significant role in reducing the body's ability to adapt to physical activity (23). Based on the described above, the purpose of this work was to study the balance of minerals of young wrestlers' bodies.

Methods

The study was approved by the decision of the Interuniversity Ethics Committee of the A. I. Evdokimov Moscow State University (protocol No. 01-19 of January 31, 2019). All individuals participating in the study provided written informed consent. The study was conducted in winter.

In the course of our experiment, 66 male youth wrestlers aged 18-20 years with at least 3 years of intensive experience in wrestling were examined. Of these, 21 athletes (subgroup 1) were engaged in Sambo, 25 athletes (subgroup 2) in freestyle wrestling, and 20 athletes (subgroup 3) in Greco-Roman wrestling. The main training process took place in the city of Moscow. All athletes lived and trained at the same sports bases. They received a standard diet developed in the Russian Federation for wrestlers. Food was prepared centrally at the athletes' places of residence and/or training. The control group consisted of 92 youth cadets of a Moscow military school aged 18-20 years, with previous sports training, but not involved in wrestling. They were selected as the control group due to receiving similar physical training including combat and they also received a standard diet with food prepared at the military school.

To determine the quantitative content of microelements for study participants in the experimental and control groups the hair was cut from the occipital part of the head for the entire length in an amount of at least 0.1 g. To remove the surface contamination and to degrease the hair, the IAEA-recommended hair sample preparation method was used (24). For this purpose the hair was treated with acetone for 10-15 minutes, and then washed three times with deionized water. The hair was dried at room temperature for 10-15 minutes.

A sample of hair, weighing 0.1 g was placed in a fluoroplastic liner and 5 ml of nitric acid was added. An autoclave with the sample in the insert, was placed in a microwave oven and the sample was decomposed using the decomposition program recommended by the manufacturer of the microwave. The following heating mode was used: raising the temperature to

200 °C for 5 minutes, keeping it for 5 minutes at 200 °C, and then cooling it to 45 °C. The cooled autoclave was shaken to mix the contents and the lid was opened to balance the pressure. A qualitatively decomposed sample after distillation of nitrogen oxides was a colorless or yellowish transparent solution with no undissolved particles at the bottom or on the walls of the liner. The dissolved sample was quantitatively transferred to a test tube, diluting the sample 1000 times. Working standard solutions were prepared by diluting standard stock solutions. The proportions and concentrations of elements in standard solutions were selected in such a way that after dilution of 20–50 times the concentrations of the same order were obtained with the upper boundaries of the range of elements in the hair decomposed according to standard methods (24, 25).

The studies were carried out using ICP-MS Agilent 7900 mass spectrometer (24). Analytical signals were processed with spectrometer software, using calibration dependences calculated by the least squares method, accounting and correction of the background, and whenever necessary, taking into account the mutual influence of the measured elements. The result on the display corresponded to the arithmetic average of 3 parallel measurements of the analyzed element. The standard software package from the EXCEL and STATGRAPH statistical software package was used for data analysis to compare the levels of macro- and microelements present in wrestlers and in people who were not involved in wrestling, but who had a high level of physical activity. Use of the Kolomogorov-Smirnov test indicated the data were not normally distributed, thus nonparametric statistics were used. Results are presented as median and interquartile range. The parameters were compared based on the Kruskal-Wallis test. Spearman correlations were calculated. The comparative analysis examined five macroelements (Na, K, Mg, P, Ca) and 30 microelements. Of the trace elements analyzed, the biological role in the following 7: [As], [Rb], [Sr], [Cs], [Hg], [Tl], [Pb] has not been studied and some authors attribute them to toxic 9 (25).

Results

As shown Table 1, wrestlers, in comparison with the control group, had higher levels of lithium, beryllium, boron, sodium, aluminum, calcium, potassium, vanadium, magnesium, cobalt, copper, germanium, rubidium, strontium, cadmium, antimony, caesium, barium and thallium. The most pronounced differences in levels including greater arsenic in subgroup 1, and silver in subgroup 2. All wrestlers had lower titanium and iodine than control group participants. Silicon was lowest among subgroup 3, and selenium was lowest in subgroup 2. No significant differences were found for the rest of the analyzed macro- and microelements.

Table 2 presents the significant correlation of mineral levels among the wrestlers. Strong positive correlations were observed for sodium with potassium and rubidium, magnesium with calcium, potassium with rubidium, and rubidium with caesium. All statistically significant correlations were positive.

Discussion

Macronutrients such as sodium, calcium, potassium, and magnesium may increase in wrestlers' bodies as a result of considerable sport activities and a higher content of these substances in their diets (26), for example, through consumption of sports supplements (27). Conversely, students in Polish sports schools had inadequate intake of potassium, calcium, sodium, copper, iron, zinc, phosphorus, iodine and a number of vitamins with food (28). It may be that differences in the standard diets of wrestlers and military cadets resulted in the differences found in this study for macro- and microelements.

A systematic review examined the effect of mineral content in food and supplements on sports performance. 17,433 articles were analyzed; and 130 experiments from 128 studies were included. Retrieved articles included iron (n = 29), calcium (n = 11), magnesium, (n = 22), phosphate (n = 17), zinc (n = 9), sodium (n = 15), boron (n = 4), selenium (n = 5), chromium (n = 12) and multi-mineral articles (n = 5). No relevant articles were identified for copper, manganese, iodine, nickel, fluorine or cobalt. Only iron and magnesium included articles of sufficient quality to be assigned as 'strong'. Currently, there is little evidence to support the use of food supplementation to improve physiological markers of athletic performance with the possible exception of iron (in particular, biological situations) and magnesium as they currently have the strongest quality evidence (23).

Our results partially overlap with those described by Zaitseva and Zaitsev (29) who found an increase in the content of macronutrients Ca, Mg, P, K, Na in young wrestlers' bodies. They believed that the increased content of macronutrients in the hair more likely indicated an enhanced "circuit" metabolism in athletes, rather than their excess (29). Indeed, a number of works describe a change in the content of mineral substances during physical exertion. For example, in adolescence, at the initial stage of sports, the development of hyperelementosis of iron, lead and selenium is noted (30). In elder men, an increase in calcium during sport activities is described (31, 32). In women, higher Ca, Cr, Fe, Co, and Zn and lower Hg are observed (31). However, in the study of cyclic sports athletes no differences in the levels of Mn, Co, Zn and Se were found, depending on the phase of the training process (32).

Many of the trace elements that were investigated in our paper have not been studied previously. Only a few studies

10.3389/fendo.2022.985297

TABLE 1 The content of the analyzed macro- and micro-elements in the hair (μg/g) of representatives of the experimental (n = 66) and control (n = 92) groups.

Elements	subgroup 1 median (Q1 ; Q3)	subgroup 2 median (Q1 ; Q3)	subgroup 3 median (Q1 ; Q3)	control median (Q1 ; Q3)
lithium	**0,20 (0,15 ; 0,22)**	**0,18 (0,08 ; 0,21)**	**0,16 (0,031;0,227)**	**0,021 (0 ; 0,065)**
beryllium	**0,00036 (0,00023 ; 0,00049)**	**0,00042 (0,00028 ; 0,00060)**	**0,00053 (0,00037 ; 0,00083)**	**0 (0 ; 0)**
boron	**1,52 (1,43 ; 1,85)**	**1,38 (1,30 ; 1,69)**	**1,71 (1,59 ; 2,29)**	**0,96 (0,58 ; 1,46)**
sodium	**459 (295 ; 1163)**	**446 (174 ; 589)**	**417 (214 ; 523)**	**141 (81,8 ; 253)**
magnesium	69,7 (53,5 ; 83,6)	52,8 (41,1 ; 74,9)	55,8 (44,1 ; 76,5)	70,7 (47,7 ; 101)
aluminum	**14,5 (11,6 ; 18,5)**	**10,3 (9,09 ; 13,1)**	**10,5 (9,19 ; 13,2)**	**9,38 (7,44 ; 11,5)**
silicon	**298 (289 ; 321)**	**255 (213 ; 331)**	**231(213 ; 249)**	**280 (219 ; 370)**
phosphorus	122 (116 ; 161)	118 (114 ; 169)	147 (130 ; 158)	138 (95,5 ; 159)
potassium	**153 (122 ; 336)**	**132 (95,7 ; 345)**	**188 (107 ; 239)**	**96,7 (54,0 ; 155)**
calcium	**793 (672 ;1062)**	**650 (417 ; 830)**	**586 (471 ; 838)**	**474(374 ; 657)**
titanium	**0,72 (0,56 ; 0,95)**	**0,73 (0,50 ; 1,04)**	**0,83 (0,61 ; 1,18)**	**1,30 (0,64 ; 3,03)**
vanadium	**0,022 (0,019 ; 0,029)**	**0,018 (0,013 ; 0,029)**	**0,021 (0,017 ; 0,026)**	**0,012 (0,008 ; 0,023)**
chromium	0,34 (0,30 ; 0,41)	0,32 (0,26 ; 0,49)	0,39 (0,31 ; 0,45)	0,35 (0,22 ; 0,78)
manganese	**0,78 (0,57 ; 0,91)**	**0,47 (0,39 ; 0,78)**	**0,38 (0,30 ; 0,70)**	**0,30 (0,19 ; 0,47)**
iron	14,4 (12,6 ; 15,7)	12,5 (9,07 ; 19,0)	13,3 (12,1 ; 16,5)	17,0 (12,5 ; 23,3)
cobalt	**0,062 (0,061 ; 0,067)**	**0,061 (0,036 ; 0,066)**	**0,056 (0,033 ; 0,075)**	**0,016 (0,013 ; 0,025)**
nickel	0,24 (0,19 ; 0,35)	0,24(0,19 ; 0,35)	0,42 (0,32 ; 0,53)	0,37 (0,19 ; 0,61)
copper	**10,6 (9,0 ; 11,1)**	**8,35 (7,95 ; 10,6)**	**10,7 (9,00 ; 11,4)**	**8,02 (6,14 ; 10,6)**
zinc	93,0 (82,6 ; 127,4)	96,7 (79,6 ;128)	117 (93,0 ; 136)	105 (83,2 ; 150)
germanium	**0,13 (0,12 ; 0,14)**	**0,13 (0,11 ; 0,14)**	**0,14 (0,13 ; 0,15)**	**0,089 (0,079 ; 0,110)**
arsenic	**0,040 (0,029 ; 0,053)**	**0,026 (0,021 ; 0,049)**	**0,025 (0,023 ; 0,031)**	**0,022 (0,0082 ; 0,037)**
selenium	**0,51 (0,49 ; 0,53)**	**0,49 (0,45 ; 0,52)**	**0,53 (0,49 ; 0,57)**	**0,66 (0,55 ; 0,79)**
rubidium	**0,15 (0,09 ; 0,24)**	**0,080 (0,065 ; 0,216)**	**0,118 (0,082 ; 0,165)**	**0,060 (0,023 ; 0,14)**
strontium	**2,01 (1,15 ; 2,83)**	**1,23 (0,81 ; 1,66)**	**1,38 (0,85 ; 1,98)**	**0,66 (0,38 ; 1,56)**
molibdenum	0,036 (0,031 ; 0,041)	0,030 (0,026 ; 0,042)	0,034 (0,027 ; 0,040)	0,032 (0,023 ; 0,044)
silver	**0,045 (0,022 ; 0,120)**	**0,054 (0,043 ; 0,130)**	**0,087 (0,061 ; 0,126)**	**0,0310 (0,0082 ; 0,0690)**
cadmium	**0,062 (0,014 ; 0,137)**	**0,0106 (0,0067 ; 0,0493)**	**0,0211 (0,0079 ; 0,0451)**	**0 (0 ; 0)**
tin	0,31 (0,27 ; 0,36)	0,25 (0,21 ; 0,33)	0,34 (0,27 ; 0,42)	0,36 (0,15 ; 0,61)
antimony	**0,057 (0,049 ; 0,117)**	**0,048 (0,030 ; 0,091)**	**0,048 (0,037 ; 0,074)**	**0,010 (0 ; 0,030)**
iodine	**0,042 (0,032 ; 0,054)**	**0,049 (0,034 ; 0,069)**	**0,051 (0,032 ; 0,059)**	**0,21 (0,17 ; 0,30)**
caesium	**0,00085 (0,00058 ; 0,00115)**	**0,00050 (0,00045 ; 0,00091)**	**0,00057 (0,00048 ; 0,00085)**	**0 (0 ; 0)**
barium	**1,61 (1,33 ; 2,00)**	**1,38 (1,10 ; 1,87)**	**1,60 (1,38 ; 2,11)**	**0,82 (0,36 ; 1,59)**
mercury	0,058 (0,043 ; 0,122)	0,055 (0,033 ; 0,085)	0,037 (0,026 ; 0,073)	0,054 (0,025 ;0,147)
thallium	**0,00048 (0,00038 ; 0,00085)**	**0,00045 (0,00022 ; 0,00078)**	**0,0006 (0,00012 ; 0,00074)**	**0 (0 ; 0)**
lead	0,89 (0,60 ; 1,60)	0,45 (0,27 ; 0,73)	0,42 (0,33 ; 0,92)	0,18 (0,12 ; 0,54)

Differences significant with $p < 0.05$ are shown in bold.

have examined changes in the content of trace elements in sports (7, 33–35). There are no systematic reviews so far. And to date, the few available research results are contradictive. In some cases, the study was made with small groups (33). Sometimes the authors do not specify the sport for which a similar study was conducted (7). There are descriptions of a higher content of tin, rubidium and antimony in individuals involved in sports, compared with non-occupants (34). These changes are especially pronounced in individuals with an aerobic-anaerobic type of exercise compared to individuals with an aerobic type (33). Long-distance runners have an increased level of Be, Cd, Cs and Pb compared to non-active sports (7). The levels of cadmium (Cd), tungsten (W), tellurium (Te), beryllium (Be), and lead (Pb) were shown to be higher in sports (34). University students with increased physical activity had decreased hair copper, vanadium, bismuth, and mercury content in comparison to low physical activity university students (35).

Elite long distance runners have higher levels of Co, Cu, Mn, Mo, Se and Zn compared to non-athletes. During a six-month training period, these differences become more significant for Co, Cu, Mo, and Se. According to the

TABLE 2 Spearman correlations of mineral content in wrestlers (N = 66).

Mineral	magnesium	potassium	calcium	titanium	vanadium	chromium	cobalt	iron	manganese	arsenic	rubidium	cadmium	tin	antimony	lead	barium	cesium
sodium	-0,06	**0,86**	-0,09	**0,55**	**0,32**	**0,53**	0,23	**0,36**	0,22	**0,48**	**0,85**	**0,43**	**0,39**	**0,55**	**0,42**	**0,51**	**0,73**
magnesium		-0,18	**0,92**	-0,15	**0,28**	-0,04	**0,40**	0,14	**0,53**	-0,07	-0,16	0,23	0,13	0,02	0,11	0,18	-0,09
potassium			-0,15	**0,65**	**0,32**	**0,62**	0,24	**0,32**	0,13	**0,49**	**0,98**	**0,51**	**0,45**	**0,54**	**0,53**	**0,56**	**0,75**
calcium				-0,14	**0,27**	-0,03	**0,39**	0,03	**0,59**	-0,12	-0,14	**0,34**	0,14	0,00	**0,24**	**0,25**	-0,07
titanium					**0,35**	**0,55**	0,06	**0,45**	0,10	**0,36**	**0,64**	**0,43**	**0,52**	**0,44**	**0,42**	**0,44**	**0,63**
vanadium						**0,58**	**0,65**	**0,61**	**0,64**	**0,40**	**0,31**	**0,56**	**0,62**	**0,53**	**0,45**	**0,36**	**0,49**
chromium							**0,45**	**0,56**	**0,35**	**0,38**	**0,61**	**0,50**	**0,60**	**0,67**	**0,48**	**0,43**	**0,56**
cobalt								**0,52**	**0,65**	0,19	0,24	**0,50**	**0,44**	**0,37**	**0,44**	**0,36**	**0,35**
iron									**0,53**	**0,38**	**0,33**	**0,37**	**0,50**	**0,58**	**0,33**	0,20	**0,45**
manganese										**0,32**	0,11	**0,52**	**0,37**	**0,48**	**0,53**	**0,35**	**0,29**
arsenic											**0,49**	**0,40**	**0,52**	**0,58**	**0,56**	**0,41**	**0,46**
rubidium												**0,51**	**0,48**	**0,57**	**0,54**	**0,56**	**0,78**
cadmium													**0,70**	**0,51**	**0,69**	**0,62**	**0,56**
tin														**0,46**	**0,62**	**0,53**	**0,52**
antimony															**0,61**	**0,38**	**0,65**
lead																**0,47**	**0,61**
barium																	**0,51**

Significant correlations with $p < 0.05$ are shown in bold.

10.3389/fendo.2022.985297

authors, such changes reflect the process of adaptation to physical activity (33).

The above articles contradict the study of Muñoz et al. (5), who showed that the concentrations of Co and Mn are higher, and Cu is lower in individuals who are not involved in sports, compared with non-athletes (36). However, a small sample size should be noted: 26 non-athletes and 21 middle and long distance runners.

Our study found lower iodine levels in wrestlers, which requires further study of thyroid function. This is important, because we propose that low iodine levels may be associated with decreased athletic performance. For example, for high efficiency of the training process, vegan athletes need iodine (37). Lower physical fitness scores for girls have been significantly correlated with lower iodine levels, although additional research is needed (38).

The literature also describes a decrease in selenium content in runners during training (39) similar to what we found in this study. Similar changes have been described in athletes of aerobic, anaerobic and mixed sports (8, 40). A non-systematic review found that during sports the level of oxidative stress increases, which can lead to a deficiency of selenium in athletes. According to the authors, those involved in sports need additional administration of selenium with food to protect the body from possible free radical damage (41).

Wrestlers in our study were characterized by higher levels of several macro- and microelements, including more toxic ones than controls. We studied a greater number of minerals than in other similar studies, which adds to the available scientific literature for examining the relationship between levels of macro and microelements and sports training. Our findings are preliminary. The study is limited by a lack of explanation regarding why these differences existed (i.e. they could be due to nutrition; training; physiological impacts of exercise; body composition difference; physical environment; and so on). As we did not track dietary intake, we are unable to state whether differences were due to what was eaten rather than the physical training done by each group. We also did not directly track the physical training of each group. Additional research is warranted to further explore underlying factors for the differences found.

Practical recommendations: It is necessary to monitor the level of trace elements in athletes-wrestlers during their training. In particular, it may be necessary to control their intake with food and/or food additives. Restoring an optimal balance of macro- and microelements can contribute to better physical performance of wrestlers. Special attention should be paid to the function of the thyroid gland in athletes-wrestlers, because we found they had a pronounced iodine deficiency. Perhaps this category of athletes needs additional iodine supplementation and/or thyroid hormones. It is necessary to consider the issue of prescribing selenium-containing drugs to wrestlers to correct the deficiency of this trace element and prevent oxidative stress associated with high-intensity training.

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 study was approved by the decision of the Interuniversity Ethics Committee of the A. I. Evdokimov Moscow State University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, KG. Formal analysis, VP and AK. Funding acquisition, IK. Investigation, MY and IS. Methodology, DK and OZ. Project administration, KG and VZ. Resources, ND and EB. Supervision, MO and AS. Writing original draft, VZ and KG. Writing—review & editing, KH. All authors have read and agreed to the published version of the manuscript. All authors agree to be accountable for the content of the work.

Acknowledgments

This paper has been supported by the RUDN University Strategic Academic Leadership Program.

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

References

1. Troegubova N, Rylova N, Gilmutdinov R. Features of the content of bioelements in saliva and hair of young athletes. *Russ Bull Perinatol Pediatr* (2016) 61(2):84–8. doi: 10.21508/1027-4065-2016-61-2-84-88

2. Stepanova E. Macro-and microelement status of the body of cyclical sports athletes. *Sport Med* (2016) 6(4):52–60. doi: 10.17238/ISSN2223-2524.2016.4.52

3. Alshammari E, Shafi S, Nurmi-Lawton J. Altered antioxidant and trace-element status in adolescent female gymnasts. *Int J Sport Nutr Exerc Metab* (2012) 20(4):291–8. doi: 10.1123/IJSNEM.20.4.291

4. Ordzhonikidze Z, Gromova O, Skalny A. The value of trace elements for achievement of high sports results and preservation of health of sportsmen. *Microelem Med* (2001) 2(2):40–5.

5. Muñoz D, Grijota F, Bartolomé I. Serum and urinary concentrations of arsenic, beryllium, cadmium and lead after an aerobic training period of six months in aerobic athletes and sedentary people. *Int Soc Sports Nutr* (2020) 17(1):43. doi: 10.1186/s12970-020-00372-7

6. Maynar M, Llerena F, Bartolomé I. Seric concentrations of copper, chromium, manganesum, nickel and selenium in aerobic, anaerobic and mixed professional sportsmen. *J Int Soc Sports Nutr* (2018) 8:13–5. doi: 10.1186/s12970-018-0212-4

7. Maynar M, Bartolomé I, Alves J. Influence of a 6-month physical training program on serum and urinary concentrations of trace metals in middle distance elite runners. *J Int Soc Sports Nutr* (2019) 16(1):53. doi: 10.1186/s12970-019-0322-7

8. Maynar M, Llerena F, Bartolomé I. Influence of an exercise until exhaustion in serum and urinary concentrations of toxic minerals among professional athletes, a preliminary approach. *Trace Elem Med Biol* (2018) 50:312–9. doi: 10.1016/j.jtemb.2018.07.022

9. McCormick R, Moretti D, McKay AKA. The impact of morning versus afternoon exercise on iron absorption in athletes. *Med Sci Sports Exerc* (2019) 51 (10):2147–55. doi: 10.1249/MSS.0000000000002026

10. Sim M, Garvican-Lewis L, Cox G. Iron considerations for the athlete: a narrative review. *Appl Physiol* (2019) 119(7):1463–78. doi: 10.1007/s00421-019-04157-y

11. McKay AKA, Goods PSR, Binnie M. Examining the decay in serum ferritin following intravenous iron infusion: a retrospective cohort analysis of Olympic sport female athletes. *Appl Physiol Nutr Metab* (2020) 45(10):1174–7. doi: 10.1139/apnm-2020-0132

12. Malczewska-Lenczowska J, Surała O, Orysiak J. Utility of novel hypochromia and microcythemia markers in classifying hematological and iron status in male athletes. *Nutrients* (2019) 11(11):2767. doi: 10.3390/nu11112767

13. Badenhorst C, Goto K, O'Brien W. Iron status in athletic females, a shift in perspective on an old paradigm. *J Sports Sci* (2021) 39(14):1565–75. doi: 10.1080/02640414.2021.1885782

14. Ponorac N, Popović M, Karaba-Jakovljević D. Professional female athletes are at a heightened risk of iron-deficient erythropoiesis compared with nonathletes. *Int J Sport Nutr Exerc Metab* (2020) 30(1):48–53. doi: 10.1123/ijsnem.2019-0193

15. Sandström G, Börjesson M, Rödjer S. Iron deficiency in adolescent female athletes - is iron status affected by regular sporting activity? *Clin J Sport Med* (2012) 22(6):495–500. doi: 10.1097/JSM.0b013e3182639522

16. Ahmadi A, Enayatizadeh N, Akbarzadeh M. Iron status in female athletes participating in team ball-sports. *Pak J Biol Sci* (2010) 13(2):93–6. doi: 10.3923/pjbs.2010.93.96

17. McClung J. Iron status and the female athlete. *J Trace Elem Med Biol* (2012) 26(2-3):124–6. doi: 10.1016/j.jtemb.2012.03.006

18. Fernández-Lázaro D, Fernandez-Lazaro C, Mielgo-Ayuso J. The role of selenium mineral trace element in exercise: antioxidant defense system, muscle performance, hormone response and athletic performance. *A systematic review Nutrients* (2020) 12(6):1790. doi: 10.3390/nu12061790

19. White S, Warren L. Submaximal exercise training, more than dietary selenium supplementation, improves antioxidant status and ameliorates exercise-induced oxidative damage to skeletal muscle in young equine athletes. *J Anim Sci* (2017) 95(2):657–70. doi: 10.2527/jas.2016.1130

20. Fernández-Lázaro D, Mielgo-Ayuso J, Seco Calvo J. Modulation of exercise-induced muscle damage, inflammation and oxidative markers by curcumin supplementation in a physically active population: A systematic review. *Nutrients* (2020) 12(2):501. doi: 10.3390/nu12020501

21. Martusevich A, Karuzin K. Metabolic estimation of efficiency of vitamin and mineral complexes in qualified athletes. *Vopr Pitan* (2021) 90(1):94–101. doi: 10.33029/0042-8833-2021-90-1-94-101

22. McCormick R, Sim M, Dawson B. Refining treatment strategies for iron deficient athletes. *Sports Med* (2020) 50(12):2111–23. doi: 10.1007/s40279-020-01360-2

23. Heffernan S, Horner K, De Vito G. The role of mineral and trace element supplementation in exercise and athletic performance: A systematic review. *Nutrients* (2019) 11(3):696. doi: 10.3390/nu11030696

24. Gurevich K. Determination of chemical elements in biological media and preparations by methods of atomic emission spectrometry with inductively coupled plasma and mass spectrometry with inductively coupled plasma. *Moscow: Russia* (2003), 120.

25. Panchenko L, Mayev I, Gurevich K. *Clinical biochemistry of trace elements* (2004). Moscow: Fgou-vunmc.

26. Irmuhametova G. Sports nutrition-harm or benefit? *J Sci* (2020) 4(9):65–8.

27. Afanasieva D, Gazetdinov R. Determination of trace elements content in sports food additives by atomic absorption spectroscopy. *Inn Sci* (2020) 3:7–8.

28. Szczepańska B, Malczewska-Lenczowska J, Wajszczyk B. Evaluation of dietary intake of vitamins and minerals in 13-15 years old boys from a sport school in Warsaw. *Rocz Panstw Zakl Hig.* (2016) 67(1):59–68.

29. Zaitseva I, Zaitsev O. The influence of professional physical activity on the element status in the hair of young athletes (wrestlers). *Hum Physiol* (2019) 45 (1):69–74. doi: 10.1134/S0362119719010158

30. Evstafyeva E, Boyarinceva Y, Evstafyeva I. Peculiarities of microelement content and hemodynamic parameters in teenagers and youths with different level of physical activity. *Rus J Physiol* (2017) 103(5):570–81.

31. Sureda A, Julibert A. Trace element contents in toenails are related to regular physical activity in older adults. *PloS One* (2017) 12(10):e0185318. doi: 10.1371/journal.pone.0185318

32. Soria M, Anson M, Escanero J. Correlation analysis of exercise-induced changes in plasma trace element and hormone levels during incremental exercise in well-trained athletes. *Biol Trace Elem Res* (2016) 170(1):55–64. doi: 10.1007/s12011-015-0466-5

33. Maynar M, Llerena F, Grijota F. Serum concentration of several trace metals and physical training. *J Int Soc Sports Nutr* (2017) 14:19. doi: 10.1186/s12970-017-0178-7

34. Llerena F, Maynar M, Barrientos G. Comparison of urine toxic metals concentrations in athletes and in sedentary subjects living in the same area of extremadura (Spain). *Eur J Appl Physiol* (2012) 112(8):3027–31. doi: 10.1007/s00421-011-2276-6

35. Zaitseva I, Skalny A, Tinkov A. The influence of physical activity on hair toxic and essential trace element content in male and female students. *Biol Trace Elem Res* (2015) 163:58–66. doi: 10.1007/s12011-014-0172-8

36. Muñoz D, Maynar M, Barrientos G. Effect of an acute exercise until exhaustion on the serum and urinary concentrations of cobalt, copper, and manganese among well-trained athletes. *Biol Trace Elem Res* (2019) 189(2):387–94. doi: 10.1007 / s12011-014-0172-8

37. Rogerson D. Vegan diets: practical advice for athletes and exercisers. *J Int Soc Sports Nutr* (2017) 14(1):36. doi: 10.1186/s12970-017-0192-9

38. Merkiel S, Chalcarz W. The relationship between physical fitness, urine iodine status, and body-mass index in 6- to 7-year-old polish children. *Int J Sport Nutr Exerc Metab* (2011) 21(4):318–27. doi: 10.1123/ijsnem.21.4.318

39. Maynar M, Muñoz D, Alves J. Influence of an acute exercise until exhaustion on serum and urinary concentrations of molybdenum, selenium, and zinc in athletes. *Biol Trace Elem Res* (2018) 186(2):361–9. doi: 10.1007/s12011-018-1327-9

40. Maynar-Mariño M, Llerena F, Bartolomé I. Effect of long-term aerobic, anaerobic and aerobic-anaerobic physical training in seric toxic minerals concentrations. *Trace Elem Med Biol* (2018) 45:136–41. doi: 10.1016/j.jtemb.2017.10.007

41. Mogulkoc R, Akil M. Review - selenium - its metabolism and relation to exercise. *Pak J Pharm Sci* (2016) 29(5):1719–25. doi: 10.1016/j.jtemb.2017.10.007

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 11 October 2022
DOI 10.3389/fnut.2022.961892

OPEN ACCESS

EDITED BY
Mor-Li Hartman,
The Forsyth Institute, United States

REVIEWED BY
Siqi Liu,
Guangxi University, China
Ping Yao,
Huazhong University of Science and Technology, China
Jiazhen Wu,
Guangzhou University of Chinese Medicine, China

*CORRESPONDENCE
Yan Zhao
amyzhaosb@163.com

SPECIALTY SECTION
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

RECEIVED 05 June 2022
ACCEPTED 08 September 2022
PUBLISHED 11 October 2022

CITATION
Zhang L, Dai X, Wang L, Cai J, Shen J, Shen Y, Li X and Zhao Y (2022) Iron overload accelerated lipid metabolism disorder and liver injury in rats with non-alcoholic fatty liver disease. *Front. Nutr.* 9:961892. doi: 10.3389/fnut.2022.961892

Iron overload accelerated lipid metabolism disorder and liver injury in rats with non-alcoholic fatty liver disease

Lijia Zhang, Xuezheng Dai, Li Wang, Jingming Cai, Jie Shen, Yang Shen, Xianan Li and Yan Zhao*

Department of Nutrition and Food Hygiene, School of Public Health, Harbin Medical University, Harbin, China

Background/aims: Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide. Iron overload has been implicated in chronic non-communicable liver diseases, but its relationship with NAFLD remains unclear. This study aimed to investigate the underlying roles of iron overload in the development of NAFLD.

Methods: Male Sprague Dawley rats were fed with a high-fat diet (HFD) and/or iron for 8, 12, and 20 weeks. Some rats fed with HFD plus iron also received intraperitoneal injection of deferoxamine (DFO) for 8 weeks. Liver steatosis, lipid metabolism and injury were evaluated.

Results: A NAFLD model, including typical liver steatosis, was established by feeding rats with a HFD, while iron overload alone is not enough to induce severe NAFL. Compared with rats fed a HFD, excess iron further increased lipid accumulation, serum levels of lipids, enzymes of liver function, and expression levels of CD36 and FAS in rat liver. In addition, iron overload decreased the activities of antioxidative enzymes in liver compared with HFD rats. The levels of CPT1 and the ratios of p-ACC/ACC were also decreased by iron overload. DFO effectively reversed the abnormal lipid metabolism and liver damage induced by a high-fat, high-iron diet.

Conclusion: A HFD plus iron overload might synergistically aggravate lipid metabolism disorders, liver injury, and oxidative damage, compared with a HFD alone. DFO might help to alleviate lipid metabolism dysfunction and improve the pathogenesis of NAFLD.

KEYWORDS

NAFLD, high-fat diet, iron overload, lipid metabolism, oxidative stress

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world (1), with a global prevalence of approximately 25% (2). NAFLD encompasses a wide spectrum of liver damage, ranging from simple steatosis to non-alcoholic steatohepatitis and advanced fibrosis, ultimately leading to hepatocellular carcinoma (1, 3). Previous studies have found that patients with obesity and type 2 diabetes are more prone to liver steatosis and inflammation; in addition, patients with NAFLD often exhibit manifestations of type 2 diabetes mellitus (T2DM), such as dyslipidemia and insulin resistance (4, 5). Therefore, a better understanding of the pathogenesis of NAFLD may provide stronger evidence for the prevention and treatment of NAFLD and concomitant metabolic diseases.

A popular theory for the pathogenesis of NAFLD is the complex "multiple hit" hypothesis based on the traditional "two-hit" hypothesis (6, 7). Insulin resistance ("first strike") promotes the entry of free fatty acids (FFAs) into hepatocytes, and then simple steatosis happens if these FFAs are unproperly metabolized or secreted. Simple steatosis leaves the liver vulnerable to second strikes, including mitochondrial dysfunction, oxidative stress, gut-derived bacterial endotoxins and inflammation (8).

Iron is an essential trace element in the human body, being a key component of hemoglobin and myoglobin, and also an essential component of cell respiration enzymes such as cytochromes, cytochrome oxidase, and catalase (CAT), which participate in crucial processes such as electron transport, redox reaction, and cell differentiation and growth (9–11). However, excess iron may initiate oxidative stress as a second "hit", *via* the generation of reactive oxygen species (ROS) by the Fenton reaction (12). The liver is the most important organ in the body in terms of iron metabolism, and circulating iron and the iron storage of patients with NAFLD were shown to be higher than those in a control population (13). Among patients with NAFLD, the probability of iron metabolism disorders in obese children was significantly higher than that in normal children (14). In a NAFLD study, liver histological evidence indicated that more than a third of biopsy samples showed iron overload (15). This phenomenon is closely related to excessive iron deposition in the liver (16–18). Iron overload may aggravate the insulin resistance and promote the progress of non-alcoholic steatohepatitis and liver fibrosis.

It is known that excess iron can lead to oxidative injury. However, a recent study found that dietary iron overload abrogated chemically induced liver cirrhosis in rats (19). Therefore, understanding the roles of iron overload in NAFLD is extremely essential. This study aimed to clarify the effects and potential molecular mechanisms of a high-fat, high-iron diet on lipid metabolism and liver injury in NAFLD rats.

Materials and methods

Animals and experimental design

Six-week-old male Sprague Dawley rats (Vital River Laboratories, Beijing, China) were maintained under a 12-h light/dark cycle at 21–23°C and provided with food and water *ad libitum*. The rats were randomly divided into control, high-iron (HI), high-fat diet (HFD) (HF), HFD with low dose of iron (HFL) and HFD with high dose of iron (HFH) groups. Rats fed with HFH diet were also given an intraperitoneal injection of deferoxamine (DFO) or saline once a day from 12 to 20 weeks. The rats received the respective diets for 20 weeks. Body weight and food intake were measured and recorded weekly. Animal care and experimental procedures in this study were approved by the Animal Experimental Committee of Harbin Medical University (permission number: SCXK 2012-0001).

Rats were deeply anesthetized using sodium pentobarbital and sacrificed at weeks 8, 12, and 20, respectively. Blood, liver tissues, and epididymal, inguinal and retroperitoneal white adipose tissues were weighed and stored at −80°C for future analysis. The liver weight/body weight × 100% was calculated as the liver index. Body fat rate was calculated as the percentage of white adipose tissues to body weight.

Dosage information

The control group was fed a 10% calorie-from-fat diet (D12450H, Research Diets). The HI group was fed a control diet plus ferrous sulfate (10 g per kilogram of diet). The HF group received a 45% calorie-from-fat diet (based on D12451 with slight modification). The rats in HFL or HFH groups were fed with a HFD plus ferrous sulfate (5 or 10 g per kilogram of diet). DFO (Novartis Pharma Stein AG, Switzerland) was given to the rats *via* intraperitoneal injection at the dose of 100 mg/kg body weight for eight consecutive weeks.

Biochemical analysis

Serum was isolated from blood samples by centrifugation (3,000 × g, 15 min). The levels of triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were analyzed using an automatic biochemical analyzer (Hitachi7100, Japan). Serum ferritin levels were detected using an enzyme-linked immunosorbent assay kit (Biotopped, Beijing, China). Hepatic lipids, superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and CAT activities and malondialdehyde (MDA) contents were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Histologic analysis

Frozen liver tissues were cut with a cryostat (Leica CM 1100), and stained with Oil Red O. Fresh hepatic tissue was fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, cut at 5 μm, and stained with hematoxylin and eosin (HE) for histopathological examination, and graded for steatosis (scored 0–3), hepatic ballooning (0–2) and lobular inflammation (0–3) according to the Kleiner classification criteria. The NAFLD activity score (NAS) was calculated as the sum of the steatosis, lobular inflammation, and ballooning scores, ranging from 0 to 8 (20). Sirius red staining was used to detect collagen deposition.

Real-time reverse transcriptase polymerase chain reaction

Total RNA samples were extracted from hepatic tissues using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. 1μg of RNA was reverse transcribed into cDNA using a High Capacity cDNA RT Kit (ABI, USA) and real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using an ABI 7,500 Fast real-time RT-PCR System with a Power SYBR Green Kit (Applied Biosystems, Foster City CA, USA), to detect mRNA levels of CD36, fatty acid synthase (FAS), acetyl-coA carboxylase (ACC)-1, and carnitine palmitoyltransferase (CPT)-1, with β-actin as an internal control for mRNA quantification. The primer sequences were shown in Table 1. 40 PCR cycles consisting of 15 s at 95°C and 1 min at 60°C were applied. Relative expression levels of mRNAs were calculated using the Ct ($2^{-\Delta\Delta Ct}$) method.

Western blot

Total protein was extracted from hepatic tissues using lysis buffer with protease inhibitors. The protein concentrations were measured with a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). Twenty microgram of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (Millipore Corporation, MA, USA), incubated with 5% skimmed milk, and probed with primary antibodies diluted in blocking buffer containing 1% bovine serum albumin (BSA) at 4°C overnight. The primary antibodies used were as follows: anti-CD36 (1:1,000; Abcam, United Kingdom), anti-FAS (1:1,000; Cell Signaling, USA), anti-ACC (1:1,000; Cell Signaling), anti-phospho-ACC (p-ACC) (1:1,000; Cell Signaling), anti-CPT1 (1:6,000; Abcam), and anti-β-actin (1:800; Santa Cruz). The membranes were washed three times with TBST, incubated with the secondary antibody (1:10,000; ZSGB-Bio, Beijing, China) diluted in TBS (containing 1% BSA) for 1 h, and reacted with ECL Prime Western Blotting Detection Reagent (Beyotime Institute of Biotechnology). Signals were detected using a FluorChem-E imaging system (Protein Simple, USA). The blot signals were quantified relative to the housekeeping protein β-actin in the same sample and normalized to control. The density of the phosphorylated protein was normalized to the total protein. Triplicates were used to calculate the average density.

TABLE 1 Primers used for real-time RT-PCR.

Gene	Primer sequence
CD36	Forward: 3′-GCAGCCTCCTTTCCACCTTT-5′
	Reverse: 3′-AAAGGCGTTGGCTGGAAGA-5′
FAS	Forward: 3′-TCCCAGGTCTTGCCGTGC-5′
	Reverse: 3′-GCGGATGCCTAGGATGTGTGC-5′
ACC1	Forward: 3′-AACATCCCGCACCTTCTTCTAC-5′
	Reverse: 3′-CTTCCACAAACCAGCGTCTC-5′
CPT1	Forward: 3′-TGCTGCATGGAAGATGCTTT-5′
	Reverse: 3′-CGTCGGTGGCCATGACATA-5′
β-actin	Forward: 3′-GAAGATCCTGACCGAGCGTG-5′
	Reverse: 3′-CGTACTCCTGCTTGCTGATCC-5′

Statistical analysis

All the data were presented as the mean ± SD. Differences were analyzed by ***t***-tests or one-way ANOVA followed by Student-Newman-Keuls test for multiple comparison. A ***P***-value < 0.05 was considered statistically significant.

Results

Iron overload aggravated lipid accumulation induced by a high-fat diet

Although there was no significant difference in body weights among the groups at the start of the experiment, body weight, liver index, and body fat percentage were all significantly higher in rats fed the HFD with or without iron than the control group after 8, 12, and 20 weeks. The difference between the HI and control groups was not significant for 12 and 20 weeks (Figures 1A–C).

The levels of ferritin in serum were elevated in rats fed with iron and/or HFD at 12 and 20 weeks, and high fat diet with high iron induced higher ferritin contents compared with control/high-fat group at the same time point (Figure 1D). The TG levels in the liver and serum were significantly increased in the HF and HFL groups compared with the control group at 8 and 12 weeks, and were also significantly higher in the HFH group compared with both the control and HF groups

10.3389/fnut.2022.961892

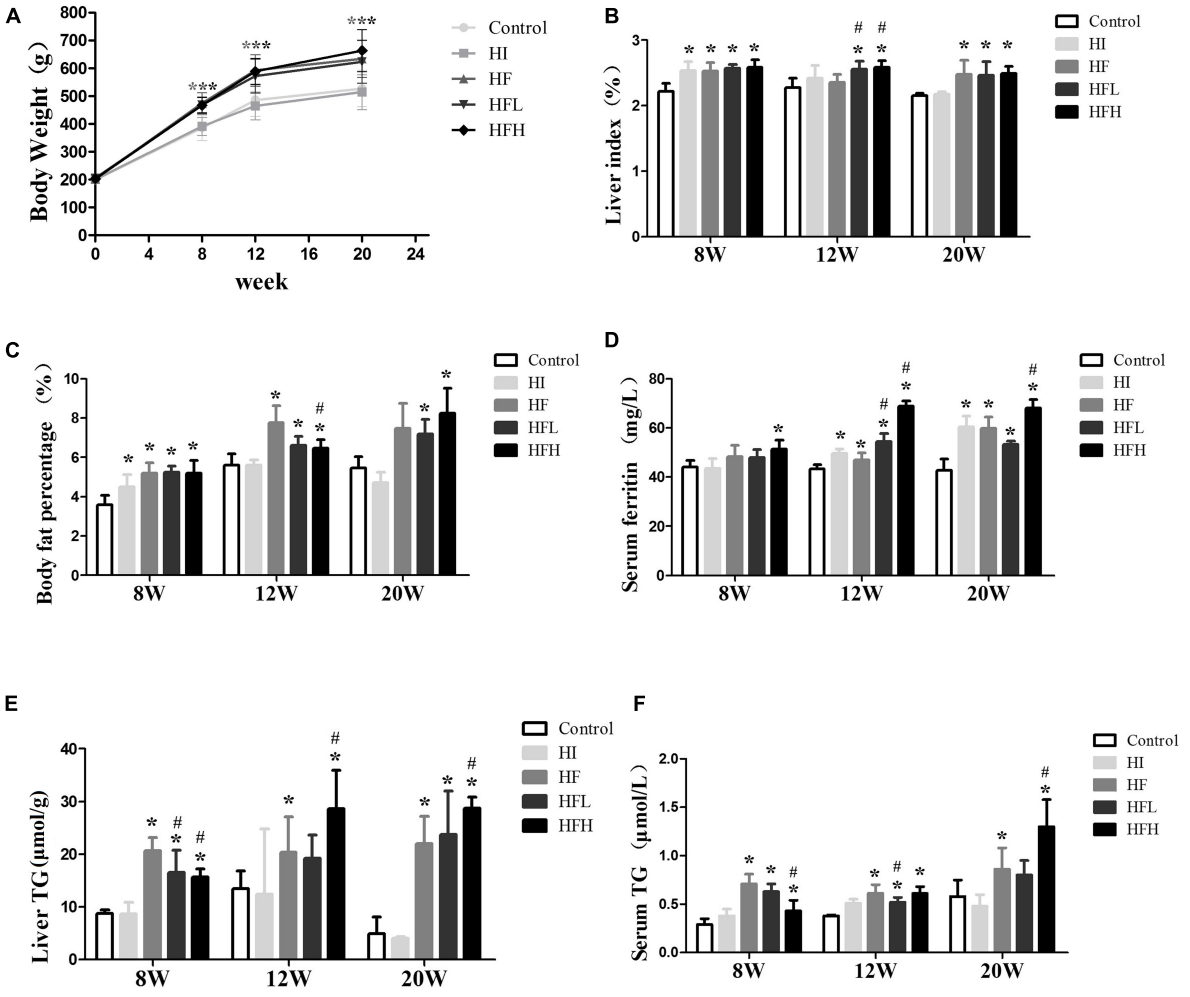


FIGURE 1
Iron overload aggravated lipid accumulation induced by a HFD. **(A)** Body weight, **(B)** liver index, **(C)** body fat percentage, **(D)** serum ferritin, **(E)** liver TG, and **(F)** serum TG levels in serum in rats at 8, 12 and 20 weeks. * and # compared with control/high-fat group at the same time point, respectively, $P < 0.05$. Only one "* and ***" refers to the HF, HFL and HFH groups compared to the control group at the same time point, respectively, $P < 0.05$.

at 20 weeks although were lower compared with HF group at 8 weeks ($P < 0.05$). The average contents of liver triglyceride in HFL increased a little while in HFH were similar from 12 to 20 weeks. Serum TG levels were lower in the HFL group at 12 weeks and in the HFH group at 8 weeks compared with the HF group (Figures 1E,F).

Iron overload aggravated hepatic steatosis and injury in non-alcoholic fatty liver disease rats induced by high-fat diet

There was extensive lipid accumulation in the livers of rats exposed to HFD with or without iron for 12 and 20 weeks, as shown by HE staining. In addition, hepatocytes in the HFH group showed steatosis, accompanied by obvious inflammation and diffuse ballooning. Fat accounted for more than 80% of hepatocytes at 20 weeks according to Oil Red O staining (Figures 2A–C). The combined administration of high-fat and high-iron induced collagen deposition in the perisinusoidal space of rat hepatocytes, while no collagen deposition was observed *via* Sirius red staining in rats fed with HI or HF diet alone (Figure 2D). The quantified NAFLD activity scores (NAS) were consistent with the HE staining results. The volumes of lipid drops were much bigger and hepatic ballooning in HFH group was clearly visible from 12 to 20 weeks (Figures 2E,F).

ALT levels were significantly higher in the HF, HFL, and HFH groups compared with the control group at 12 weeks

10.3389/fnut.2022.961892

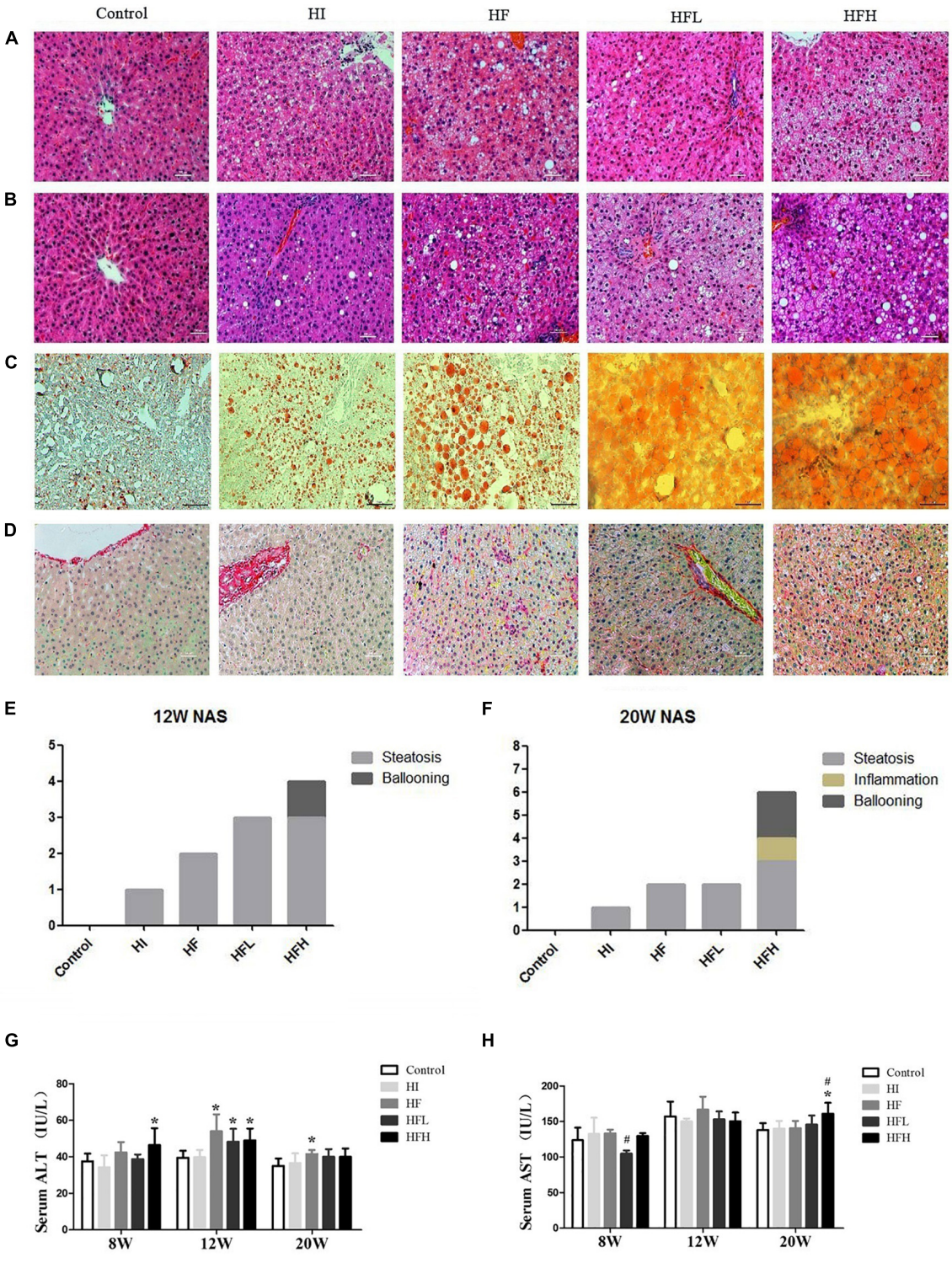


FIGURE 2
Dietary iron overload aggravated hepatic steatosis and liver injury in rats with a HFD-induced NAFLD. HE staining at **(A)** 12 and **(B)** 20 weeks. **(C)** Oil red O staining at 20 weeks. **(D)** Sirius red staining at 20 weeks (magnification × 200). NAFLD activity scores in rats at **(E)** 12 and **(F)** 20 weeks. Serum **(G)** ALT and **(H)** AST levels in rats at each time point. * and # compared with control/high-fat group at the same time point, respectively, $P < 0.05$.

($P < 0.05$), while obvious increase of AST levels occurred at 20 weeks in HFH group compared with the control or HF groups (Figures 2G,H).

Deferoxamine improved lipid metabolism disorder and liver injury induced by iron overload plus a high-fat diet

Treatment with DFO for 8 weeks had no significant effect on body weight, liver index, or body fat percentage compared with the HFH-fed rats after 20weeks (Figures 3A–C). However, DFO significantly decreased the levels of TG in both the liver and serum, serum AST and ferritin levels compared with the HFH group ($P < 0.05$) (Figures 3D–H).

DFO treatment also reduced the amount and area of lipid droplets in the liver during the last 8 weeks of the study, as shown by HE and Oil red O staining. Collagen deposition in hepatocytes was also reduced as shown by Sirius red staining (Figure 3I).

Iron overload plus high-fat diet impaired antioxidant capacity and induced oxidative damage

The activities of antioxidative enzymes (CAT, SOD, GSH-Px) were significantly reduced in all groups, compared with the controls, while the levels of MDA were increased at 12 and 20 weeks. Similar changes were seen in the HFH compared with the HF group ($P < 0.05$) (Figures 4A–D). Although there was no significant difference in MDA levels between the DFO and HFH groups, DFO attenuated oxidative damage in hepatocytes of NAFLD rats induced by a high-fat, high-iron diet ($P < 0.05$) (Figures 4E–H).

High-fat and high-iron diets affected fatty acid intake and synthesis

The levels of CD36 and FAS mRNA and protein were significantly higher in the HF, HFL, and HFH groups compared with the control group, while the levels of CPT1 mRNA and protein and the ratio of p-ACC/ACC protein were significantly lower when exposed to HFD with or without iron compared with the controls. In addition, CD36 and FAS expression were significantly higher in the HFH compared with the HF group (Figures 5A–J).

Deferoxamine attenuated the effect of a high-fat, high-iron diet on lipid metabolism

CD36 and FAS mRNA and protein levels were significantly decreased after treatment with DFO, compared with the HFH group (Figures 6A–D,G). In addition, β-oxidation of fatty acids in the liver was aggravated in rats fed a high-fat, iron-rich diet. DFO also increased CPT-1 and p-ACC expression was significantly in the compared with the HFH group (Figures 6E,F,H,I).

Discussion

Dietary administration of iron is usually applied to induce iron overload in animals, similar as genetic mutations such as hereditary hemochromatosis. Iron overload is also due to repeated blood transfusions, increased dietary iron, iron supplementation and aging. But the effects of iron overload on the liver and their underlying mechanisms as well as the origin of excess iron remain unclear. This study found that a long-term high fat diet induced steatosis, lipid accumulation, antioxidant capacity damage and lipid metabolism disorders; whereas, dietary iron overload aggravated the abnormal lipid metabolism and liver injury in NAFLD rats, and iron removal therapy by DFO efficiently attenuated this phenomenon.

Ferritin is an iron storage protein and its concentration in the serum reflects iron stores; elevated ferritin reflects risk of iron overload. In our study, the levels of serum ferritin were increased in the presence of high fat diet and/or iron at 12 and 20 weeks. Especially, high fat diet with high iron induced higher ferritin contents compared with control/high-fat group at the same time point, and DFO decreased the ferritin levels, indicating the occurrence of iron overload induced by high fat or high iron diets, and even more iron overload by synergistic administration of high fat and high iron diet.

The roles of iron in the metabolic diseases including NAFLD were still controversial (21). A series of recent studies showed that hepatic iron overload contributed to the progression of NAFLD. In NAFLD patients, excessive nutrition leads to endoplasmic reticulum stress in hepatocytes and the acceleration of intracellular iron deposition (22). However, Atarashi et al. showed that dietary iron overload could also improve chemical-induced hepatic cirrhosis (19). According to the results from the present study, HFD induced extensive lipid accumulation in the hepatocytes. The average contents of liver triglyceride in HFL were a little higher at 20 weeks than those at 12 weeks, while in HFH were similar at 12 and 20 weeks, implicating serious hepatic steatosis lasted for a long time. Liver TG levels were also significantly higher in the HFH group compared with both the control and HF groups

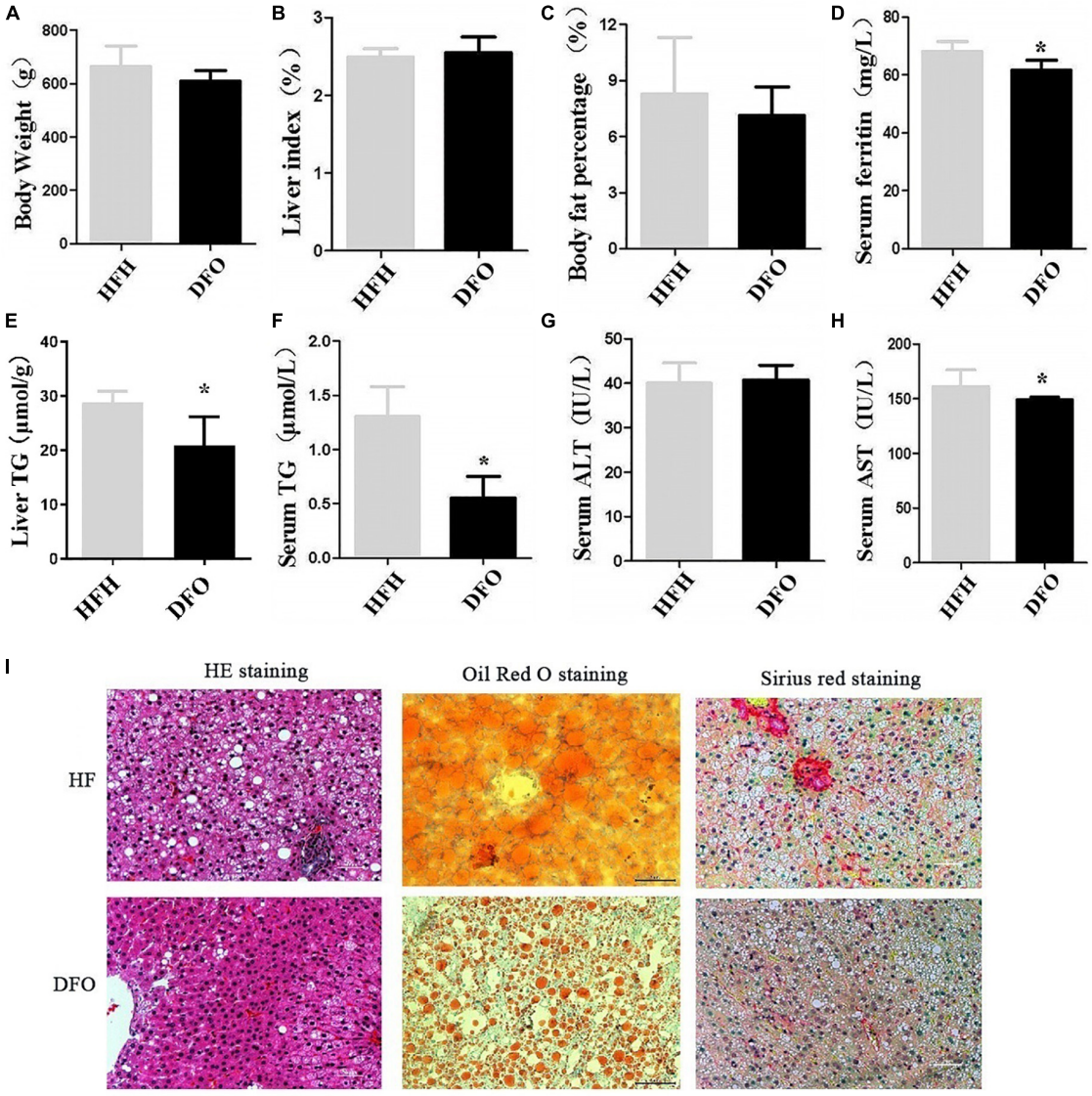


FIGURE 3
DFO treatment improved lipid metabolism and liver injury induced by iron overload plus a HFD. **(A)** Body weight, **(B)** liver index, **(C)** body fat percentage, **(D)** serum ferritin, **(E)** liver TG, **(F)** serum TG, **(G)** ALT, and **(H)** AST levels in rats. **(I)** HE, Oil red O, and Sirius red staining at 20 weeks. * Compared with the HFH group, $P < 0.05$.

at 20 weeks. Hepatic ballooning in HFH was clearly visible from 12 to 20 weeks. Therefore, the hepatocytes were easy to rupture and lipid droplets were dispersed when sliced. From this point of view, it was demonstrated that long-term excess dietary iron could lead to acceleration of hepatic steatosis and injury caused by HFD, supporting most recent findings. Additionally, we found that the administration of iron at low dose for 12 weeks and at high dose for 8 weeks obviously reduced HFD-triggered elevation of TG levels, in accordance with the recognition that appropriate iron was beneficial to the health. But iron overload could lead to oxidative stress and liver damage, a risk factor for the onset and progression of NAFLD (23).

Oxidative stress is defined as an imbalance between the production of free radicals and the antioxidant system responsible for maintaining homeostasis in the organism. Oxidative stress can lead to damage by the activation of inflammation, the secretion of proteases and the production of large amounts of oxidative products (24). Antioxidant enzyme systems, including SOD, CAT and GSH-Px, catalyze reactions to neutralize free radicals and ROS. MDA is the end-product of the peroxidation between free radicals and lipids, and can directly reflect the degree of lipid oxidation. In the present study, a combined high-fat, high-iron diet weakened the antioxidant capacity of the liver tissues and increased the MDA contents

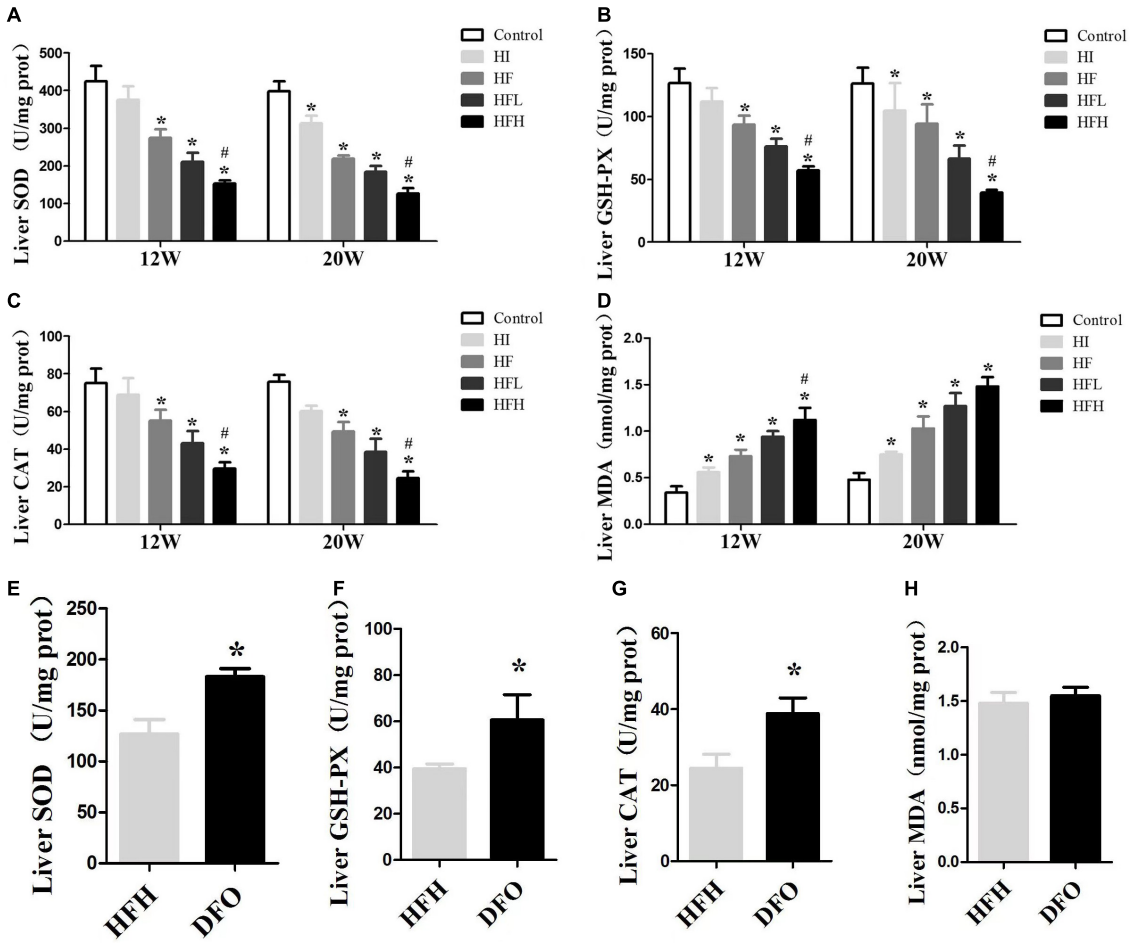


FIGURE 4
Iron overload impaired antioxidant capacity and increased oxidative damage in HFD-induced NAFLD rats while DFO attenuates this effect. **(A–D)** The activities of SOD, GSH-PX, CAT and the levels of MDA in the liver of rats. * and # compared with control/high-fat group at the same time point, respectively, $P < 0.05$. **(E–H)** The activities of SOD, GSH-PX, CAT and the levels of MDA in the liver of rats after DFO treatment. * Compared with the HFH group, $P < 0.05$.

compared with a HFD alone. Notably, lipid peroxidation and antioxidant capacity had been shown to be raised or reduced, respectively, in many metabolic diseases, such as NAFLD (25). Oxidative stress and lipid peroxidation were associated with the development of NAFLD (26). Iron is an important promoter of ROS production that can initiate or catalyze the Fenton reaction and produce free radicals to destroy liver cells. At the same time, ROS produced by excess iron will further aggravate lipid peroxidation and oxidative damage (**Figure 7**) (27, 28).

Lipid metabolism is an important part of the pathogenesis of NAFLD. CD36 is a cell membrane transporter that plays an important role in promoting the absorption of FFAs into muscle and adipose tissue (29). In the current study, CD36 levels of mRNA and protein in the liver was significantly increased in rats fed a HFD. Previous studies showed that a HFD-induced increase in the expression of CD36 might contribute to the uptake of FFAs and the accumulation of TG in the liver (30). Expression levels of CD36 in the liver were also significantly higher in rats fed with high-fat, high-iron diets compared with those fed high-fat alone, and levels were significantly decreased after iron isolation treatment. These results suggested that long-term iron overload might promote the uptake of FFAs in the liver.

Energy produced by β-oxidation of fatty acids is an important source of energy. CPT1 is a rate-limiting enzyme for fatty acid β-oxidation and occurs in three forms in the body, of which only CPT1a is expressed in the liver (31). ACC and FAS are related to fatty acid synthesis. The present results showed that CPT1 expression levels were significantly decreased and FAS levels were increased in livers of rats fed a HFD. Moreover, the ratio of p-ACC/ACC was significantly reduced. These effects were exacerbated in rats fed with high-fat and high-iron diets. During the *de novo* fatty acid synthesis,

10.3389/fnut.2022.961892

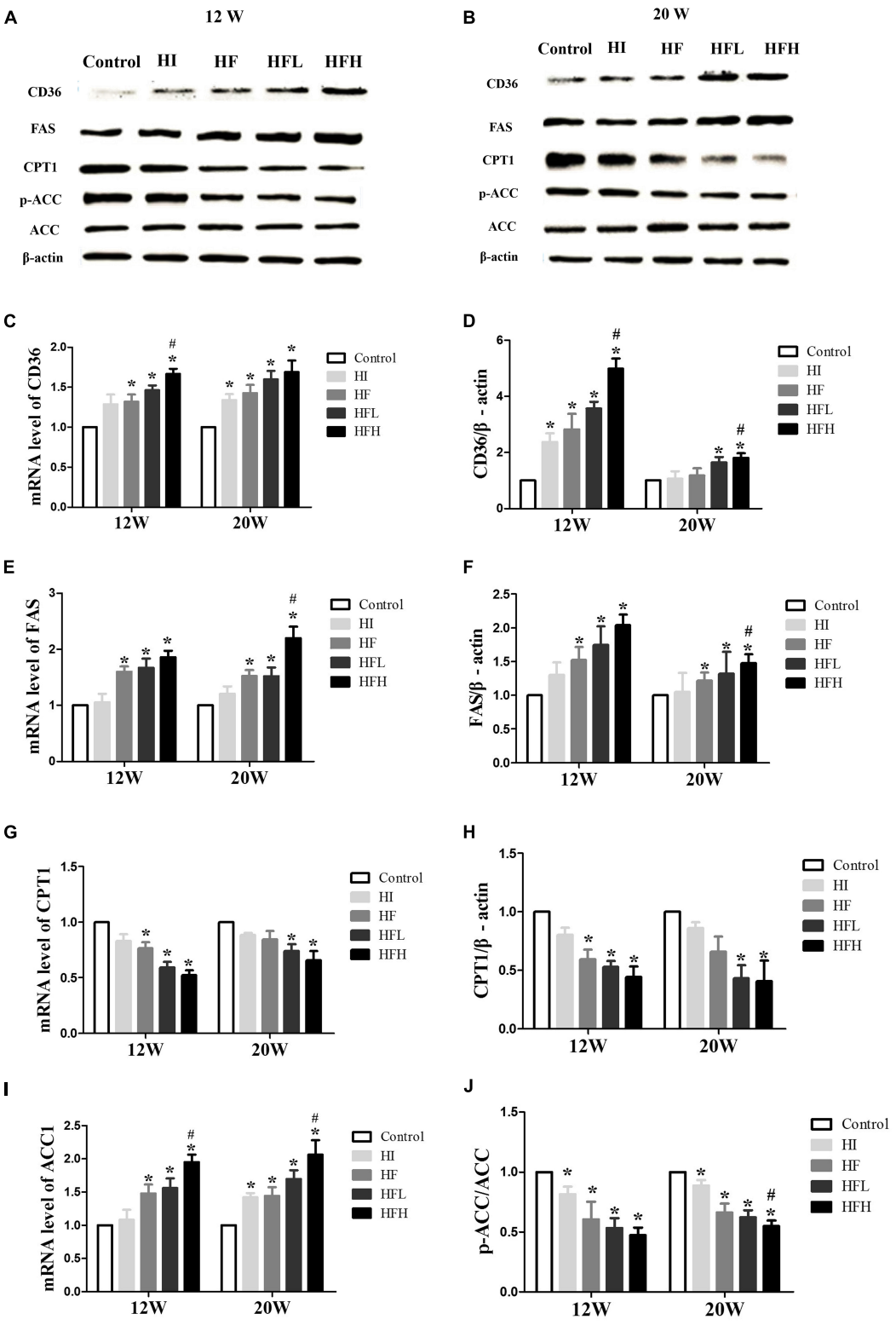


FIGURE 5
High-fat and high-iron diets affected fatty acid uptake and synthesis. **(A–J)** CD36, FAS, CPT1, and ACC mRNA and protein expression levels in the liver of rats. * and # compared with control/high-fat group at the same time point, respectively, $P < 0.05$.

10.3389/fnut.2022.961892

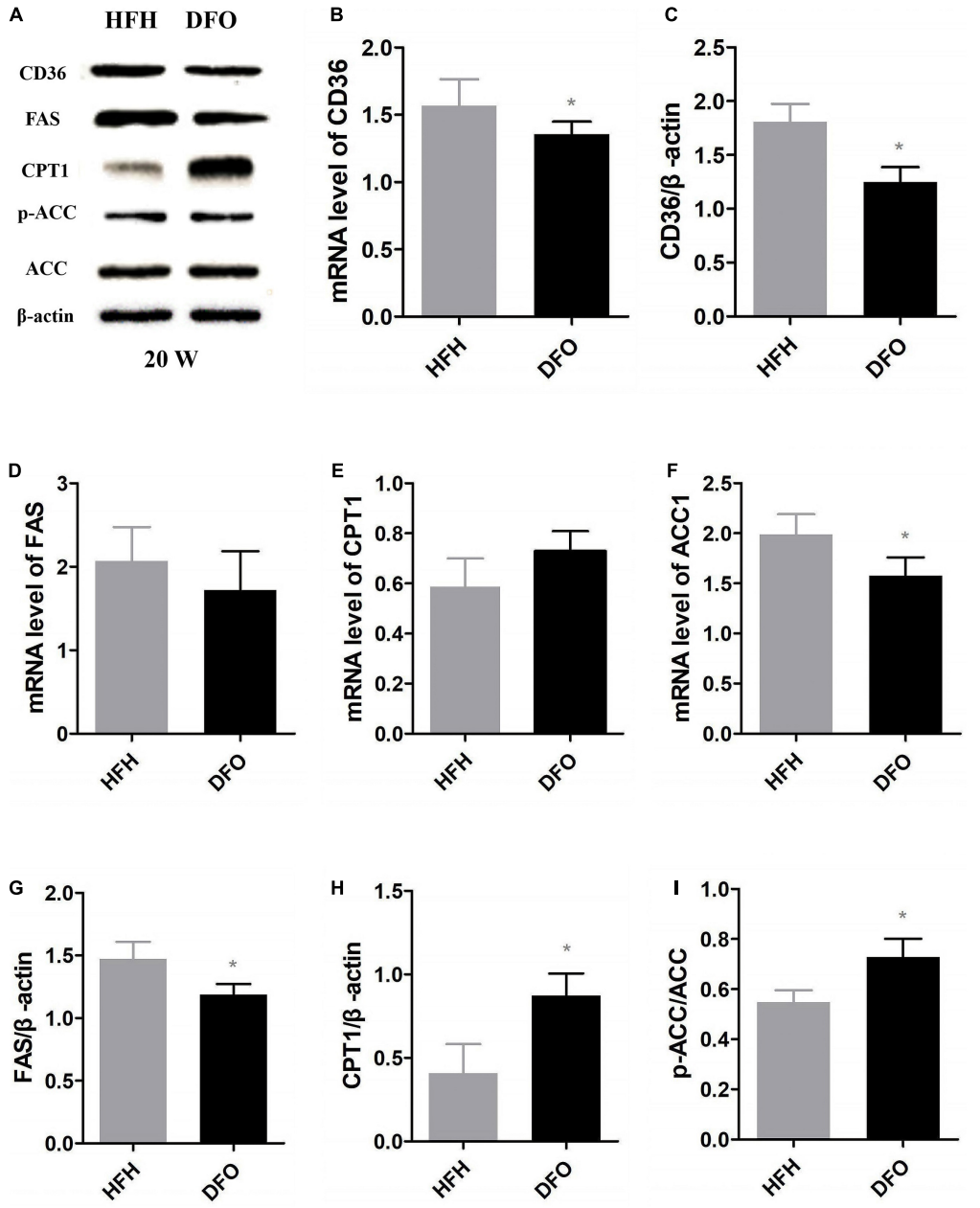


FIGURE 6
DFO attenuated the effect of a high-fat, high-iron diet on lipid metabolism. **(A–I)** CD36, FAS, CPT1 and ACC mRNA and protein expression levels in the liver after DFO treatment. * Compared with HFH group, $P < 0.05$.

malonyl coenzyme A could inhibit the activity of CPT1 *via* the catalyzation by ACC, a central enzyme involved in fatty acid β-oxidation and inactivated on phosphorylation (32). Inhibition of ACC phosphorylation by high fat and high iron diet could increase ACC activities, leading to the subsequent lipogenesis and accumulation. On the basis of "two-hit" hypothesis, iron overload as a multiple hit might aggravate lipid peroxidation and oxidative stress, leading to the release of inflammatory factors. All these formed multiple hits to promote the progress of non-alcoholic steatohepatitis and liver fibrosis. Interestingly, iron chelation reversed the levels of these lipid metabolism-related genes and proteins, implicating that long-term iron overload might promote the synthesis of FFAs, whereas inhibit the consumption of FFAs in the liver, leading to more serious lipid metabolism disorder than NAFLD itself (**Figure 7**).

Several studies also showed that hepatic iron deposition could play a role in the pathogenesis of NAFLD (33–35). In our study, both iron overload and high fat induced liver steatosis in

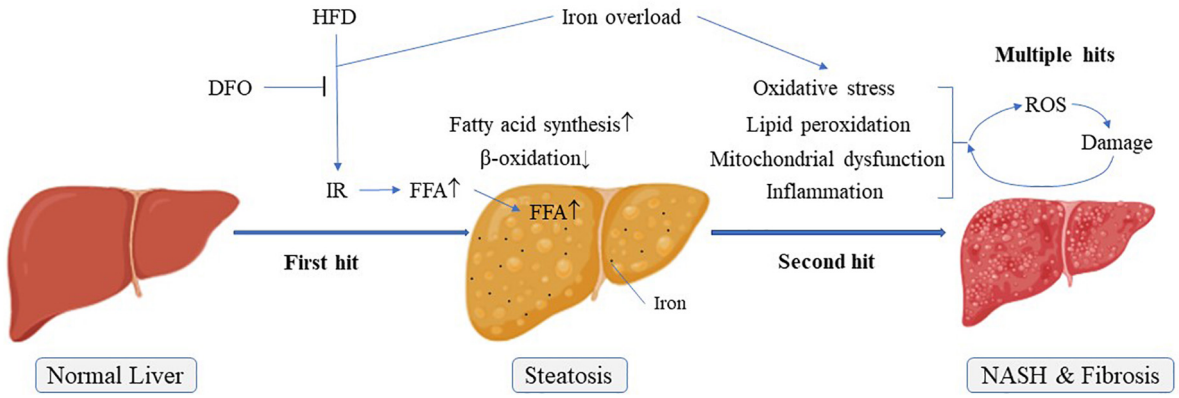


FIGURE 7
Possible pathogenesis of NAFLD "multiple hit theory". IR, Insulin resistance; NASH, Non-alcoholic steatohepatitis.

rats, and their synergistic effects further aggravated this damage with obvious inflammation and fibrosis (Figure 7). DFO, an iron chelator, has been proved to be helpful for the protection of nerves and diabetic wound healing (36–38), DFO can also have a beneficial effect on improving adiposity by inhibiting oxidative stress and inflammation (39). The use of DFO significantly reversed this combined effect of iron, mainly by inhibiting the liver's damage resulting from lipid peroxidation and oxidative stress, thereby alleviating lipid metabolism disorder in the liver caused by iron overload, not by HFD. Therefore, our findings provided powerful evidence for the involvement of iron overload in the pathogenesis of NAFLD. Meanwhile, DFO might be considered as a potential candidate for the treatment of NAFLD.

Interestingly, the iron overload in the HI group significantly increased body weight, liver index and fat content at 8 weeks, but there was no difference at 12 and 20 weeks, compared with the control group. It was supposed that iron supplementation for rats at a period of rapid growth for short time might help to function as a growth promoter. But as time went, the body could be adapted to iron supplementation and the promotion disappeared. In addition, the HI group was also overloaded with iron, but no adverse effects were observed when exposure to iron alone for 20 weeks in our study, except high levels of serum ferritin and lower antioxidant capacity. It was not paradoxical that excess iron may initiate oxidative stress as a second "hit" in the presence of lipid accumulation.

There were also some shortcomings in the present study. First, iron toxicity can influence major tissues involved in glucose and lipid metabolism and organs attacked by related complications. It was demonstrated that iron overload resulted in the disturbance of the lipid metabolism. However, there was an extensive interaction network or among various kinds of factors in the body. The elevation of iron storage might be associated with other factors or specific nutrition. Second, this study made a preliminary exploration on the roles of DFO in the treatment. DFO was applied after the accumulation of excess lipid and iron in the liver. It was worth investigating further whether DFO could be used at the beginning of the experiment for the prevention of iron overload and NAFLD or even liver damage. In summary, we demonstrated that a HFD caused NAFLD in rats, and that concurrent iron overload could further aggravate lipid metabolism disorders by promoting the transport of FFAs to the liver, the synthesis of endogenous fatty acids, and inhibiting fatty acid β-oxidation, resulting in lipid accumulation and oxidative damage in the liver during the development of NAFLD. Iron removal may help to relieve lipid metabolism dysfunction and improve NAFLD. These findings may provide new insights into the prevention and treatment of NAFLD.

Data availability statement

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

Ethics statement

This animal study was reviewed and approved by the Animal Experimental Committee of Harbin Medical University.

Author contributions

LZ performed the experiments and wrote the manuscript. XD and LW wrote the manuscript. JC and JS performed the experiments. YS and XL contributed to review and editing. YZ conceived and designed the experiments and wrote the

manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the National Natural Science Foundation of China (Grant Nos. 81273062 and 81573136).

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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.961892/full#supplementary-material

References

1. Powell EE, Wong VW, Rinella M. Non-alcoholic fatty liver disease. *Lancet.* (2021) 397:2212–24. doi: 10.1016/S0140-6736(20)32511-3

2. Cotter TG, Rinella M. Nonalcoholic fatty liver disease 2020: The state of the disease. *Gastroenterology.* (2020) 158:1851–64. doi: 10.1053/j.gastro.2020.01.052

3. Sayiner M, Koenig A, Henry L, Younossi ZM. Epidemiology of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis in the united states and the rest of the world. *Clin Liver Dis.* (2016) 20:205–14. doi: 10.1016/j.cld.2015.10.001

4. Pafili K, Maltezos E, Papanas N. Ipragliflozin and sodium glucose transporter 2 inhibitors to reduce liver fat: Will the prize we sought be won? *Expert Opin Pharmacother.* (2018) 19:185–7. doi: 10.1080/14656566.2017.1413346

5. Pafili K, Roden M. Nonalcoholic fatty liver disease (NAFLD) from pathogenesis to treatment concepts in humans. *Mol Metab.* (2021) 50:101122. doi: 10.1016/j.molmet.2020.101122

6. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism.* (2016) 65:1038–48. doi: 10.1016/j.metabol.2015.12.012

7. Basaranoglu M, Basaranoglu G, Sentürk H. From fatty liver to fibrosis: A tale of "second hit". *World J Gastroenterol.* (2013) 19:1158–65. doi: 10.3748/wjg.v19.i8.1158

8. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. *Hepatology.* (2010) 52:1836–46. doi: 10.1002/hep.24001

9. Hirota K. An intimate crosstalk between iron homeostasis and oxygen metabolism regulated by the hypoxia-inducible factors (HIFs). *Free Radic Biol Med.* (2019) 133:118–29. doi: 10.1016/j.freeradbiomed.2018.07.018

10. Ravingerová T, Kindernay L, Barteková M, Ferko M, Adameová A, Zohdi V, et al. The molecular mechanisms of iron metabolism and its role in cardiac dysfunction and cardioprotection. *Int J Mol Sci.* (2020) 21:7889. doi: 10.3390/ijms21217889

11. Gattermann N, Muckenthaler MU, Kulozik AE, Metzgeroth G, Hastka J. The evaluation of iron deficiency and iron overload. *Dtsch Arztebl Int.* (2021) 118:847–56. doi: 10.3238/arztebl.m2021.0290

12. Mancardi D, Mezzanotte M, Arrigo E, Barinotti A, Roetto A. Iron overload, oxidative stress, and ferroptosis in the failing heart and liver. *Antioxidants.* (2021) 10:1864. doi: 10.3390/antiox10121864

13. Corradini E, Buzzetti E, Dongiovanni P, Scarlini S, Caleffi A, Pelusi S, et al. Ceruloplasmin gene variants are associated with hyperferritinemia and increased liver iron in patients with NAFLD. *J Hepatol.* (2021) 75:506–13. doi: 10.1016/j.jhep.2021.03.014

14. Kowdley KV, Belt P, Wilson LA, Yeh MM, Neuschwander-Tetri BA, Chalasani N, et al. NASH clinical research network. serum ferritin is an independent predictor of histologic severity and advanced fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology.* (2012) 55:77–85. doi: 10.1002/hep.24706

15. Hayashi T, Saitoh S, Takahashi J, Tsuji Y, Ikeda K, Kobayashi M, et al. Hepatic fat quantification using the two-point Dixon method and fat color maps based on non-alcoholic fatty liver disease activity score. *Hepatol Res.* (2017) 47:455–64. doi: 10.1111/hepr.12767

16. Nemeth E, Ganz T. Hepcidin-ferroportin interaction controls systemic iron homeostasis. *Int J Mol Sci.* (2021) 22:6493. doi: 10.3390/ijms22126493

17. Vogt AS, Arsiwala T, Mohsen M, Vogel M, Manolova V, Bachmann MF. On iron metabolism and its regulation. *Int J Mol Sci.* (2021) 22:4591. doi: 10.3390/ijms22094591

18. Yeo YH, Lai YC. Redox regulation of metabolic syndrome: Recent developments in skeletal muscle insulin resistance and non-alcoholic fatty liver disease (NAFLD). *Curr Opin Physiol.* (2019) 9:79–86. doi: 10.1016/j.cophys.2019.05.003

19. Atarashi M, Izawa T, Mori M, Inai Y, Kuwamura M, Yamate J. Dietary iron overload abrogates chemically-induced liver cirrhosis in rats. *Nutrients.* (2018) 10:1400. doi: 10.3390/nu10101400

20. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Nonalcoholic steatohepatitis clinical research network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology.* (2005) 41:1313–21. doi: 10.1002/hep.20701

21. Kucukazman M, Ata N, Yavuz B, Dal K, Sen O, Deveci OS, et al. Evaluation of early atherosclerosis markers in patients with nonalcoholic fatty liver disease. *Eur J Gastroenterol Hepatol.* (2013) 25:147–51. doi: 10.1097/MEG.0b013e32835a58b1

22. González-Domínguez Á, Visiedo-García FM, Domínguez-Riscart J, González-Domínguez R, Mateos RM, Lechuga-Sancho AM. Iron metabolism in obesity and metabolic syndrome. *Int J Mol Sci.* (2020) 21:5529. doi: 10.3390/ijms21155529

23. Yuan P, Qi X, Song A, Ma M, Zhang X, Lu C, et al. LncRNA MAYA promotes iron overload and hepatocyte senescence through inhibition of YAP in non-alcoholic fatty liver disease. *J Cell Mol Med.* (2021) 25:7354–66. doi: 10.1111/jcmm.16764

24. Mohamed J, Nazratun Nafizah AH, Zariyantey AH, Budin SB. Mechanisms of diabetes-induced liver damage: The role of oxidative stress and inflammation. *Sultan Qaboos Univ Med J.* (2016) 16:e132–41. doi: 10.18295/squmj.2016.16.02.002

25. Lubrano V, Balzan S. Enzymatic antioxidant system in vascular inflammation and coronary artery disease. *World J Exp Med.* (2015) 5:218–24. doi: 10.5493/wjem.v5.i4.218

26. Jennison E, Patel J, Scorletti E, Byrne CD. Diagnosis and management of non-alcoholic fatty liver disease. *Postgrad Med J.* (2019) 95:314–22. doi: 10.1136/postgradmedj-2018-136316

27. Fischer C, Volani C, Komlódi T, Seifert M, Demetz E. Valente de Souza L, et al. Dietary iron overload and Hfe-/- related hemochromatosis alter hepatic mitochondrial function. *Antioxidants* (2021) 10:1818. doi: 10.3390/antiox10111818

28. Tomasello G, Mazzola M, Leone A, Sinagra E, Zummo G, Farina F, et al. Nutrition, oxidative stress and intestinal dysbiosis: Influence of diet on gut microbiota in inflammatory bowel diseases. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* (2016) 160:461–6. doi: 10.5507/bp.2016.052

29. Asterholm IW, Mundy DI, Weng J, Anderson RG, Scherer PE. Altered mitochondrial function and metabolic inflexibility associated with loss of caveolin-1. *Cell Metab.* (2012) 15:171–85. doi: 10.1016/j.cmet.2012.01.004

30. Luukkonen PK, Qadri S, Ahlholm N, Porthan K, Männistö V, Sammalkorpi H, et al. Distinct contributions of metabolic dysfunction and genetic risk factors in the pathogenesis of non-alcoholic fatty liver disease. *J Hepatol.* (2022) 76:526–35. doi: 10.1016/j.jhep.2021.10.013

31. Wang M, Ma LJ, Yang Y, Xiao Z, Wan JB. n-3 Polyunsaturated fatty acids for the management of alcoholic liver disease: A critical review. *Crit Rev Food Sci Nutr.* (2019) 59(suppl. 1):S116–29. doi: 10.1080/10408398.2018.1544542

32. Vergani L. Fatty acids and effects on in vitro and in vivo models of liver steatosis. *Curr Med Chem.* (2019) 26:3439–56. doi: 10.2174/0929867324666170518101334

33. Yu L, He M, Liu S, Dou X, Li L, Gu N, et al. Fluorescent Egg white-based carbon dots as a high-sensitivity iron chelator for the therapy of nonalcoholic fatty liver disease by iron overload in zebrafish. *ACS Appl Mater Interfaces.* (2021) 13:54677–89. doi: 10.1021/acsami.1c14674

34. Xue H, Chen D, Zhong YK, Zhou ZD, Fang SX, Li MY, et al. Deferoxamine ameliorates hepatosteatosis via several mechanisms in ob/ob mice. *Ann N Y Acad Sci.* (2016) 1375:52–65. doi: 10.1111/nyas.13174

35. Feldman A, Aigner E, Weghuber D, Paulmichl K. The potential role of iron and copper in pediatric obesity and nonalcoholic fatty liver disease. *Biomed Res Int.* (2015) 2015:287401. doi: 10.1155/2015/287401

36. Farr AC, Xiong MP. Challenges and opportunities of deferoxamine delivery for treatment of Alzheimer's disease, Parkinson's disease, and intracerebral hemorrhage. *Mol Pharm.* (2021) 18:593–609. doi: 10.1021/acs.molpharmaceut.0c00474

37. Kosyakovsky J, Fine JM, Frey WH II, Hanson LR. Mechanisms of intranasal deferoxamine in neurodegenerative and neurovascular disease. *Pharmaceuticals.* (2021) 14:95. doi: 10.3390/ph14020095

38. Gao SQ, Chang C, Li JJ, Li Y, Niu XQ, Zhang DP, et al. Co-delivery of deferoxamine and hydroxysafflor yellow A to accelerate diabetic wound healing via enhanced angiogenesis. *Drug Deliv.* (2018) 25:1779–89. doi: 10.1080/10717544.2018.1513608

39. Grasselli E, Canesi L, Portincasa P, Voci A, Vergani L, Demori I. Models of non-alcoholic fatty liver disease and potential translational value: The effects of 3,5-L-diiodothyronine. *Ann Hepatol.* (2017) 16:707–19. doi: 10.5604/01.3001.0010.2713

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 30 November 2022
DOI 10.3389/fendo.2022.973078

OPEN ACCESS

EDITED BY
Mor-Li Hartman,
The Forsyth Institute, United States

REVIEWED BY
Jiancheng Xu,
First Affiliated Hospital of Jilin University, China
Elena Kovaleva,
Endocrinology Research Center, Russia

*CORRESPONDENCE
Yue Qi
qiyue_bjcn@163.com
qiyue_bjcn@mail.ccmu.edu.cn
Jing Liu
jingliu@ccmu.edu.cn

SPECIALTY SECTION
This article was submitted to Cardiovascular Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 19 June 2022
ACCEPTED 16 November 2022
PUBLISHED 30 November 2022

CITATION
Li J, Zhao D, Deng Q, Hao Y, Wang M, Sun J, Liu J, Ren G, Li H, Qi Y and Liu J (2022) Reduced serum calcium is associated with a higher risk of retinopathy in non-diabetic individuals: The Chinese Multi-provincial Cohort Study.
Front. Endocrinol. 13:973078.
doi: 10.3389/fendo.2022.973078

Reduced serum calcium is associated with a higher risk of retinopathy in non-diabetic individuals: The Chinese Multi-provincial Cohort Study

Jiangtao Li[1,2,3], Dong Zhao[1,2,3], Qiuju Deng[1,2,3], Yongchen Hao[1,2,3], Miao Wang[1,2,3], Jiayi Sun[1,2,3], Jun Liu[1,2,3], Guandi Ren[4], Huiqi Li[4], Yue Qi[1,2,3]* and Jing Liu[1,2,3]*

[1]Center for Clinical and Epidemiologic Research, Beijing Anzhen Hospital, Capital Medical University, Beijing Institute of Heart, Lung and Blood Vessel Diseases, Beijing, China, [2]The Key Laboratory of Remodeling-Related Cardiovascular Diseases, Ministry of Education, Beijing, China, [3]Beijing Municipal Key laboratory of Clinical Epidemiology, Beijing, China, [4]School of Information and Electronics, Beijing Institute of Technology, Beijing, China

Aims: As a common micro-vascular disease, retinopathy can also present in non-diabetic individuals and increase the risk of clinical cardiovascular disease. Understanding the relationship between serum calcium and retinopathy would contribute to etiological study and disease prevention.

Methods: A total of 1836 participants (aged 55–84 years and diabetes-free) from the Chinese Multi-Provincial Cohort Study-Beijing Project in 2012 were included for analyzing the relation between serum calcium level and retinopathy prevalence. Of these, 1407 non-diabetic participants with data on serum calcium in both the 2007 and 2012 surveys were included for analyzing the association of five-year changes in serum calcium with retinopathy risk. The retinopathy was determined from retinal images by ophthalmologists and a computer-aided system using convolutional neural network (CNN). The association between serum calcium and retinopathy risk was assessed by multivariate logistic regression.

Results: Among the 1836 participants (male, 42.5%), 330 (18.0%) had retinopathy determined by CNN. After multivariate adjustment, the odds ratio (OR) comparing the lowest quartiles (serum calcium < 2.38 mmol/L) to the highest quartiles (serum calcium ≥ 2.50 mmol/L) for the prevalence of retinopathy determined by CNN was 1.58 (95% confidence interval [CI]: 1.10 – 2.27). The findings were consistent with the result discerned by ophthalmologists, and either by CNN or ophthalmologists. These relationships are preserved even in those without metabolic risk factors, including hypertension, high hemoglobin A1c, high fasting blood glucose, or high low-density lipoprotein cholesterol. Over 5 years, participants with the sustainably low levels of serum calcium (OR: 1.58; 95%CI: 1.05 – 2.39) and those who experienced a decrease in serum calcium (OR: 1.56; 95%CI: 1.04 –

2.35) had an increased risk of retinopathy than those with the sustainably high level of serum calcium.

Conclusions: Reduced serum calcium was independently associated with an increased risk of retinopathy in non-diabetic individuals. Moreover, reduction of serum calcium could further increase the risk of retinopathy even in the absence of hypertension, high glucose, or high cholesterol. This study suggested that maintaining a high level of serum calcium may be recommended for reducing the growing burden of retinopathy. Further large prospective studies will allow more detailed information.

KEYWORDS

serum calcium, retinopathy, microvascular disease, non-diabetic, convolutional neural network

Introduction

Retinopathy is generally considered to be a clinical presentation of diabetes mellitus, commonly termed diabetic retinopathy, which is the leading cause of vision impairment and blindness in working-aged people globally (1). However, typical retinopathy lesions, such as microaneurysms and retinal hemorrhages, can also be seen in middle-aged and elderly adults without diabetes, with a prevalence rate of up to 10% (2). As a common micro-vascular disease, recent studies have suggested that retinopathy could significantly increase the risk of clinical cardiovascular disease in non-diabetic individuals (2). Although hyperglycemia had been a target in patients with diabetes, there were no effective prevention and treatment approach to reduce the growing burden of retinopathy in non-diabetic individuals. Thus, identifying novel risk factors related to retinopathy is of utmost importance to understand the etiology and prevent the disease burden.

Calcium, the most abundant metallic element in the human body, is an important component of bone and teeth that participates in many important biological processes, including cell metabolism and neurotransmission (3). A recent single-cell RNA sequencing study reported that abnormal calcium and other metal ion response pathways were involved in mouse diabetic retinopathy (4). A recent study in patients with type 2 diabetes mellitus suggested the relation between circulating calcium levels and vision-threatening diabetic retinopathy (5). Yet, it remains unclear whether serum calcium and its changes are associated with the risk of retinopathy in non-diabetic individuals, due to the different local microenvironments.

Automatic retinal image analysis is an important screening tool for the detection of retinopathy, which can reduce the workload of manual grading and save diagnosis cost and time (6). Convolutional neural networks (CNN) have been proved to have high sensitivity and specificity for detecting diabetic retinopathy (7), and thus we have developed a computer-aided system based on CNN with similar high accuracy (8) for automated detection of retinopathy in retinal fundus photographs from a community-based cohort study. Thereafter, this study aimed to explore the association of serum calcium level and five-year changes in calcium with the risk of retinopathy prevalence, and further investigate the joint impact of serum calcium and metabolic risk factors on the retinopathy in non-diabetic individuals.

Abbreviations: AMD, age-related macular degeneration; BMI, body mass index; CI, confidence interval; Conv, convolutional; CNN, convolutional neural network; DR, diabetic retinopathy; DBP, diastolic blood pressure; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C reactive protein; IPTW, the inverse probability of treatment weighting; LDL-C, low-density lipoprotein cholesterol; NDR, nonproliferative diabetic retinopathy; OR, odds ratio; PDR, proliferative diabetic retinopathy; ps, propensity score; SD, standard deviation; SMD, standardized mean differences; SBP, systolic blood pressure; eGFR, the estimated glomerular filtration rate; TC, total cholesterol; TG, triglyceride.

Materials and methods

Study design and study population

The study population was recruited from the Chinese Multi-Provincial Cohort Study-Beijing Project. The design and selection criteria have been previously described (9, 10). A total of 1941 participants without diabetes who participated in the examinations on demographic characteristics and traditional risk factors in 2012 were included in this study. Retinal images

were collected from 1901 participants (97.9%). After excluding those with incomplete data (n=65), 1836 participants were included for analyzing the relation between serum calcium level and retinopathy prevalence. Of these, 1407 non-diabetic participants with data on serum calcium in both the 2007 survey and the 2012 survey were included for analyzing the association of five-year changes in serum calcium with retinopathy (Figure S1).

The study was approved by the Ethics Committee of Beijing An Zhen Hospital, Capital Medical University, and written informed consent was obtained from all participants. All research adhered to the tenets of the Declaration of Helsinki.

Measurement of serum calcium

Serum levels of total calcium were determined by Arsenazo III colorimetry (Beckman Coulter, Brea, America) using fresh samples on the day of collection. This assay has a functional sensitivity of 0.01 mmol/L. The mean coefficient of variation was 0.57%. Albumin levels were tested by the bromocresol green colorimetric method (Beckman Coulter, Brea, America), and the mean coefficient of variation was 1.30%. Serum levels of total calcium and albumin were measured using the same method in both the 2007 and 2012 surveys. The levels of albumin-corrected calcium were calculated according to the following formula (11): *albumin-corrected calcium (mmol/L) = measured total calcium (mmol/L) + 0.02 × [40 – albumin (g/L)]*.

Retinal photography and definition of retinopathy

A standardized ophthalmologic examination was performed by a trained ophthalmologist. After 5 minutes of dark adaptation, macular-centered 45° digital retinal photographs of each eye were obtained using a color fundus camera (Canon EOS) without pharmacological mydriasis. Images were saved in JPEG format with a resolution of 3888×2592 pixels. Retinopathy was determined by a trained ophthalmologist in the ocular examination and defined as the presence of any lesions of micro hemangioma, hemorrhage, exudation, cotton spots, and neovascularization.

Furthermore, a computer-aided system based on CNN was developed to detect retinopathy and lesion area marking. The design and methods have been previously described (8). Briefly, 35 092 retinal images were obtained from the Kaggle database. The images were already classified into five grades (grades 0: no apparent retinopathy; grade 1: mild nonproliferative diabetic retinopathy; grade 2: moderate nonproliferative diabetic retinopathy; grade 3: severe nonproliferative diabetic retinopathy; grade 4: proliferative diabetic retinopathy) using international clinical diabetic retinopathy severity scales (12) as the reference standard. At first, two CNNs were constructed and trained as a weak learner. The main difference between the two network models was that the convolution kernels had different sizes. Next, the features of the last pooling layers were extracted repeatedly using different data augmentation. The mean and standard deviation of the features were stored as the input of a strong learner; the output of the strong learner was the final classification results (Figure 1). In the test set, the classification accuracy was 80%, and the kappa value was 84%. In the present study, retinopathy grade was obtained from all the retinal images using this computer-aid system, and retinopathy was defined as grades 1–4 in any retinal images of the participant. Retinopathy severity was defined as the maximum grade in all images of one participant. In the sensitivity analyses, retinopathy was also defined as meeting any diagnostic criteria of retinopathy of CNN or ophthalmologist.

Assessment of relevant covariates

Information on demographic characteristics, lifestyle factors, and personal medical history were collected using a standardized questionnaire. Anthropometric measurements, including height, weight, and blood pressure, were obtained by trained physicians during physical examination. Body mass index (BMI) was calculated according to the following formula: weight in kilograms divided by height in meters squared. Blood pressure was calculated by averaging three consecutive recordings, measured at the right-side brachial artery with the participants in a sitting position using a mercury sphygmomanometer after resting for at least 5 minutes. Hypertension was defined as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, and/or antihypertensive treatment in the last two weeks. Smoking was defined as smoking one or more cigarettes per day for more than 3 months. Diabetes was determined as any of the following criteria (1): fasting blood glucose (FBG) ≥ 7.1mmol/L (2), hemoglobin A1c (HbA1c) ≥ 6.5% (only in the 2012 survey) (3), the previous diagnosis by a physician, or (4) use of insulin or glucose-lowering medications during the past month. History of cardiovascular disease were defined as presence of coronary heart disease or stroke events.

Laboratory measurements were performed by using fresh samples on the day of collection. FBG levels were measured using enzymatic methods (Beckman Coulter, Brea, America). HbA1c levels were determined using ion-exchange high-performance liquid chromatography (BIO-RAD Turbo, Hercules, America). Triglyceride and total cholesterol levels were tested by enzymatic methods (Sekisui Medical Co., Ltd, Tokyo, Japan). High-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels were measured using a homogeneous method (Sekisui Medical Co., Ltd, Tokyo, Japan). High-sensitivity C reactive protein (hs-CRP) levels were analyzed using immunoturbidimetric methods

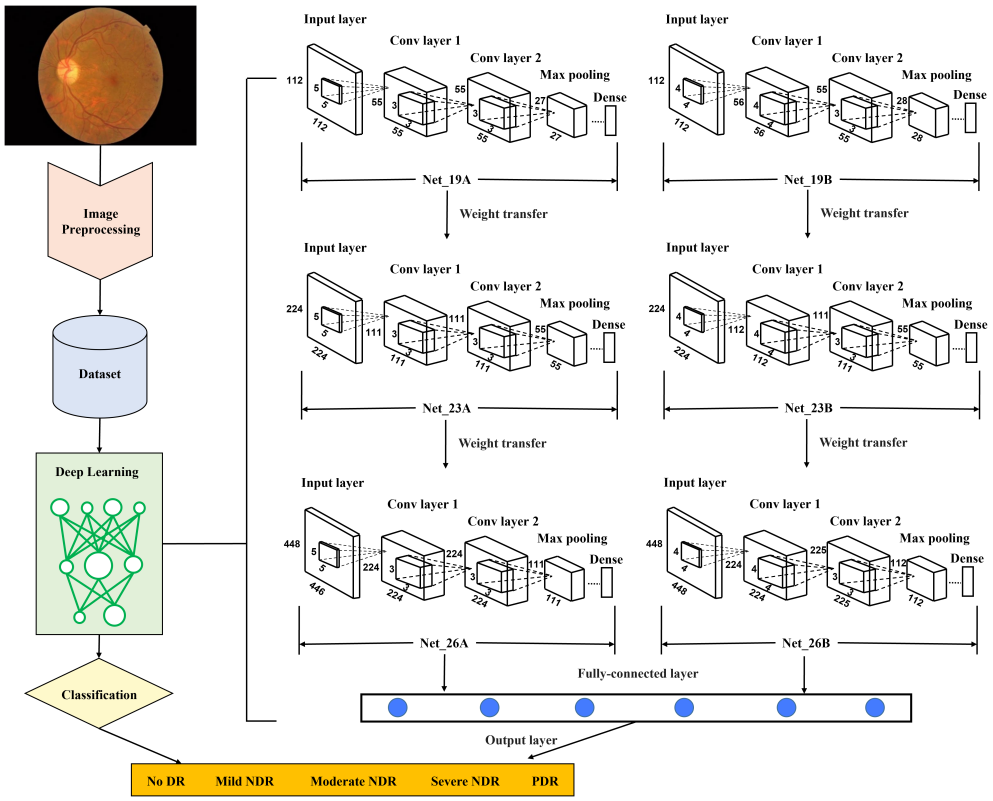


FIGURE 1
Flow diagram of the proposed retinopathy grading system. Conv, convolutional; CNN, convolutional neural network; DR, diabetic retinopathy; NDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy. First, in the preprocessing stage, the retinal images as the training set were re-cut, and the training set was expanded to balance the proportion of five grades of images. In addition, the resolution of images was adjusted, and the preprocessed images will be unified into three different resolutions for training CNN with different layers. Second, images with a resolution of 112×112 were used to train two CNNs with 19 layers, Net_19A and Net_19B. Using the transfer learning method, assign the weights of the first eleven layers obtained by its training to the corresponding first eleven layers in the two CNNs with 23 layers, Net_23A and Net_23B. Images with a resolution of 224×224 were used to train Net_23A and Net_23B. The weights of the first fifteen layers obtained after training are assigned to the first fifteen layers of the two CNNs with 26 layers, Net_26A and Net_26B. Then images with a resolution of 448×448 were used to train Net_26A and Net_26B. Third, after all the training of the CNN was completed, the output features of the last pooling layer in the two network models of Net_26A and Net_26B were extracted and saved. Finally, the extracted features were used as input data to train the final fully connected network model.

(Diasys Diagnostic Systems (Shanghai) Co., Ltd, Shanghai, China). Creatinine levels were measured using the enzymic method (Sekisui Medical Co., Ltd, Tokyo, Japan). Magnesium levels were tested by the dimethyl aniline blue colorimetric method (Beckman Coulter, Brea, America). The estimated glomerular filtration rate (eGFR) was calculated by the following formula (13): $eGFR\ (mL/min/1.73m^2) = 186 \times creatinine\ (mg/dL)^{-1.154} \times age^{-0.203} \times 1.233\ (\times 0.742\ in\ females)$.

Sample power estimation

Until now, no studies have investigated the association between serum calcium and retinopathy prevalence in non-diabetic adults. In the present study, the prevalence of retinopathy was 18.0%, and the R-squared value for serum calcium with other covariates was 0.201. The risk estimate of serum calcium for retinopathy was 1.58. Assuming an alpha (probability of type I error) of 0.05, the actual sample size of 1836 enabled sufficient statistical power (power = 0.87).

Statistical analysis

Participants' characteristics were described using percentages for categorical variables and mean ± standard deviation (SD) or medians (interquartile range) for continuous variables across quartiles of serum calcium (cutoff points: 2.38, 2.44, and 2.50 mmol/L). Categorical variables were compared using the chi-square test and continuous variables using the student *t*-test or Wilcoxon rank-sum test. Normality was

analyzed by Kolmogorov- Smirnov test (Table S1). Correlations between the levels of serum calcium and other covariate were estimated using partial correlations controlling for age and sex. *P* for trend was test by binary logistic regression for categorical variables and linear regression for continuous variables, and quartiles of serum calcium levels were included in the models as the independent variable.

Odds ratio (OR) for the risk of retinopathy prevalence associated with serum calcium levels were calculated using the binary logistic regression model controlling for traditional risk factors of retinopathy and variables associated with calcium levels, including: age (per 1 years), sex, BMI (per 1 kg/m^2), smoking, systolic blood pressure (< 140 mmHg, 140–159 and ≥ 160 mmHg), antihypertensive treatment, HDL-C (≥ 1.04 [male]/ 1.30 [female] mmol/L and < 1.04 [male]/1.30 [female] mmol/L), LDL-C (< 3.4 mmol/L and ≥ 3.4 mmol/L), natural log-transformed triglyceride, lipid-lowering treatment, natural log-transformed hs-CRP, albumin (per 1 g/L), eGFR (per 1 mL/min/ 1.73m^2), serum magnesium (per 0.1 mmol/L decrease), and FBG (< 5.19 [median] mmol/L and ≥ 5.19 mmol/L) or HbA1c (< 5.6% [median] and ≥ 5.6%). Serum calcium levels as a continuous variable (per SD decrease) and a categorical variable (the highest quartile of calcium as the reference) were included in the analyses, respectively.

To investigate the dose-response relationship between retinopathy prevalence risk and serum calcium as a continuous variable, the restricted cubic splines in logistic regression were performed, with three knots (10th, 50th, and 90th percentiles of calcium) recommended according to Loic Desquilbet and FrançoisMariotti (14, 15) and the first quartile of calcium (2.38 mmol/L) as the reference.

Subgroup analyses were performed using traditional risk factors, including age (< 65 years, ≥ 65 years), sex, BMI (< 24 kg/ m^2, ≥ 24 kg/m^2), smoking, hypertension, hs-CRP (< 1mg/L, ≥ 1 mg/L), HDL-C (< 1.04 [male]/1.30 [female] mmol/L, ≥ 1.04 [male]/1.30 [female] mmol/L), LDL-C (< 3.4 mmol/L, ≥ 3.4 mmol/L), triglyceride (< 1.7 mmol/L, ≥ 1.7 mmol/L), lipid-lowering treatment, albumin (< 45g/L [median], ≥ 45g/L), HbA1c (< 5.6% [median], ≥ 5.6%), FBG (< 5.19 mmol/L [median], ≥ 5.19 mmol/L), and serum magnesium (< 0.92 mmol/L [median], ≥ 0.92 mmol/L). Serum calcium levels as a categorical variable (< 2.38 mmol/L *vs.* ≥ 2.38 mmol/L) were included in the analyses separately. Beside the grouping factors, other factors were used as covariates. *P* for interaction was assessed by including a multiplicative interaction term in the logistic regression models to estimate the interaction on retinopathy between calcium and subgroup variables.

Several sensitivity analyses were performed. First, the association between serum calcium levels and retinopathy defined by the ophthalmologist, and by CNN or ophthalmologist was investigated. Second, participants with pre-diabetes (FBG ≥ 6.1 mmol/L) or those with cardiovascular disease and whose calcium levels were not within the normal reference range (2.25–2.75 mmol/L) were excluded. Third, total serum calcium was substituted with albumin-corrected calcium for analyzing the association with retinopathy, as it may better reflect the physiological state of calcium in the body (16). Fourth, the association between serum calcium and retinopathy risk was also assessed by a propensity score-based inverse probability of treatment weighting (IPTW) method. The propensity score (ps) was calculated using a logistic regression model in which the level of serum calcium (< 2.38 mmol/L vs. ≥ 2.38 mmol/L) was the dependent variable and other covariates included age, sex, BMI, smoking, systolic blood pressure, antihypertensive treatment, HDL-C, LDL-C, natural log-transformed triglyceride, lipid-lowering treatment, natural log-transformed hs-CRP, albumin, eGFR, magnesium, and FBG. The IPTWs were 1/ps and 1/(1-ps) for calcium < 2.38 mmol/L and ≥ 2.38 mmol/L respectively. Group differences were evaluated by standardized mean differences (SMD), with SMDs ≤0.10 indicating balance characteristics. The SMD was calculated with IPTW unweighted and weighted (Figure S2). In the IPTW model, only calcium level was included, and the model was weighted by IPTW.

Joint analysis for the association of serum calcium and metabolic risk factors with retinopathy was performed by dividing participants into four groups according to calcium levels and metabolic risk factors. Participants with calcium ≥ 2.38 mmol/L and without metabolic risk factors as the reference group. Metabolic risk factors in the present study included high levels of HbA1c (≥ 5.6%), FBG (≥ 5.19 mmol/L), LDL-C (≥ 3.4 mmol/L), and hypertension.

To explore the association of five-year changes in serum calcium with retinopathy, participants were divided into the following four groups: maintaining a high level of serum calcium during five years (serum calcium ≥ 2.34 mmol/L [first quartile] in the 2007 survey and ≥ 2.38 mmol/L [first quartile] in 2012 survey), serum calcium decreased from high level to low level during five years (serum calcium ≥2.34 mmol/ L in 2007 survey and <2.38 mmol/L in 2012 survey), serum calcium increased from low level to high level during five years (serum calcium <2.34 mmol/L in 2007 survey and ≥2.38 mmol/ L in 2012 survey), and maintaining a low level of serum calcium during five years (serum calcium <2.34 mmol/L in 2007 survey and <2.38 mmol/L in 2012 survey). The first group was defined as the reference group, and binary logistic regression models were performed to calculate the ORs of the other three groups for the retinopathy risk by controlling for age, sex, BMI, smoking, systolic blood pressure, antihypertensive treatment, HDL-C, LDL-C, natural log-transformed triglyceride, lipid-lowering treatment, natural log-transformed hs-CRP, albumin, eGFR, serum magnesium, and FBG.

To investigate whether missing data would lead to potential bias, comparisons were performed between participants included in the study and those excluded due to incomplete

data (Tables S2, S3). No significant differences in general characteristics were observed.

All statistical analyses were performed using the R software (version 3.6.2, R Foundation for Statistical Computing). A *P* value of < 0.05 on the two-sided test was considered statistically significant. Sample size estimation was calculated using the PASS software (version 11.0, NCSS, Kaysville, UT).

Results

General characteristics of the participants

Among 1836 non-diabetic participants, the mean age was 66.4 (± 7.8) years, and 42.5% were males. The mean (range) level of serum calcium was 2.44 (2.06, 2.85) mmol/L, with an SD of 0.11. The mean (range) level of albumin-corrected calcium was 2.34 (1.93, 2.71) mmol/L. The participants' characteristics, when stratified into serum calcium quartile, are shown in Table 1. Statistically significant trends were found for all known cardiovascular risk factors (all $P < 0.05$), except for eGFR and age. Participants with a lower level of serum calcium were more likely to be smokers. In addition, individuals with the lowest quartiles of serum calcium had higher BMI and hs-CRP, but lower blood pressure, lipids, glucose, and rates of related treatments. Weak correlations were found between serum calcium and cardiovascular risk factors after controlling for age and sex (Figure S3).

The association of serum calcium with retinopathy

The prevalence of retinopathy determined by CNN and ophthalmologists was 18.0% and 5.9% in non-diabetic participants, respectively (Figure 2). Serum calcium was negatively associated with retinopathy prevalence (Figure 2 and Figure S4). After multivariate adjustment, participants with the lowest quartiles (<2.38mmol/L) of serum calcium had a significantly higher risk for the prevalence of retinopathy discerned by CNN (OR: 1.58; 95% confidence interval [CI]:

TABLE 1 The characteristics of participants across the quartiles of serum calcium.

Characteristic [a]	Quartiles of serum calcium (mmol/L)				*P* for trend
	<2.38 (n = 435)	2.38~2.44 (n = 457)	2.44~2.50 (n = 434)	≥2.50 (n = 510)	
Calcium, mmol/L	2.32 ± 0.04	2.41 ± 0.02	2.46 ± 0.02	2.56 ± 0.06	< 0.001
Albumin-corrected calcium, mmol/L	2.24 ± 0.05	2.30 ± 0.05	2.36 ± 0.05	2.43 ± 0.06	< 0.001
Age, years	64 (59, 74)	64 (59, 72)	67 (59, 74)	66 (59, 73)	0.104
Male, n (%)	227 (52.2)	187 (40.9)	183 (42.2)	184 (36.1)	< 0.001
Body mass index, kg/m^2	24.7 (22.6, 27.1)	24.3 (22.4, 26.7)	23.8 (22.0, 26.1)	23.9 (21.9, 26.1)	< 0.001
Smoking, n (%)	64 (14.7)	59 (12.9)	49 (11.3)	41 (8.0)	0.001
Systolic blood pressure, mmHg	135.3 (125.3, 147.7)	135.0 (124.5, 145.0)	135.3 (125.3, 145.3)	138.0 (128.0, 148.3)	0.005
Diastolic blood pressure, mmHg	77.3 (72.3, 84.7)	79.0 (72.7, 84.7)	78.2 (72.3, 84.7)	80.3 (73.3, 85.8)	0.017
Hypertension, n (%)	245 (56.3)	248 (54.3)	249 (57.4)	328 (64.3)	0.006
Antihypertensive treatment, n (%)	163 (37.5)	161 (35.2)	164 (37.8)	240 (47.1)	< 0.001
Fasting blood glucose, mmol/L	5.08 (4.81, 5.42)	5.16 (4.87, 5.50)	5.22 (4.90, 5.54)	5.30 (4.97, 5.63)	< 0.001
Hemoglobin A1c, %	5.6 (5.4, 5.8)	5.7 (5.4, 5.9)	5.7 (5.4, 5.9)	5.7 (5.4, 5.9)	0.010
Pre-diabetes, n (%)	27 (6.2)	29 (6.3)	22 (5.1)	52 (10.2)	0.031
Total cholesterol, mmol/L	5.09 ± 0.95	5.31 ± 1.01	5.27 ± 1.01	5.44 ± 1.09	< 0.001
LDL-C, mmol/L	2.98 ± 0.81	3.11 ± 0.88	3.07 ± 0.81	3.18 ± 0.92	0.001
HDL-C, mmol/L	1.27 (1.08, 1.47)	1.28 (1.11, 1.49)	1.33 (1.13, 1.56)	1.37 (1.16, 1.59)	< 0.001
Triglyceride, mmol/L	1.25 (0.87, 1.75)	1.37 (0.97, 1.98)	1.37 (0.98, 1.85)	1.40 (1.01, 2.02)	< 0.001
Lipid-lowering treatment, n (%)	62 (14.3)	83 (18.2)	113 (26.0)	142 (27.8)	< 0.001
Hs-CRP, mg/L	1.16 (0.59, 2.52)	0.99 (0.52, 1.94)	0.85 (0.43, 1.76)	0.78 (0.39, 1.59)	< 0.001
Albumin, g/L	44.31 ± 2.24	45.2 ± 2.29	45.31 ± 2.34	46.25 ± 2.26	< 0.001
eGFR, mL/min/1.73m^2	109.40 (96.14, 121.99)	108.75 (95.86, 119.79)	105.22 (95.74, 119.59)	105.42 (92.80, 117.82)	0.131
Magnesium, mmol/L	1.27 (1.08, 1.47)	1.28 (1.11, 1.49)	1.33 (1.13, 1.56)	1.37 (1.16, 1.59)	0.026
History of cardiovascular disease, n (%)	32 (7.4)	33 (7.2)	31 (7.1)	35 (6.9)	<0.001

eGFR, the estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C reactive protein; LDL-C, low-density lipoprotein cholesterol.
[a]Data are expressed as mean (standard deviation) for continuous variables in the case of normal distributions and median (interquartile range); otherwise, as number (percent) for categorical variables.

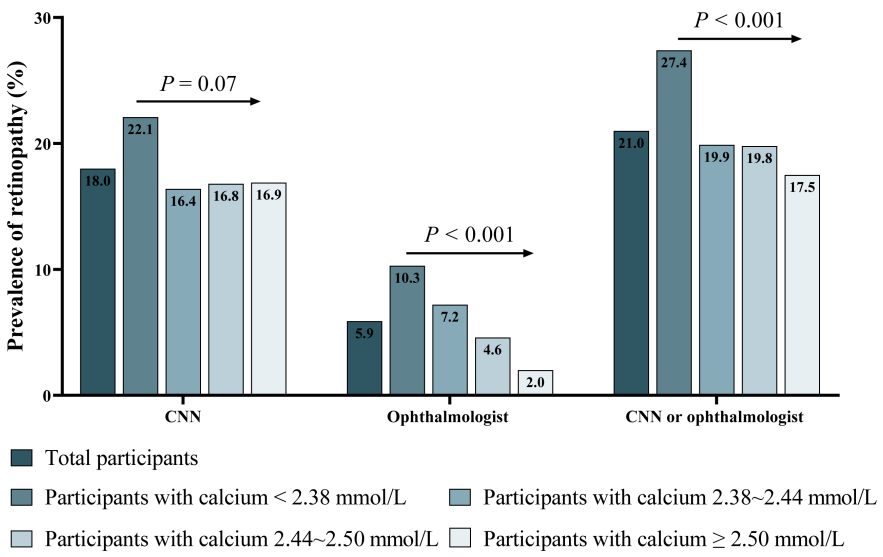


FIGURE 2
Prevalence of retinopathy discerned by CNN, ophthalmologist, and by either CNN or ophthalmologist. CNN, convolutional neural network. *P*: *P* for trend tested by chi-square test for trend.

1.10-2.27; P =0.015) than those with the highest quartiles (Table 2 and Table S4). The ORs remained unchanged after the adjustment of HbA1c substituting for FBG. These findings were consistent with data provided by the ophthalmologist, and by either CNN or the ophthalmologist (Table 2). The relationship between serum calcium and retinopathy prevalence remained significant after excluding participants with pre-diabetes, history of cardiovascular disease, or those without a normal reference range of serum calcium (Table S5), as well as substituting total calcium for albumin-corrected calcium (Table S6). In a IPTW sample, participants with calcium < 2.38 mmol/L had a 56% increased risk of retinopathy compared to those with calcium ≥ 2.38 mmol/L (OR: 1.56; 95% CI: 1.32-1.84; P <0.001).

TABLE 2 Logistic regression analyses of serum calcium quartiles and per SD decrease with retinopathy.

Diagnostic methods	Calcium (mmol/L)	Unadjusted	Model 1	Model 2
CNN	≥2.50	Reference	Reference	Reference
	2.44-2.50	1.00 (0.71, 1.40)	1.05 (0.74, 1.50)	1.05 (0.74, 1.49)
	2.38-2.44	0.97 (0.69, 1.36)	1.09 (0.76, 1.56)	1.08 (0.75, 1.54)
	<2.38	1.40 (1.01, 1.93)	1.58 (1.10, 2.27)	1.55 (1.08, 2.23)
	Per SD	1.14 (1.00, 1.29)	1.20 (1.04, 1.38)	1.19 (1.03, 1.37)
Ophthalmologist	≥2.50	Reference	Reference	Reference
	2.44-2.50	2.42 (1.12, 5.22)	2.85 (1.30, 6.26)	2.80 (1.28, 6.14)
	2.38-2.44	3.89 (1.90, 7.99)	5.05 (2.39, 10.66)	4.89 (2.32, 10.31)
	<2.38	5.77 (2.87, 11.59)	8.48 (4.00, 18.02)	8.14 (3.84, 17.25)
	Per SD	1.89 (1.52, 2.35)	2.30 (1.77, 2.97)	2.26 (1.74, 2.92)
CNN or ophthalmologist [a]	≥2.50	Reference	Reference	Reference
	2.44-2.50	1.17 (0.84, 1.62)	1.28 (0.91, 1.79)	1.27 (0.90, 1.78)
	2.38-2.44	1.18 (0.85, 1.63)	1.39 (0.99, 1.96)	1.37 (0.97, 1.93)
	<2.38	1.78 (1.31, 2.43)	2.22 (1.56, 3.14)	2.17 (1.53, 3.07)
	Per SD	1.26 (1.11, 1.42)	1.39 (1.21, 1.60)	1.38 (1.20, 1.58)

CNN, convolutional neural network; SD, standard deviation; OR, odds ratio.
[a]Retinopathy is defined as meeting any diagnostic criteria of retinopathy of CNN or ophthalmologist. Model 1: adjusted for age, sex, body mass index, smoking, systolic blood pressure, antihypertensive treatment, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, natural log-transformed triglyceride, lipid-lowering treatment, fasting blood glucose, natural log-transformed high-sensitivity C-reactive protein, albumin, the estimated glomerular filtration rate, and magnesium. Model 2: model 1 + hemoglobin A1c substituting for fasting blood glucose.

No differences were found between serum calcium and retinopathy prevalence among various subgroups (Figure S5). Further joint analysis showed that a low level of serum calcium exaggerated the impact of high glucose or high cholesterol on retinopathy risk. Compared with participants with HbA1c <5.6% and calcium ≥2.38 mmol/L, participants with HbA1c level ≥5.6% but serum calcium <2.38 mmol/L had 62% higher risk for retinopathy (OR: 1.62; 95%CI: 1.09-2.40; P =0.023). However, participants with HbA1c ≥5.6% and serum calcium ≥2.38 mmol/L had a comparable risk as those with a low level of HbA1c (<5.6%) but the same level of serum calcium (Figure S6). Similar results were found when analyzing FBG and LDL-C.

The association of five-year changes in serum calcium with retinopathy

Changes in serum calcium over 5 years and their association with the risk of retinopathy prevalence were further investigated among participants without diabetes over 5 years. There are 11.8% of participants maintained a low level of serum calcium over 5 years, and they had a 58% increased risk for retinopathy discerned by CNN (OR: 1.58; 95%CI: 1.05-2.39, P =0.030), compared with those who maintained their serum calcium at a high level. Another 12.4% of participants experienced a decrease in serum calcium from high level to low level over the period, and they had a 56% increased risk (OR: 1.56; 95%CI: 1.04-2.35, P =0.033). However, participants whose serum calcium increased from low level to high level over 5 years did not have a heightened risk for retinopathy (OR: 1.27; 95%CI: 0.82-1.97, P =0.278) (Table 3). Similar results were found in the association of changes in serum calcium with retinopathy discerned by the ophthalmologist, and by either CNN or the ophthalmologist.

Discussion

In this large community-based study, we examined the association between serum calcium levels and changes with retinopathy, based on reliable and repeated measurements of serum calcium, as well as a clear diagnosis of retinopathy by the ophthalmologist and deep learning. After controlling for covariates, reduced levels of serum calcium were significantly associated with an increased risk of retinopathy prevalence among non-diabetic individuals. Moreover, these relationships are preserved even among those without hypertension, high glucose, or high cholesterol levels. These findings suggested important implications of calcium metabolism for current retinopathy management strategies. However, as the potential for confounding may exist, further studies are warranted.

Limited evidence reported the association between serum calcium and the risk of retinopathy in non-diabetic individuals, a common micro-vascular disease that may precede macrovascular disease. The present study explored the contribution of serum calcium concentrations to the risk of retinopathy and suggested that reduced serum calcium is

TABLE 3 The association between five-year changes in serum calcium and retinopathy.

The five-year changes in serum calcium levels	n/N (%)	OR (95%CI)[a]	P
Retinopathy discerned by CNN			
Sustainably high-level[b]	155/912 (17.0)	Reference	
Decreased from high level to low level[c]	41/174 (23.6)	1.56 (1.04, 2.35)	0.033
Increased from low level to high level[d]	31/154 (20.1)	1.27 (0.82, 1.97)	0.278
Sustainably low level[e]	41/167 (24.0)	1.58 (1.05, 2.39)	0.030
Retinopathy discerned by the ophthalmologist			
Sustainably high-level[b]	41/912 (4.5)	Reference	
Decreased from high level to low level[c]	17/174 (9.8)	2.62 (1.41, 4.88)	0.002
Increased from low level to high level[d]	10/154 (6.5)	1.52 (0.74, 3.13)	0.254
Sustainably low level[e]	24/167 (14.4)	4.14 (2.35, 7.32)	<0.001
Retinopathy discerned by either CNN or the ophthalmologist			
Sustainably high-level[b]	177/912 (19.4)	Reference	
Decreased from high level to low level[c]	48/174 (27.6)	1.71 (1.16, 2.53)	0.007
Increased from low level to high level[d]	35/154 (22.7)	1.27 (0.83, 1.92)	0.268
Sustainably low level[e]	54/167 (32.3)	2.14 (1.46, 3.14)	<0.001

CI, confidence interval; CNN, convolutional neural network; N, number; OR, odds ratio.
[a]ORs were calculated by logistic regressions after adjusting for age, sex, body mass index, smoking, systolic blood pressure, antihypertensive treatment, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, natural log-transformed triglyceride, lipid-lowering treatment, fasting blood glucose, natural log-transformed high-sensitivity C-reactive protein, albumin, the estimated glomerular filtration rate, and serum magnesium.
[b]Serum calcium ≥2.34 mmol/L (first quartile) in the 2007 survey and ≥2.38 mmol/L (first quartile) in the 2012 survey.
[c]Serum calcium ≥2.34 mmol/L in the 2007 survey and <2.38 mmol/L in the 2012 survey.
[d]Serum calcium <2.34 mmol/L in the 2007 survey and ≥2.38 mmol/L in the 2012 survey.
[e]Serum calcium <2.34 mmol/L in the 2007 survey and <2.38 mmol/L in the 2012 survey.

independently associated with a higher risk of retinopathy in non-diabetic individuals, and this impact remained significant in participants without other reported influential factors separately. With the extension, our study further investigated the association between five-year changes in serum calcium and retinopathy and found that the sustainable decrease of serum calcium levels was significantly associated with an increased risk of retinopathy.

Limited evidence was found on the impact of serum calcium on retinopathy among non-diabetic adults. Calcium balance in circulation changes during life stages and is mostly determined by body calcium requirement and dietary calcium intake. Previous studies have reported that decreased serum vitamin D, as the influential factor of body calcium requirement, was associated with an increased risk of retinopathy prevalence among 5120 non-diabetic participants in the Rotterdam Study (17). The reduction of vitamin D could lead to low serum calcium levels by reducing the reabsorption of calcium by renal tubules and inhibiting the absorption of calcium by intestinal mucosal epithelium (18), suggesting the potential role of serum calcium on the risk of retinopathy in non-diabetic individuals. Meanwhile, the Blue Mountains Eye Study (19) and Age-Related Eye Disease Study (20) all found that decreased dietary calcium intake was associated with a higher risk of late age-related macular degeneration (AMD) incidence, suggesting the possibility of targeting calcium to prevent retinopathy. Previous studies found that smoking was associated with a higher risk of retinopathy (21). In our study, participants with a lower levels of serum calcium were more likely to be smokers, suggesting that the potential impact of smoking on the association between the risk of retinopathy and serum calcium levels.

However, a recent study conducted in patients with type 2 diabetes has shown the opposite findings compared to the present study among non-diabetic individuals, with a high level of serum calcium independently associated with the increased risk of diabetic retinopathy (5). Several reasons may account for the difference. First, the study patients were all from hospitals and recruited over a 10-year period, which was different from the population without diabetes recruited from the community in the present study. Second, the underlying pathogenesis of retinopathy may be different between diabetic and non-diabetic individuals (22). Third, calcium metabolism may be different in individuals with and without diabetes. Compared with non-diabetic individuals, the calcium ion metabolism of diabetic patients is in a negative balance state (23), mainly manifested as increased calcium excretion in urine. Finally, the association of high glucose or high cholesterol with retinopathy was not found in non-diabetic individuals in the present study. Reduction of serum calcium could unexpectedly exaggerate the impact of high glucose or high cholesterol on the risk of retinopathy, even at a normal level. By these findings, elevated glucose may alter calcium homeostasis in the retina (24) and enhance constriction of retinal venules through activation of the reverse-mode sodium-calcium exchanger (25). On the other hand, abnormalities in circulating calcium levels could have a crucial role in insulin release and glucose homeostasis (26). The impact of decreased calcium on the relation between high glucose and retinopathy risk warrants prospective assessment in the future.

Several *in vivo* and *in vitro* studies support the association between lower serum calcium levels and a higher risk of retinopathy. When serum calcium levels decrease, the intracellular calcium increases, which is known as "the abnormal calcium influx" (27), thus causing the vascular smooth muscle to contract, and eventually increasing vascular resistance (28). Low serum calcium levels may also stimulate the release of parathyroid hormone and renin, thereby increasing calcium in smooth muscle cells and leading to vasoconstriction (28). The increasing vascular resistance and vasoconstriction further lead to the reduction of blood flow, which may be crucial for retinopathy where blood flow regulation is disrupted. In addition, previous animal studies demonstrated that the reduction of glial calcium signaling could cause capillary contraction, thereby reducing capillary blood flow in the mouse retina (29). However, the potential mechanism between calcium and retinopathy remains unknown and requires external validation to corroborate its utility.

This study has a few limitations. First, this cross-sectional analysis from a community-based cohort study suggested a possible association between low calcium and a higher risk of retinopathy; however, the causal-effect relationship cannot be inferred. Consequently, further prospective studies are needed. Second, some potential confounding factors, which may regulate or affect serum calcium levels and changes, were unavailable, including dietary calcium, serum parathyroid hormone, serum vitamin D, several drugs (calcium salts, calcium supplements, vitamin D supplements, glucocorticosteroids, antiresorptive drugs, recombinant parathyroid hormone) (30), and the circadian variation in serum calcium (31). Third, the ionized calcium level was not measured in the present study, which represents the physiologically active fraction of serum calcium. Although total calcium levels and albumin-adjusted calcium levels have been reported to be reasonably correlated to ionized calcium levels (32, 33), further studies are needed to explore the association between retinopathy and ionized calcium levels in non-diabetic individuals.

In conclusion, our study suggested that a low level of serum calcium and its sustainably decrease over five years were independently associated with an increased risk of retinopathy prevalence in non-diabetic individuals. Moreover, reduction of serum calcium can increase the risk of retinopathy even among individuals without high glucose, high LDL-C, or hypertension. This study provides an important clue on the risk of retinopathy associated with reduced calcium and foundational data for future intervention studies, which are essential if targeting calcium is to be considered to prevent disease burden. Yet,

more studies are needed to obtain detailed information and determine causal-effect relationship, and to investigate the role of altered calcium homeostasis in the pathogenesis of the microvascular disease.

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 studies involving human participants were reviewed and approved by Ethics Committee of Beijing An zhen Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DZ, QD, YH, MW, QY, and JiL designed the study. JTL, JS, JuL, GR, and HL cleaned the data. JTL analyzed the data. JTL, YQ, and JiL wrote the manuscript. All authors reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Beijing Natural Science Foundation (No. 7212006); the National Natural Science Foundation of China (grant numbers 82073635, 82103962,and 81570409); Beijing municipal medical research institutes pilot reform project (grant number 2021-07); the National Key Research and Development Program of China (grant number 2016YFC0900902); the National Science & Technology Pillar Program (grant numbers 2011BAI09B01, 2011BAI11B03, 2006BAI01A01, and 2006BAI01A02); and the National Key R&D Program of China (2019YFE0116000). The sponsors had no role in the design or conduct of the 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/fendo.2022.973078/full#supplementary-material

References

1. Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. *Lancet (London England)* (2010) 376(9735):124–36. doi: 10.1016/s0140-6736(09)62124-3

2. Gupta P, Lamoureux EL, Sabanayagam C, Tham YC, Tan G, Cheng CY, et al. Six-year incidence and systemic associations of retinopathy in a multi-ethnic Asian population without diabetes. *Br J Ophthalmol* (2022) 106(6):845–51. doi: 10.1136/bjophthalmol-2020-318126

3. da Silva Lopes K, Abe SK. Polymorphisms contributing to calcium status: A systematic review. *Nutrients* (2021) 13(8):2488. doi: 10.3390/nu13082488

4. Van Hove I, De Groef L, Boeckx B, Modave E, Hu TT, Beets K, et al. Single-cell transcriptome analysis of the akimba mouse retina reveals cell-Type-Specific insights into the pathobiology of diabetic retinopathy. *Diabetologia* (2020) 63 (10):2235–48. doi: 10.1007/s00125-020-05218-0

5. Hu Y, Zhou C, Shi Y, She X, Zhao S, Gu C, et al. A higher serum calcium level is an independent risk factor for vision-threatening diabetic retinopathy in patients with type 2 diabetes: Cross-sectional and longitudinal analyses. *Endocr Pract* (2021) 27(8):826–33. doi: 10.1016/j.eprac.2021.05.003

6. Valverde C, Garcia M, Hornero R, Lopez-Galvez MI. Automated detection of diabetic retinopathy in retinal images. *Indian J Ophthalmol* (2016) 64(1):26–32. doi: 10.4103/0301-4738.178140

7. Gulshan V, Peng L, Coram M, Stumpe MC, Wu D, Narayanaswamy A, et al. Development and validation of a deep learning algorithm for detection of diabetic retinopathy in retinal fundus photographs. *Jama* (2016) 316(22):2402–10. doi: 10.1001/jama.2016.17216

8. Zhao C, Li H. Diabetic retinopathy detection and lesion area marking in retinal images: Beijing institute of technology. (2018). Available at: https://kns.cnki.net/kcms/detail/detail.aspx?dbcode=CMFD&dbname=CMFD202102&filename=1021568876.nh&uniplatform=NZKPT&v=DQrWjKw28uAy0Z1LpeXbOpTvUMq0DoHfk-CRMm6ytNGYKjxs8y0IR0WQou5BgwzG

9. Qi Y, Han X, Zhao D, Wang W, Wang M, Sun J, et al. Long-term cardiovascular risk associated with stage 1 hypertension defined by the 2017 Acc/Aha hypertension guideline. *J Am Coll Cardiol* (2018) 72(11):1201–10. doi: 10.1016/j.jacc.2018.06.056

10. Zhou M, Liu J, Qi Y, Wang M, Wang Y, Zhao F, et al. The association between helicobacter pylori seropositivity and risk of new-onset diabetes: A prospective cohort study. *Diabetologia* (2018) 61(2):300–7. doi: 10.1007/s00125-017-4465-2

11. Bushinsky DA, Monk RD. Electrolyte Quintet: Calcium. *Lancet* (London, England) (1998) 352(9124):306–11. doi: 10.1016/s0140-6736(97)12331-5.

12. Wilkinson CP, Ferris FL3rd, Klein RE, Lee PP, Agardh CD, Davis M, et al. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. *Ophthalmology* (2003) 110(9):1677–82. doi: 10.1016/s0161-6420(03)00475-5

13. Kong X, Ma Y, Chen J, Luo Q, Yu X, Li Y, et al. Evaluation of the chronic kidney disease epidemiology collaboration equation for estimating glomerular filtration rate in the Chinese population. *Nephrol dialysis Transplant Off Publ Eur Dialysis Transplant Assoc - Eur Renal Assoc* (2013) 28(3):641–51. doi: 10.1093/ndt/gfs491

14. Zhang X, Li Y, Wang Y, Hu K, Tu R, Zhang H, et al. Contribution of serum lipids as effect modifiers to a relationship between mean arterial pressure and Coronary Heart Disease in Chinese rural population: The henan rural cohort study. *BMJ Open* (2019) 9(11):e029179. doi: 10.1136/bmjopen-2019-029179

15. Desquilbet L, Mariotti F. Dose-response analyses using restricted cubic spline functions in public health research. *Stat Med* (2010) 29(9):1037–57. doi: 10.1002/sim.3841

16. National Kidney Foundation. K/Doqi clinical practice guidelines for bone metabolism and disease in chronic kidney disease. *Am J Kidney Dis Off J Natl Kidney Foundation* (2003) 42(4 Suppl 3):S1–201. doi: 10.1016/S0272-6386(03)00905-3

17. Mutlu U, Ikram MA, Hofman A, de Jong P, Uitterlinden AG, Klaver CCW, et al. Vitamin d and retinal microvascular damage: The Rotterdam study. *Medicine* (2016) 95(49):e5477. doi: 10.1097/md.0000000000005477

18. Ariganjoye R. Pediatric hypovitaminosis d: Molecular perspectives and clinical implications. *Global Pediatr Health* (2017) 4:2333794x16685504. doi: 10.1177/2333794x16685504

19. Gopinath B, Flood VM, Louie JC, Wang JJ, Burlutsky G, Rochtchina E, et al. Consumption of dairy products and the 15-year incidence of age-related macular degeneration. *Br J Nutr* (2014) 111(9):1673–9. doi: 10.1017/s000711451300408x

20. Tisdale AK, Agrón E, Sunshine SB, Clemons TE, Ferris FL3rd, Chew EY. Association of dietary and supplementary calcium intake with age-related macular degeneration: Age-related eye disease study report 39. *JAMA Ophthalmol* (2019) 137(5):543–50. doi: 10.1001/jamaophthalmol.2019.0292

21. Hu R, Zhang XX, Wang WQ, Lau CP, Tse HF. Smoking, homocysteine and degree of arteriolar retinopathy. *Atherosclerosis* (2005) 183(1):95–100. doi: 10.1016/j.atherosclerosis.2005.01.053

22. Munch IC, Kessel L, Borch-Johnsen K, Glümer C, Lund-Andersen H, Larsen M. Microvascular retinopathy in subjects without diabetes: The Inter99 eye study. *Acta ophthalmologica* (2012) 90(7):613–9. doi: 10.1111/j.1755-3768.2011.2148.x

23. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes–a meta-analysis. *Osteoporosis Int J established as result cooperation between Eur Foundation Osteoporosis Natl Osteoporosis Foundation USA* (2007) 18(4):427–44. doi: 10.1007/s00198-006-0253-4

24. Santiago AR, Rosa SC, Santos PF, Cristóvão AJ, Barber AJ, Ambrósio AF. Elevated glucose changes the expression of ionotropic glutamate receptor subunits and impairs calcium homeostasis in retinal neural cells. *Invest Ophthalmol Visual Sci* (2006) 47(9):4130–7. doi: 10.1167/iovs.06-0085

25. Chen YL, Xu W, Rosa RHJr., Kuo L, Hein TW. Hyperglycemia enhances constriction of retinal venules *Via* activation of the reverse-mode sodium-calcium exchanger. *Diabetes* (2019) 68(8):1624–34. doi: 10.2337/db19-0069

26. Kotnik P, Knapič E, Kokošar J, Kovač J, Jerala R, Battelino T, et al. Identification of novel alleles associated with insulin resistance in childhood obesity using pooled-DNA genome-wide association study approach. *Int J Obes (2005)* (2018) 42(4):686–95. doi: 10.1038/ijo.2017.293

27. Campillo-Soto A, Lirón-Ruiz R, Gervasio Martín-Lorenzo J, Flores-Pastor B. [Deconstructing the myth of using serum parathormone as a predictor of hypocalcaemia after total thyroidectomy]. *Cirugia espanola* (2009) 86(6):391–2. doi: 10.1016/j.ciresp.2009.03.029

28. Ephraim RK, Osakunor DN, Denkyira SW, Eshun H, Amoah S, Anto EO. Serum calcium and magnesium levels in women presenting with pre-eclampsia and pregnancy-induced hypertension: A case-control study in the cape coast metropolis, Ghana. *BMC pregnancy childbirth* (2014) 14:390. doi: 10.1186/s12884-014-0390-2

29. Biesecker KR, Srienc AI, Shimoda AM, Agarwal A, Bergles DE, Kofuji P, et al. Glial cell calcium signaling mediates capillary regulation of blood flow in the retina. *J Neurosci Off J Soc Neurosci* (2016) 36(36):9435–45. doi: 10.1523/jneurosci.1782-16.2016

30. Poudel K, Shah AM, Michos ED, Folsom AR, Konety S, Lutsey PL. Association of serum calcium and phosphorus with measures of left ventricular structure and function: The aric study. *Nutrition metabolism Cardiovasc Dis NMCD* (2020) 30(5):758–67. doi: 10.1016/j.numecd.2020.01.003

31. Touitou Y, Touitou C, Bogdan A, Reinberg A, Motohashi Y, Auzéby A, et al. Circadian and seasonal variations of electrolytes in aging humans. *Clinica chimica acta; Int J Clin Chem* (1989) 180(3):245–54. doi: 10.1016/0009-8981(89)90006-5

32. Kim KN, Oh SY, Hong YC. Associations of serum calcium levels and dietary calcium intake with incident type 2 diabetes over 10 years: The Korean genome and epidemiology study (Koges). *Diabetol Metab syndrome* (2018) 10:50. doi: 10.1186/s13098-018-0349-y

33. Gebreyohannes RD, Abdella A, Ayele W, Eke AC. Association of dietary calcium intake, total and ionized serum calcium levels with preeclampsia in Ethiopia. *BMC pregnancy childbirth* (2021) 21(1):532. doi: 10.1186/s12884-021-04005-y

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 11 March 2024
DOI 10.3389/fendo.2023.1174299

OPEN ACCESS

EDITED BY
Yuanyuan Wang,
Monash University, Australia

REVIEWED BY
Emmanouella Magriplis,
Agricultural University of Athens, Greece
Ayukafangha Etando,
Eswatini Medical Christian University, Eswatini
Sergio Perez-Burillo,
Public University of Navarre, Spain
Rahnuma Ahmad,
Medical College for Women and Hospital, Bangladesh

*CORRESPONDENCE
Muhanad Alhareky
malhareky@iau.edu.sa
Mor-Li Hartman
Morlihartman2000@gmail.com

RECEIVED 10 March 2023
ACCEPTED 17 October 2023
PUBLISHED 11 March 2024

CITATION
Alhareky M, Goodson JM, Tavares M and Hartman M-L (2024) Beverage consumption and obesity in Kuwaiti school children.
Front. Endocrinol. 14:1174299.
doi: 10.3389/fendo.2023.1174299

Beverage consumption and obesity in Kuwaiti school children

Muhanad Alhareky[1]*, Jo Max Goodson[2], Mary Tavares[3] and Mor-Li Hartman[2]*

[1]Department of Preventive Dental Sciences, Imam Abdulrahman Bin Faisal, College of Dentistry, Dammam, Saudi Arabia, [2]Department of Applied Oral Sciences, the Forsyth Research Institute, Cambridge, MA, United States, [3]The Forsyth Institute, Cambridge, MA, United States

Sweetened beverage consumption is particularly important in countries such as Kuwait, where the prevalence of obesity is high, and most children drink sweetened beverages daily. To assess the relationship between three most commonly consumed beverages, (soda, milk, and juice) and the incidence of obesity among Kuwaiti children at the critical age of 10-12 year, Longitudinal cohort data of 6,305 children on initial presentation in 2012 (age, 10 years) and follow-up in 2014 (age, 12 years) were obtained from the Kuwait Healthy Life Study. The servings for the three beverages (soda, juice, and milk) were calculated as servings per day groups (0, 1-2, and 3 servings/day or more). Multivariate logistic regression was performed to assess the relationship between developing obesity during 2012-2014 and soda, juice, and milk consumption. Model selection was based on clinically relevant covariates and potential confounders using stepwise model selection. Six percent children become obese between baseline and follow-up visits. High soda drinking showed significant association with developing obesity. High milk consumption (more than 3 servings a day) was also significantly associated with developing obesity. Potential confounders included in the final model were age, sex, governorates, and fitness level, of which none were significant confounders or effect modifiers for the association. Children with high soda consumption had significantly higher prevalence of obesity. High obesity prevalence was observed with high milk consumption at a lower significance level but not with high juice consumption.

KEYWORDS

adolescent obesity, beverage, weight, BMI - body mass index, SSB products

1 Introduction

The effect of sugar-sweetened beverage (SSB) consumption on obesity development is an increasing healthcare concern (1). Although epidemiological data for adults reveal that sugar consumption is associated with type 2 diabetes (T2D) directly and indirectly (2), limited comparable data are available for children. Obesity and type 2 diabetes have a strong association. The risk and severity of type 2 diabetes are connected to body mass

index (BMI). Obese people are seven times more likely to get diabetes than normal-weight people, whereas overweight people are three times more likely (3).

Nutrition evolution, fast socioeconomic developments, and westernization have all had a substantial impact on Kuwaiti adolescents' lifestyle and dietary preferences during the last few decades, which has led in a significant shift in eating patterns, with fast food becoming an integral component of the Kuwaiti diet (4). Another study on Kuwaiti adolescents proposed the factors of weight gaining would be that Kuwaiti teenagers prefer high-energy snacks such as soft drinks and sugar-sweetened beverages, sweets, chocolate, potato chips, French fries, and fast food, which replace nutritious foods such as vegetables, whole grain products, and milk (5). Adolescents may gain weight if they consume unhealthy snacks instead of main meals on a regular basis.

Kuwait has one of the world's highest percentage of adults with obesity (6, 7) and diabetes (8, 9). In 2005, a survey showed that 75.5% of Kuwaiti adults were overweight and 42.1% were obese (10), although these percentages were high, the more alarming observation was the rapid increase to 80.4% of overweight individuals and 47% of obese individuals for both sexes after 2 years (11). In 2009 a study reported, 10-14-year-old Kuwaiti children had an overall prevalence of 30.7% and 14.6% with respect to being overweight and obese (12).

In 2015, the World Health Organization (WHO) made several recommendations to reduce sugar intake, mainly because of its role in obesity and dental caries (13). Several studies have examined the relationship between SSB and obesity (14, 15). Soft drinks, in particular, have been assessed because they represent the primary source of added sugar in the diet, accounting for approximately 36% of the total added sugar consumed (16).

The dietary habits of Kuwaitis has major changed because of their lifestyle transformation, particularly after the Gulf War in 1990 (17). Honkala et al. showed that 13-year-old Kuwaiti children consume a higher proportion of soft drink consumption than children from 34 countries participating in their study (18). Approximately 75% of Kuwaiti children in that study consumed soft drinks every day (18). To our knowledge, no longitudinal studies have assessed the effect of beverage consumption of children on obesity development in the Middle East and North Africa (MENA) region (19).

With such an alarming percentage of obesity and high soft drink consumption, this longitudinal cohort study was conducted to assess the relationship between three most commonly consumed beverages, (soda, milk, and juice) and the incidence of obesity among Kuwaiti children over 2 years.

2 Methods

2.1 Subjects and study design

We obtained data of Kuwaiti children enrolled in Kuwait public schools. All participants were Kuwaiti nationals, and only 4th- and 5th -grade Kuwaiti students were included in the study. Participants were selected to represent each of the six Kuwait governorates. All children provided a signed parent/guardian informed consent in Arabic, and assent for adolescents was obtained on the day of the school visit. The first school visit (baseline) occurred in 2012, in which 8,317 students participated. In 2014, 6,316 adolescents from the original sample enrolled in the follow-up visit. Longitudinal data were collected from this second group. Data from 11 subjects were excluded owing to incomplete information, and thus, the final sample size was 6,305 subjects. Both the Dasman Diabetes Institute Ethical Review Committee in Kuwait and the Forsyth Institutional Review Board reviewed and approved the study. The study used a longitudinal observational prospective analysis. We describe further details of the study elsewhere (20–22).

2.2 Beverage scoring

A questionnaire was prepared and administered in Arabic and English *via* iPads. Children were asked to select what they usually ate and drank with each meal and as a snack. The list of food items was based on responses from a pilot study conducted among 95 Kuwaiti schoolgirls before launching the survey (23). The dietary preference questions included 79 food and beverage items with accompanying pictures, and food selection options were modified to reflect the regularly consumed foods in Kuwait. Interviewers queried the children regarding the food items they usually ate for breakfast, lunch, dinner, and snacks. Following the questions on food preferences, questions on portions were presented, with pictures provided to assess the difference between portion sizes, e.g., one can, two cans, and three or more cans of soda with each meal. At the end of food selection, we asked the children if they preferred diet or regular soda and if they drink flavored or unflavored milk. The beverage for breakfast, lunch, dinner, and snacks was added to give a total number of servings. By this procedure, we obtained total servings per day for each of the three commonly used beverages (soda, juice, and milk). We excluded coffee and tea owing to a minimal number of subjects consuming these beverages. We computed the servings for each of the three beverages (soda, juice, and milk) as servings per day categories (0, 1-2, and 3 servings/day or more). Those who reported 0 serving a day were considered to have no consumption of the beverage. We analyzed consumption of drinks in three categories, i.e., non-consumers (0 serving/day), moderate consumers (1-2 servings/day), and high consumers (≥ 3 servings/day).

2.3 Obesity measurements

During the two visits, both weight and height were measured. Weight (kg) and height (cm) were used to compute the body mass index (BMI) of each participant. At each visit, we categorized the participants as either obese or nonobese utilizing the WHO definition of obesity with the use of BMI Z-score obesity cutoff (24). If the participant had a Z-score higher than 2 standard deviations, they were considered obese. Children were either obese or nonobese at baseline and follow-up. Based on these two visits, participants were placed in one of four groups, i.e., became obese, remained nonobese, remained obese, and became nonobese.

2.4 Data management and statistical analysis

At both visits, the four groups based on obesity status were used to identify children who developed obesity during the study period. The children who were nonobese at baseline and became obese at follow-up were identified as the "became obese group" (Group 1). Children in this group developed obesity during the two-year monitoring period. Children who were nonobese at baseline and remained nonobese at follow-up were identified as the "remained nonobese group" (Group 2). Children who were obese at baseline and remained obese at follow-up were identified as the "remained obese group" (Group 3). Children who were obese at baseline and became nonobese at follow-up were identified as the "became nonobese group" (Group 4). To analyze the effect of age, we divided the children into two groups, i.e., above and below the median age (9.9 years) at baseline visit. Fitness level was measured using the Queens College step test (25) as the increase in heart rate (beats/minute) following a standard exercise. The chi-square test was used to determine the significance level between the children who developed obesity and the other groups combined. Binary group differences in sex, age, and fitness level, along with consumption percentages of different levels of soda, juice, and milk consumed, were tabulated.

We analyzed the association between the three beverages consumed and developing obesity within the two-year study period using univariate logistic regression. The univariate logistic regression analysis was performed to assess the crude association between each beverage and the odds of developing obesity. To identify confounders, the following variables were tested separately using logistic regression based on clinical relevance and possibility of being confounders: age, sex, governorate, blood pressure, fitness level, salivary glucose level, and salivary high-density lipoprotein cholesterol (HDLC) level. None of these variables had an odds ratio (OR) of ≥10% difference compared to the crude association OR with any of the three beverages. We added variables to the stepwise selection model keeping only significant variables at a significance level of p = 0.05. Age, governorate, and fitness level were significant, but sex was not. All other tested variables were not statistically significant and therefore were not added to the completely adjusted multivariate model. We created interaction terms for all variables and found no significant effect modification. We examined the goodness of fit for all of the three beverages using the Hosmer-Lemshow test, and all three were found to have sufficient goodness of fit. The crude and completed adjusted models are found in Table 1. The children who developed obesity (Group 1) were the group of interest. We tested these children against all the other three groups combined. Table 2 demonstrates the crude association and the fully adjusted model for Group 1 against each group separately, first against the "remained nonobese group" (Group 2) alone, followed by the "remained obese group" (Group 3) alone, and finally against the "become a non-obese group" (Group 4) alone. To test the trend between the categories for each beverage in both crude and adjusted models, we used the categorical beverage variable coded as a continuous variable. The p-value was ≤0.05 which showed a statistically significant trend between categories.

A cross-sectional analysis of the baseline data was conducted using multivariate logistic regression analysis after adjusting for age, sex, governorate, and fitness level to compare it to longitudinal analysis.

3 Results

The study included 6,305 children (Figure 1). At baseline, 4,171 (66.1%) were non-obese and 2,134 (33.9%) were obese. The target population in Group 1 (n = 378, 6%) developed obesity between baseline and follow-up visits. Group 2 (n = 3,793, 60.2%) were non-obese at baseline and remained non-obese at follow-up visit. Group 3 (n = 1,827, 28.9%) were obese at baseline and remained obese at follow-up. Group 4 (n = 307, 4.9%) became non-obese at follow-up.

Table 1 illustrates the characteristics of participants who became obese (Group 1) and those who did not (Groups 2, 3, and 4). Girls from all age groups, and children (boys and girls) less than 9.9 years old had a higher tendency to develop obesity ($p < 0.001$). Fitness level did not differ significantly between those who developed obesity and those who did not ($p = 0.143$). We found none of the comparisons of moderate consumption (1-2 servings) of juice and milk to be significantly associated with obesity. Children who developed obesity during the study period reported the highest percentage of individuals with high consumption of soda (12.43% compared to 7.4%, p = 0.001). By this analysis, high milk consumption was also not found statistically significant with the obese. (5.6% compared to 3.3%, p = 0.057). Children consuming juice did not differ significantly in the percentage of becoming obese (17.2% compared to 15.7%, p = 0.116).

The association of the different beverages and the odds of becoming obese (Table 2) demonstrated that participants who reported to have high soda consumption had an OR of 1.68 in becoming obese compared to those who reported without soda consumption (p = 0.004). Subjects who reported high consumption of milk had odds of 1.78 times than that of becoming obese compared to those who reported without milk consumption (p = 0.018). Adjustment for potential confounders did not alter this association between obesity and soda consumption. Milk had a similar effect, as those who reported with high consumption had an OR of 1.77 in becoming obese compared to those who reported with no milk consumption at all (p = 0.019). Consumption of juice (OR = 1.11) did not significantly affect the percentage of children who became obese (p = 0.494).

A comparison of the obese group (Group 1) with each of the other three groups is shown in Table 3. By this analysis, we found that children who reported high soda consumption showed significantly higher odds of being obese than those who reported with no soda consumption for every group comparison. We found the highest odds of becoming obese in those who became obese compared to the children who became non-obese at follow-up (OR = 2.42, p = 0.005). Milk was only significant when we compared children who became obese (Group 1) to children who were nonobese at both visits (OR= 1.87, p = 0.013). We did not find that consumption of milk was significantly associated with any of the other groups. Consumption of juice was also not significantly associated with any group. None of the tests in Table 3 any significant trend.

10.3389/fendo.2023.1174299

TABLE 1 Characteristics of Kuwaiti children by obesity change status from 2012 to 2014 using the chi-square test.

Variable	Those who became obese (Group 1, n = 378, 6%)$^{\Delta}$	Those who did not become obese (Group 2,3 and 4, n = 5927, 94%)$^{\Delta}$	P value
Sex			**<0.001****
Female 3,958 (62.8%)	229 60.6%	3729 62.9%	
Male 2,347 (37.2%)	149 39.4%	2198 37.1%	
Age $^{\alpha}$			**<0.001****
Younger (≤9.9 years) 3,157 (50.1%)	217 57.4%	872 47.7%	
Older (>9.9 years) 3,148 (49.9)	161 42.6%	955 52.3%	
Fitness level $^{\beta}$			**0.143**
Low fitness level (≥23.5 bpm) 3,149 (49.94%)	175 46.3%	2,974 50.18%	
High fitness level (<23.5 bpm) 3,156 (50.06)	203 53.7%	2953 49.82%	
Soda consumption			**0.001****
No soda 2,170 (34.4%)	130 34.39%	2040 34.4%	
Moderate soda consumption 3,649 (57.9%)	201 53.17%	3448 58.2%	
High soda consumption 486 (7.7%)	47 12.43%	439 7.4%	
Juice consumption			**0.116**
No juice 2,670 (42.3%)	158 41.8%	2512 42.4%	
Moderate juice consumption 2,640 (41.9%)	155 41.0%	2485 41.9%	
High juice consumption 995 (15.8%)	65 17.2%	930 15.7%	
Milk consumption			**0.057**
No milk 2,766 (43.9%)	157 41.5%	2609 44.0%	
Moderate milk consumption 3,322 (52.7%)	200 52.9%	3122 52.7%	
High milk consumption 217 (3.4%)	21 5.6%	196 3.3%	

p values listed are computed for the ≥3-serving group for soda, juice, and milk consumption. α = age divided into younger and older age groups around the median age at baseline (9.9 years). β = fitness level divided into low and high fitness level groups around the median fitness level at baseline (23.5 bpm). Δ = obesity as defined by WHO obesity Z-score. bpm = beats/minute.
* Significant (p ≤ 0.05) by chi-square test. ** significant (p ≤0.001).

Figure 2 summarizes the association between high consumption of any of the three beverages and the odds of being obese, comparing Group 1 to all and each of the other three groups.

4 Discussion

Our findings suggested that soda and to a lesser extent, milk but not juice consumption led to Kuwaiti children becoming obese (Table 1). Only high beverage consumption affected obesity. This association was only evident when we assessed longitudinal data but not cross-sectional data.

As per authors knowledge, this study is the first study to be done on grade 4th and 5th of school going children under big research that looks for salivary biomarkers. Some of the effects described in this analysis were likely associated with the climate in Kuwait. Most longitudinal observational studies are in North American (14, 26–34) and in European countries (35–38). Furthermore, we believe

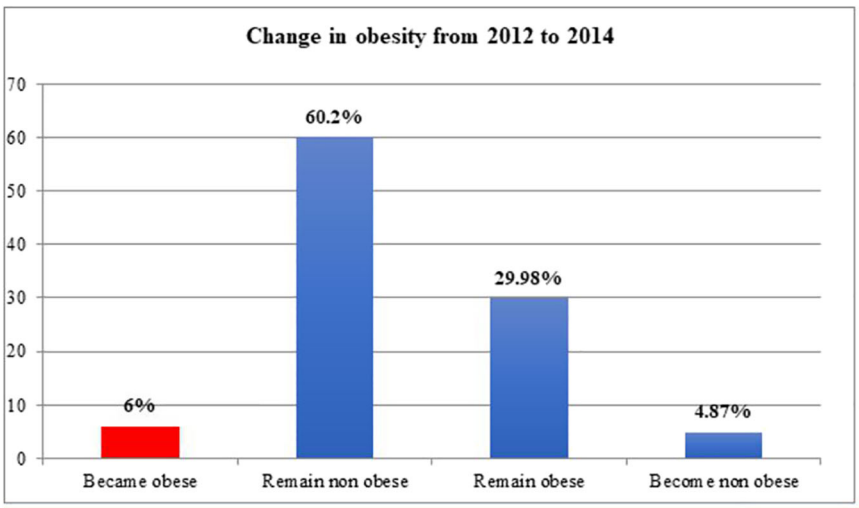
10.3389/fendo.2023.1174299

TABLE 2 Logistic regression model for the relationship between children who developed obesity (Group 1) and those who did not (Groups 2, 3, and 4).

Variable	1. Crude			2. Adjusted		
	Group 1 vs. Groups 2, 3, and 4			Group 1 vs. Groups 2, 3, and 4		
	OR	95% CI	P-value	OR	95% CI	p value
Soda	Trend p value = 0.107			Trend p value = 0.111		
No soda	–	–	–	–	–	–
Moderate soda consumption	0.91	0.73-1.15	0.442	0.91	0.73-1.14	0.426
High soda consumption	1.68	1.18-2.38	0.004*	1.68	1.19-2.39	0.004*
Juice	Trend p value = 0.582			Trend p value = 0.552		
No juice	–	–	–	–	–	–
Moderate juice consumption	0.99	0.79-1.25	0.943	1.01	0.80-1.27	0.936
High juice consumption	1.11	0.82-1.50	0.489	1.11	0.82-1.50	0.494
Milk	Trend p value = 0.109			Trend p value = 0.110		
No milk	–	–	–	–	–	–
Moderate milk consumption	1.06	0.86-1.32	0.569	1.07	0.86 -1.32	0.565
High milk consumption	1.78	1.10-2.87	0.018*	1.77	1.10-2.87	0.019*

Adjusted values are for age, sex, governorate, and fitness level. OR = odds ratio. 95% CI = 95% confidence interval *Significant (p≤ 0.05).

this is the first time that this association has been investigated in one of the MENA countries that have a high prevalence of both sugary drink consumption (18) and (11) obesity. The majority of studies investigating SSB and weight gain in children showed a positive association between the two (15). SSB includes a spectrum of beverages such as sugar-added soft drinks/sodas, energy drinks, flavored juice beverages, sports drinks, coffee and tea with added caloric sweeteners, and electrolyte replacement drinks (39). Some of



Change in obesity status from 2012 to 2014			N (%) 6,305
Non-obese at baseline 4,171 (66.1%)	Obese 2014 (become obese)	Group 1*	378 (6%)
	Non-obese 2014 (remain non obese)	Group 2	3,793 (60.2%)
Obese at baseline 2,134 (33.9%)	Obese 2014 (remain obese)	Group 3	1,827 (28.9%)
	Non-obese 2014 (become non-obese)	Group 4	307 (4.9%)

FIGURE 1
Change in obesity status from 2012 to 2014. Group 1 is the interest group. Children in Group 1 who developed obesity during the two-year study period. We compared the other groups with the interest group. *Interest group.

10.3389/fendo.2023.1174299

TABLE 3 The association between consumption of three beverages and developing obesity over a two-year period.

Variable	Group 1 vs. Group 2			Group 1 vs. Group 3			Group 1 vs. Group 4		
	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI	p value
Soda	Trend p value = 0.162			Trend p value = 0.174			Trend p value = 0.078		
No soda	–	–	–	–	–	–	–	–	–
Moderate soda consumption	0.91	0.72 -1.15	0.432	0.93	0.73-1.18	0.538	0.92	0.66- 1.28	0.615
High soda consumption	1.68	1.17-2.42	0.005*	1.58	1.08-2.32	0.019*	2.42	1.31-4.47	0.005*
Juice	Trend p-value = 0.646			Trend p value = 0.259			Trend p value = 0.891		
No juice	–	–	–	–	–	–	–	–	–
Moderate juice consumption	0.98	0.78-1.24	0.864	1.07	0.841.37	0.564	0.92	0.651.28	0.607
High juice consumption	1.06	0.78-1.43	0.720	1.21	0.87-1.67	0.257	1.1	0.69-1.74	0.699
Milk	Trend p value = 0.059			Trend p value = 0.318			Trend p value = 0.367		
No milk	–	–	–	–	–	–	–	–	–
Moderate milk consumption	1.08	0.87-1.35	0.489	1.01	0.80-1.27	0.932	1.05	0.76 -1.44	0.773
High milk consumption	1.87	1.14- 3.06	0.013*	1.63	0.96-2.77	0.070	1.68	0.75-3.74	0.205

This assesses children who developed obesity (Group 1) with each group, i.e., the groups comprising children who remained nonobese (Group 2), obese children who became nonobese (Group 3), and those who were obese and remained obese (Group 4) * Significantly associated at 0.05 level.

the studies investigated these beverages combined as one group (14, 29, 31, 35), and some separated these beverages investigating each beverage separately accounting for a different source of sugar (25, 32, 33, 35). We found segregating the beverages to be beneficial as we found juice to be not associated with developing obesity, but soda and milk were. In other studies that segregated different SSB, soda was regularly associated with weight gain more than the other beverages investigated (32, 38). Striegel-Moore et al. (32) found that high soda consumption was the most reliable predictive factor of weight gain compared to other beverages including diet and regular

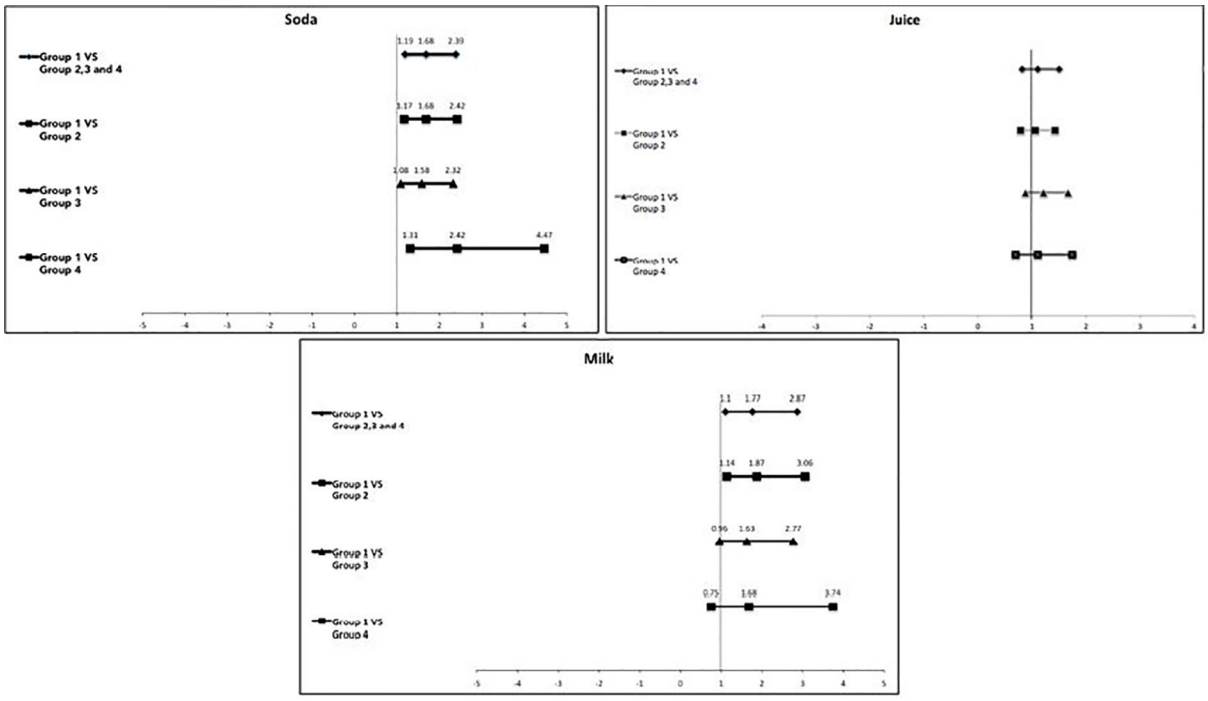


FIGURE 2
OR and 95% confidence intervals showing the association between high soda, juice, and milk consumption in children who developed obesity (interest group) and all the other three groups. If the bar passes through the solid vertical line, it means there is no significant association. Group 1: Became obese; Group 2: Remained nonobese; Group 3: Remain obese; Group 4: Became nonobese.

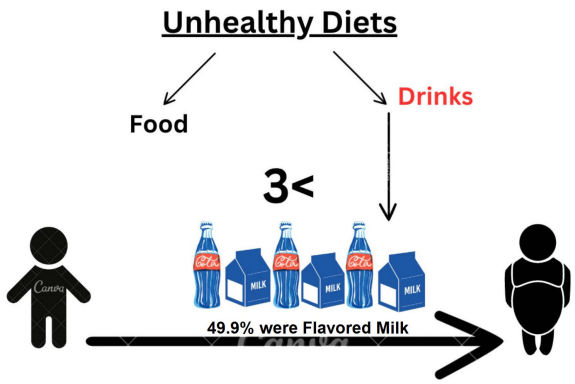


FIGURE 3
Representation of higher consumption.

soda, milk, coffee/tea, fruit juice, and fruit-flavored drinks. Viner et al. (38). also showed significant weight gain in children who reported high consumption of soda. Another study on young kids (5 years to 11 years) showed that the high frequency of SSB consumption had a roughly threefold increased chance of being overweight between the ages of 5/6 and 10/11 (40). Our study had the same finding that children with high soda consumption had higher odds of becoming obese. Soda is an excellent source of phosphate in diet, and high phosphate or phosphorus intake association with obesity has been reported in other epidemiological studies (41, 42), but the mechanism is still unclear (43). In our research group, Hartman et al. (44) found in a cross-sectional investigation of 77 children that salivary phosphate was significantly elevated in obese children compared to healthy weight children. Our data suggest that the combination of phosphorus with sugar in soda may synergize in the development of obesity. However, this hypothesis will require further investigation.

In contrast, high milk consumption resulted in children having significantly higher odds in becoming obese but only when the children were compared with those who were nonobese and remained nonobese (Group 2). Our findings concerning milk do not agree with findings from several longitudinal studies on its relationship with weight (45–47). Furthermore, some studies did not find any association between milk and changes in weight (48). On the other hand, our findings were consistent with the findings by Berkey et al. (49) as they found in a longitudinal study on 12,829 US children that drinking 3 or more servings of milk was associated with weight gain. In their findings, they also found that weight gain is attributed to the added calories from the milk because the association attenuated when they adjusted for the total energy intake. Another study on the different age group of children in Kuwait published recently, sweetened beverage consumption was linked to being overweight and having a higher BMI-for-age z score, respectively (19). In our study, we were unable to adjust for the total energy intake to identify if the association between milk and weight gain would remain the same or would disappear. Similar to the suggested contribution of phosphorus in the soda, we also, may suggest as a hypothesis that the fat content in milk in combination with added sugar would have contributed to the association between weight gain and obesity. Strong recommendations to limit sugar intake for adults and children were published by WHO (11) in 2015 due to its widely documented association with obesity and dental caries. We find it valuable to investigate some other ingredients added in some beverages that make them more associated with obesity. Phosphorus in soda beverages and fat in flavored or unflavored milk drinks are two good examples of some of these additives.

Considering that juice consumption is described to be associated with obesity by many (50), it was a surprise that compared to soda and milk, fruit juice was not found to have a significant effect on obesity. However, our findings on fruit juice are in agreement with other studies investigating fruit juice and obesity, Gustavo in 2017 concluded that moderate consumption of fruit juices has a lower risk of metabolic syndrome (51).

A vast majority of studies concerning dietary intake are usually self-reported; therefore, they are subject to reporter accuracy, bias, and recall especially in children and children (52). Our study is no exception, although we believe that using pictures for selection of both the beverages and portion size may help in improving the accuracy. As bottles, cans, and cups come in different sizes, the servings computed in the survey represent a proxy for the expected consumption pattern.

The questions about beverage consumption did not discriminate between regular and diet soda, but in a follow-up question at the end of the survey, children were asked if they chose regular or diet soda, and only 283 children (4.5%) reported drinking diet soda. Hence, we decided to keep these 283 children in the sample due to their small number. Conversely, another research on preschool children to investigate the connection of beverage intake with BMI revealed that higher beverage consumption (Milk, Soda, fruit drinks and fruit juices) correlated with an increase in the children's overall calorie intake rather than their BMI (53).

Questions regarding milk did not inquire about the type of milk consumed (whole, low fat, skim, flavored, or unflavored). However, at the end of the survey, children were asked if they drink flavored milk and 3,152 (49.9%) children responded positively. Therefore, it is important to include flavored milk (with added sugar) in the milk category.

5 Conclusions

High consumption (3 or more servings) of soda and to a lesser degree, milk but not fruit juice was significantly associated with obesity development in Kuwaiti children, thus clearly indicating that there is more to obesity than simple sugar consumption. Neither high nor moderate juice consumption was significantly associated with obesity. Only high soda or milk consumption (≥3 servings/day) was associated with increased prevalence of obesity. Consumption of moderate amounts of any of these beverages (1-2 servings/day) was not associated with significantly increased prevalence of obesity.

In our results, we were able to find significant association between high soda and high milk (49.9% were flavored milk) consumption with developing obesity in Kuwaiti children. Figure 3 is presenting that the higher consumption of sugar sweetened milk (49.9% were flavored milk) and soda may lead to the obesity.

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 humans were approved by Both the Dasman Diabetes Institute Ethical Review Committee in Kuwait and the Forsyth Institutional Review Board reviewed and approved the study. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MA, MH, and JG designed and conducted the study. MA and MT analyzed the data. MA wrote the paper with the assistance of all authors. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by Dasman 10 from the Dasman Diabetes Institute, Dasman, Kuwait.

Acknowledgments

The authors wish to recognize these individuals for helping to conduct the study in Kuwait from 2011 - 2012: Dr Sarah Ahmed, Dr Sumaiah, Dr Yasmeen, Rabab Al Khajah, Massouma Saket, Laila Khaja, Laila Diab, Mariyam Sayer, Nadia, Mahmoud Bader, Amna Ahmed, Eman Adnan, Shymaa Al Fadly. We gratefully acknowledge the participation of dentists Rithna Shajahan, Safura Mohammed, Hemalatha Vasudevaraj, Enas Mahmoud Ismail and Noha Syed Musthafa; nurses Wisam Hassan, Neda Saleh Hamad, Amal Kamel Ebrahim, Abeer Abdul Hameed, Amani Ata'allah, Flavia D. Ferrao, July K. Kunjumon, Reji Thomas, Wedad Al Shamary, Hanady Salem, Asma Al Said, AbdulHameed Said, Bashaer Eid Al Enezin, Jenny Mol Kurian, Laila Hamed Habib, Kholoud Kazem Jaber, Rawan Salem, Halima Malek Hussein, Marwa Ibraheem, Priya, Deslin, Suad Ahmed Al Deyab, Zakiya Abdul Mohsen, Rasha Mohammed, Kawthar Sa'ad Jaber, Afaf Eid Tharesh, Nawal Abdullah, Shaima Syed Mohammed; field coordinators Eman Sayed Khadmi and Fatma Al Fadhli; secretary Raseena Valamvayal; drivers A. K. Jamaluddeen, V. M. Kunhammed, K. K. Basheer and O. T. Shaishad; and setup helpers P. Soopy and Shameer Porayil.

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

References

1. Evans CEL. Sugars and health: a review of current evidence and future policy. *Proc Nutr Soc* (2017) 76:400–7. doi: 10.1017/S0029665116002846

2. Stanhope KL. Sugar consumption, metabolic disease, and obesity: The state of the controversy. *Crit Rev Clin Lab Sci* (2016) 53(1):52–67. doi: 10.3109/10408363.2015.1084990

3. Gatineau M, Hancock C, Holman N, Outhwaite H, Oldridge L, Christie A, et al. Adult obesity and type 2 diabetes. *Oxford: Public Health Engl* (2014), 5–14. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/338934/Adult_obesity_and_type_2_diabetes_.pdf.

4. Zaghloul S, Al-Hooti SN, Al-Hamad N, Al-Zenki S, Alomirah H, Alayan I, et al. Evidence for nutrition transition in Kuwait: over-consumption of macronutrients and obesity. *Public Health Nutr* (2013) 16(4):596–60. doi: 10.1017/S1368980012003941

5. Zafar TA, Alkazemi D. Correlates of food intake and mealtime behaviors among Kuwaiti adolescents. *Int J Adolesc Med Health* (2022) 34(2):19–30. doi: 10.1515/ijamh-2021-0005

6. Walpole SC, Prieto-Merino D, Edwards P, Cleland J, Stevens G, Roberts I. The weight of nations: an estimation of adult human biomass. *BMC Public Health* (2012) 12:1. doi: 10.1186/1471-2458-12-439

7. Carlson T. *Top 10 Fattest Countries In The World List Gazette Review*. Available at: https://gazettereview.com/top-10-fattest-countries-in-world/.

8. ATLAS ID. *Kuwait Seventh Edition* (2015). Available at: https://www.diabetesatlas.org/upload/resources/previous/files/7/IDF%20Diabetes%20Atlas%207th.pdf.

9. IDF. *Atlas, IDF Diabetes*. Available at: https://diabetesatlas.org/.

10. Al-Nesf Y KM, El-Shazly MK, Makboul GM, Sadek AA, El-Sayed AM, El-Fararji A. *Kuwait STEPS 2006*. Kuwait: Kuwait Ministry of Health, GCC, WHO (2006).

11. Al Rashdan I, Al Nesef Y. Prevalence of overweight, obesity, and metabolicsyndrome among adult Kuwaitis: results from community-based national survey. *Angiology* (2010) 61:42–8. doi: 10.1177/0003319709333226

12. El-Bayoumy I, Shady I, Lotfy H. Prevalence of obesity among adolescents (10 to 14 years) in Kuwait. *Asia-Pacific J Public Health* (2009) 21(2):153–9. doi: 10.1177/1010539509331786

13. Guideline W. *sugars intake for adults and children*. Geneva: World Health Organization (2015).

14. Ludwig DS, Peterson KE, Gortmaker SL. Relation between consumption of sugar-sweetened drinks and childhood obesity: a prospective, observational analysis. *Lancet* (2001) 357:505–8. doi: 10.1016/S0140-6736(00)04041-1

15. Malik VS, Pan A, Willett WC, Hu FB. Sugar-sweetened beverages and weight gain in children and adults: a systematic review and meta-analysis. *Am J Clin Nutr* (2013) 98:1084–102. doi: 10.3945/ajcn.113.058362

16. McGuire S. US Department of Agriculture and US Department of Health and human services, dietary guidelines for Americans, 2010. Washington, DC: US government printing office, January 2011. *Adv Nutrition: Int Rev J* (2011) 2:293–4. doi: 10.3945/an.111.000430

17. Al-Ansari JM, Al-Jairan LY, Gillespie GM. Dietary habits of the primary to secondary school population and implications for oral health. *J Allied Health* (2006) 35:75–80.

18. Honkala S, Behbehani JM, Honkala E. Daily consumption of sugary drinks and foods as a behavioural risk for health of adolescents in Kuwait. *Oral Health Prev dentistry* (2012) 15(2):10.

19. Alqaoud N, Al-Jawaldeh A, Al-Anazi F, Subhakaran M, Doggui R. Trend and causes of overweight and obesity among pre-school children in Kuwait. *Children* (2021) 8(6):524. doi: 10.3390/children8060524

20. Alqaderi H, Tavares M, Hartman M, Goodson J. Effect of sleep and salivary glucose on gingivitis in children. *J Dental Res* (2016) 95:1387–93. doi: 10.1177/0022034516661509

21. Goodson JM, Shi P, Mumena CH, Haq A, Razzaque MS. Dietary phosphorus burden increases cariogenesis independent of vitamin D uptake. *J Steroid Biochem Mol Biol* (2017) 167:33–8. doi: 10.1016/j.jsbmb.2016.10.006

22. Goodson JM, Tavares M, Wang X, Niederman R, Cugini M, Hasturk H, et al. Obesity and dental decay: inference on the role of dietary sugar. *PloS One* (2013) 8:74461. doi: 10.1371/journal.pone.0074461

23. Barake R, Goodson JM, Cucini M, Hasturk H, Soparkar P, Tavares M, et al. *Nutrition of Kuwaiti School Girls - Unpublished work*. (2012).

24. Onis Md, Onyango AW, Borghi E, Siyam A, Nishida C, Siekmann J. Development of a WHO growth reference for school-aged children and adolescents. *Bull World Health Organ* (2007) 85:660–7. doi: 10.2471/BLT.07.043497

25. Suriano K, Curran J, Byrne SM, Jones TW, Davis EA. Fatness, fitness, and increased cardiovascular risk in young children. *J Pediatr* (2010) 157:552–8. doi: 10.1016/j.jpeds.2010.04.042

26. Berkey CS, Rockett HR, Field AE, Gillman MW, Colditz GA. Sugar-added beverages and adolescent weight change. *Obes Res* (2004) 12:778–88. doi: 10.1038/oby.2004.94

27. Blum JW, Jacobsen DJ, Donnelly JE. Beverage consumption patterns in elementary school aged children across a two-year period. *J Am Coll Nutr* (2005) 24:93–8. doi: 10.1080/07315724.2005.10719449

28. Carlson JA, Crespo NC, Sallis JF, Patterson RE, Elder JP. Dietary-related and physical activity-related predictors of obesity in children: a 2-year prospective study. *Childhood Obes* (2012) 8:110–5. doi: 10.1089/chi.2011.0071

29. Laska MN, Murray DM, Lytle LA, Harnack LJ. Longitudinal associations between key dietary behaviors and weight gain over time: transitions through the adolescent years. *Obesity* (2012) 20:118–25. doi: 10.1038/oby.2011.179

30. Laurson K, Eisenmann JC, Moore S. Lack of association between television viewing, soft drinks, physical activity and body mass index in children. *Acta Paediatrica* (2008) 97(6):795–800. doi: 10.1111/j.1651-2227.2008.00713.x

31. Mundt CA, Baxter-Jones A, Whiting SJ, Bailey DA, Faulkner RA, Mirwald RL. Relationships of activity and sugar drink intake on fat mass development in youths. *Med Sci sports Exercise* (2006) 38:1245–54. doi: 10.1249/01.mss.0000227309.18902.fe

32. Newby P, Peterson KE, Berkey CS, Leppert J, Willett WC, Colditz GA. Beverage consumption is not associated with changes in weight and body mass index among low-income preschool children in North Dakota. *J Am Dietetic Assoc* (2004) 104:1086–94. doi: 10.1016/j.jada.2004.04.020

33. Striegel-Moore RH, Thompson D, Affenito SG, Franko DL, Obarzanek E, Barton BA, et al. Correlates of beverage intake in adolescent girls: the National Heart, Lung, and Blood Institute Growth and Health Study. *J Pediatr* (2006) 148:183–7. doi: 10.1016/j.jpeds.2005.11.025

34. Vanselow MS, Pereira MA, Neumark-Sztainer D, Raatz SK. Adolescent beverage habits and changes in weight over time: findings from Project EAT. *Am J Clin Nutr* (2009) 90:1489–95. doi: 10.3945/ajcn.2009.27573

35. Johnson L, Mander AP, Jones LR, Emmett PM, Jebb SA. Is sugar-sweetened beverage consumption associated with increased fatness in children? *Nutrition* (2007) 23(7):557–63. doi: 10.1016/j.nut.2007.05.005

36. Libuda L, Alexy U, Sichert-Hellert W, Stehle P, Karaolis-Danckert N, Buyken AE, et al. Pattern of beverage consumption and long-term association with body-weight status in German adolescents–results from the DONALD study. *Br J Nutr* (2008) 99:1370–9. doi: 10.1017/S0007114507862362

37. Olsen NJ, Andersen LB, Wedderkopp N, Kristensen PL, Heitmann BL. Intake of liquid and solid sucrose in relation to changes in body fatness over 6 years among 8-to 10-year-old children: the European Youth Heart Study. *Obes facts* (2012) 5:506–12. doi: 10.1159/000341631

38. Viner RM, Cole TJ. Who changes body mass between adolescence and adulthood? Factors predicting change in BMI between 16 year and 30 years in the 1970 British Birth Cohort. *Int J Obes* (2006) 30:1368–74. doi: 10.1038/sj.ijo.0803183

39. CDC. *The CDC guide to strategies for reducing the consumption of sugar-sweetened beverages*. Atlanta: CDC (2010).

40. Vinke PC, Blijleven KA, Luitjens MHHS, Corpeleijn E. Young children's sugar-sweetened beverage consumption and 5-year change in BMI: lessons learned from the timing of consumption. *Nutrients* (2020) 12(8):2486. doi: 10.3390/nu12082486

41. Beydoun MA, Gary TL, Caballero BH, Lawrence RS, Cheskin LJ, Wang Y. Ethnic differences in dairy and related nutrient consumption among US adults and their association with obesity, central obesity, and the metabolic syndrome. *Am J Clin Nutr* (2008) 87:1914–25. doi: 10.1093/ajcn/87.6.1914

42. de Carvalho Pereira D, Lima RPA, de Lima RT, Gonçalves M, de Morais LCSL, Franceschini S, et al. Association between obesity and calcium: phosphorus ratio in the habitual diets of adults in a city of Northeastern Brazil: an epidemiological study. *Nutr J* (2013) 12:90. doi: 10.1186/1475-2891-12-90

43. Anderson JJ. Potential health concerns of dietary phosphorus: cancer, obesity, and hypertension. *Ann New York Acad Sci* (2013) 1301:1–8. doi: 10.1111/nyas.12208

44. Hartman M-L, Groppo F, Ohnishi M, Goodson JM, Hasturk H, Tavares M, et al. Can salivary phosphate levels be an early biomarker to monitor the evolvement of obesity. *Phosphate Vitamin D Chronic Kidney Disease. 180: Karger Publishers;* (2013) 10:138–48. doi: 10.1159/000346793

45. Carruth B, Skinner J. The role of dietary calcium and other nutrients in moderating body fat in preschool children. *Int J Obes* (2001) 25:559. doi: 10.1038/sj.ijo.0801562

46. Pereira MA, Jacobs DR Jr., Van Horn L, Slattery ML, Kartashov AI, Ludwig DS. Dairy consumption, obesity, and the insulin resistance syndrome in young adults: the CARDIA Study. *Jama* (2002) 287:2081–9. doi: 10.1001/jama.287.16.2081

47. Skinner JD, Bounds W, Carruth BR, Ziegler P. Longitudinal calcium intake is negatively related to children's body fat indexes. *J Am Dietetic Assoc* (2003) 103:1626–31. doi: 10.1016/j.jada.2003.09.018

48. Phillips S, Bandini L, Cyr H, Colclough-Douglas S, Naumova E, Must A. Dairy food consumption and body weight and fatness studied longitudinally over the adolescent period. *Int J Obes* (2003) 27:1106–13. doi: 10.1038/sj.ijo.0802370

49. Berkey CS, Rockett HR, Willett WC, Colditz GA. Milk, dairy fat, dietary calcium, and weight gain: a longitudinal study of adolescents. *Arch Pediatr Adolesc Med* (2005) 159:543–50. doi: 10.1001/archpedi.159.6.543

50. Sonneville KR, Long MW, Rifas-Shiman SL, Kleinman K, Gillman MW, Taveras EM. Juice and water intake in infancy and later beverage intake and adiposity: Could juice be a gateway drink? *Obesity* (2015) 23:170–6. doi: 10.1002/oby.20927

51. Velasquez-Melendez G, Molina M, Benseñor IM, Cardoso LO, Fonseca M, Moreira AD, et al. Sweetened soft drinks consumption is associated with metabolic syndrome: cross-sectional analysis from the Brazilian longitudinal study of adult health (ELSA-Brasil). *J Am Coll Nutr* (2016) 15(2):1–9. doi: 10.1080/07315724.2016.1191975

52. Livingstone M, Robson P, Wallace J. Issues in dietary intake assessment of children and adolescents. *Br J Nutr* (2004) 92:213–22. doi: 10.1079/BJN20041169

53. O'Connor TM, Yang SJ, Nicklas TA. Beverage intake among preschool children and its effect on weight status. *Pediatrics* (2006) 118(4):e1010–8. doi: 10.1542/peds.2005-2348

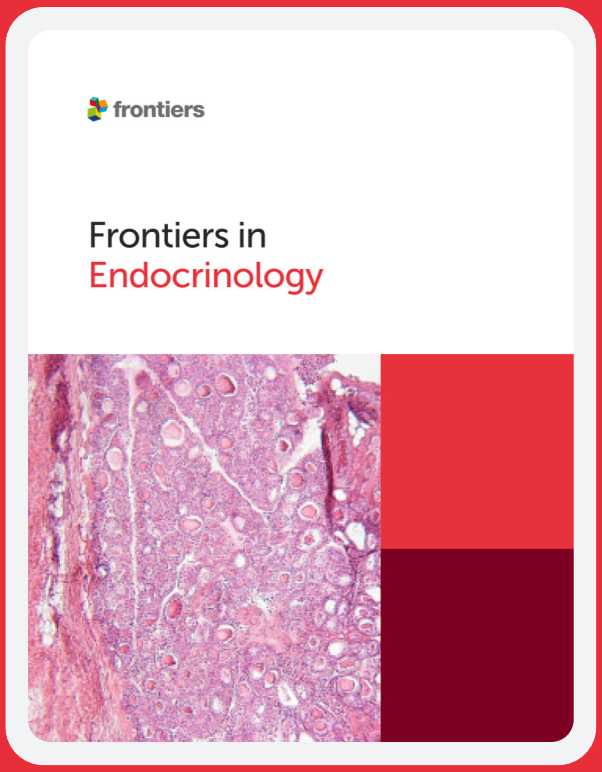
Frontiers in Endocrinology

Explores the endocrine system to find new therapies for key health issues

The second most-cited endocrinology and metabolism journal, which advances our understanding of the endocrine system. It uncovers new therapies for prevalent health issues such as obesity, diabetes, reproduction, and aging.

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