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Insights in nutrition and metabolism

Edited by
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Published in
Frontiers in Nutrition

ISSN 1664-8714
ISBN 978-2-8325-2708-5
DOI 10.3389/978-2-8325-2708-5

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Insights in nutrition and metabolism

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Citation
Blaak, E. E., ed. (2023). *Insights in nutrition and metabolism*.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2708-5

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frontiers in Nutrition

ORIGINAL RESEARCH
published: 17 February 2022
doi: 10.3389/fnut.2022.828824

Association of Serum β-Hydroxybutyrate and Coronary Artery Disease in an Urban Chinese Population

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Ketone bodies, including β-hydroxybutyrate (BHB), acetoacetate (AA), and acetone, can substitute and alternate with glucose under conditions of fuel/food deficiency. Ketone-body metabolism is increased in a myriad of tissue-metabolism disorders. Perturbations in metabolism are major contributors to coronary artery disease (CAD). We investigated the association of BHB with CAD. A total of 2,970 people of Chinese Han ethnicity were enrolled. The Gensini score was calculated for all patients who had positive findings. The serum level of BHB and other laboratory parameters were measured. The association of serum levels of metabolites with traditionally risk factors and CAD severity was analyzed. The BHB was found to be associated with some traditional risk factors of CAD and CAD severity, as determined by the Gensini score or the number of diseased regions. Moreover, BHB was associated with the T3/T1 tertiles of the Gensini score after the adjustment for traditional risk factors by multivariable logistic regression analysis. The association of BHB with CAD severity was more obvious in women. Taken together, these data suggest that the circulating BHB level is independently associated with CAD severity, and that this association is more pronounced in women.

Keywords: coronary artery disease, ketone bodies, β-hydroxybutyrate, Gensini score, metabolic dysfunction

OPEN ACCESS

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

Received: *04 December 2021*
Accepted: *17 January 2022*
Published: *17 February 2022*

Citation:
Mu H, Yang R, Wang S, Zhang W, Wang X, Li H, Dong J, Chen W, Yu X and Ji F (2022) Association of Serum β-Hydroxybutyrate and Coronary Artery Disease in an Urban Chinese Population. Front. Nutr. 9:828824. doi: 10.3389/fnut.2022.828824

INTRODUCTION

Coronary artery disease (CAD) caused by atherosclerosis, has been reported to be the leading cause of death worldwide. Although new medical therapies have emerged in recent years, the risk of death from CAD is still high (1). Therefore, there is a need for the development of more accurate diagnostic methods, identification of novel biomarkers, more efficacious drugs and identification of new therapeutic targets to reduce the risk of death from CAD (2, 3).

Metabolic dysfunction is a hallmark of CAD pathophysiology, reflecting not only the altered metabolism of the myocardium but also the overall contributions from peripheral tissues and organs (4, 5). These metabolic changes influence disease pathophysiology directly, and may serve as earlier and more sensitive markers of CAD (6, 7). Abnormal accumulation or deficiency of specific metabolites within the circulation can be powerful markers in the management of CAD, with regard to the detecting disease, evaluating disease progression, and assessing therapeutic efficacy (8, 9).

Ketone bodies [e.g., β-hydroxybutyrate (BHB), acetoacetate (AA), and acetone] are water-soluble molecules that contain the ketone groups produced from fatty acids by the liver (ketogenesis). Ketone bodies are derived from increased beta oxidation of free fatty acid, BHB is a major component of the ketone body (10). During perturbed tissue metabolism, an increase in BHB may help circumvent the metabolic malfunctions that directly contribute to disease pathophysiology (11). Recent studies have shown that BHB level was increased in a myriad of pathological conditions, such as type 2 diabetes mellitus (T2DM) (12), arrhythmogenic cardiomyopathy (13), and heart failure (14). In addition, it was reported that BHB was increased in patients with non-ST segment elevation acute coronary syndrome, which reflects the oxidative stress and hypoxic state that myocardial cells suffer. Hence, BHB could be used for the early diagnosis of acute coronary syndrome (15). Obokata and colleagues showed that an increased serum level of BHB was independently associated with cardiovascular events and all-cause death in patients undergoing hemodialysis (16). Those studies implied a potential association of BHB with CAD. However, studies on the associations between BHB and CAD in Chinese Han populations have not been carried out. Here, we evaluated the association of BHB with CAD, as well as the severity of CAD, and risk factors of CAD in a case–control study.

MATERIALS AND METHODS

Ethical Approval of the Study Protocol

The protocol for this cross-sectional study was approved (2016BJYYEC-121-03) by the Ethics Committee of Beijing Hospital (Beijing, China). Written informed consent was provided by all participants.

Exclusion Criteria

The exclusion criteria were patients: (i) with severe congenital heart disease, severe cardiac insufficiency, primary pulmonary hypertension, hepatic/renal dysfunction, severe peripheral arterial disease, or related conditions which are contraindications to cardiac catheterization; (ii) receiving radiotherapy or chemotherapy; (iii) who were pregnant or nursing a baby; (iv) suffering from alcoholism or drug abuse; and (v) under treatment for mental illness.

Study Cohort

Patients suspected of having CAD (or adjudicated to have a CAD history) and subjected to coronary angiography in Beijing Hospital between March 2017 and October 2020 were enrolled. The demographic characteristics (e.g., age and gender), medical history (e.g., DM, hypertension, and dyslipidemia), cigarette smoking, and body mass index (BMI) of the study cohort were recorded. Before coronary angiography, samples of venous blood were collected after an overnight fast. The serum was isolated, aliquoted, and stored at −80°C until analyses.

Abbreviations: BHB, β-hydroxybutyrate; BMI, body mass index; CAD, coronary artery disease; Crea, creatinine; DBP, diastolic blood pressure; DM, diabetes mellitus; FBG, fasting blood glucose; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; UA, uric acid.

Coronary Angiography

Coronary angiography was performed for CAD assessment by experienced cardiologists. All targeted coronary lesions of enrolled patients were analyzed by the built-in QCA software of the Allura Xper FD20 Angiography System (Philips Healthcare, Amsterdam, the Netherlands). Fifteen coronary segments were analyzed quantitatively based on the American Heart Association classification. According to current guidelines, "coronary stenosis" was defined as a reduction of >50% in the arterial diameter, and if this was not present, was diagnosed as non-CAD. In addition, individuals with negative findings on CT of the coronary arteries or stress myocardial perfusion imaging were categorized as the non-CAD group.

Gensini Score

The Gensini score was used to assess the severity of damage to coronary arteries (17). The Gensini score was computed by assigning a severity score for each coronary stenosis depending on the degree of luminal narrowing and its importance based on location. Luminal stenoses of 25, 50, 75, 90, 99%, and total occlusion were given a Gensini score of 1, 2, 4, 8, 16, and 32, respectively. Then, these scores were multiplied by a factor according to location: 5, left main coronary artery; 2.5, proximal segment of the left anterior descending coronary artery and proximal segment of the circumflex artery; 1.5, mid-segment of the left anterior descending coronary artery; 1.0, right coronary artery, distal segment of the left anterior descending coronary artery, posterior descending artery, and obtuse marginal artery; and 0.5, other segments. Finally, the Gensini score was calculated by summation of the scores for individual coronary segments. The Gensini score was calculated in 2,047 patients who had not undergone percutaneous arterial intervention previously.

Liquid Chromatography-Tandem Mass Spectrometry Measurement (LC-MS/MS) of the BHB Level

The serum concentration of BHB was quantified using an LC-MS/MS system comprising a 1,260 Infinity II Series high-performance liquid chromatography (HPLC) (Agilent, Santa Clara, CA, USA) coupled with a Sciex 5,500 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA). MS parameters were set as previously described (18). BHB standards, serum samples, and quality controls were, respectively, mixed with the isotopically labeled internal standard (BHB-D4) solution and proteins were precipitated with methanol containing 0.5% formic acid. After vortexing and centrifuging, the metabolites were separated by a Kinetex™ hydrophilic interaction LC column (2.6 μm, 150 mm × 2.1 mm, Phenomenex, Torrance, CA, USA) and eluted with a mobile phase of 75% methanol, containing 5 mmol/L ammonium formate, and 0.05% formic acid at a flow rate of 0.3 ml/min. BHB and internal standard were detected with positive electrospary ionization in multiple reaction monitor mode using characteristic precursor–product ion transitions of m/z 103.0→ 59.0 and m/z 107.0→ 59.0, respectively. The

concentration of BHB was calculated using standard curves. The analytical coefficients of variation for BHB measurements were below 3%. Applied Biosystems Analyst version 1.6.2 software was used for system control, data collection, and processing.

Other Parameters and Laboratory Testing

In addition, the serum samples were tested for fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), creatinine (Crea), and uric acid (UA) using the respective assay kits (Sekisui Medical Technologies, Osaka, Japan) on a chemistry analyzer (7,180 series; Hitachi, Tokyo, Japan). Two quality control materials, prepared by mixed fresh serum samples, were analyzed with patient samples in each run in LC-MS/MS and the laboratory assays to monitor the performance of the measurements.

Statistical Analysis

The normal distribution of samples was tested. Parameters with normal distribution are expressed as mean ± SD, while parameters with a skewed distribution are presented as the median and percentile (25–75th). Count data are reported as frequencies and percentages. The one-way ANOVA was conducted to compute the differences for continuous variables, while the non-parametric Jonckheere-Terpstra test was used to determine the differences when the data did not have a normal distribution. For categorical variables, the Chi-square test was used. Spearman's correlation analysis was employed to examine the associations between BHB and traditional CAD risk factors. The multivariable logistic regression analysis was employed to determine the relationship between the BHB level and the Gensini score. Potential confounding variables (age, gender, smoking status, obesity or overweight, hypertension, dyslipidemia, DM, history of stroke, and family history of

TABLE 1 | Comparison of baseline characteristics of study population according to serum BHB tertile.

Characteristic[a]	Tertile of serum level of BHB (μM)			
	Low	Intermediate	High	Trend *p*-value
N	989	992	989	–
Age, years	65.0 ± 10.6	65.7 ± 10.8	65.9 ± 11.3	0.058
Male, *n* (%)	638 (64.5)	618 (62.3)	599 (60.6)	0.066
BMI, kg/m2	25.7 ± 3.6	25.9 ± 3.3	25.5 ± 3.6	0.097
SBP, mmHg	135.6 ± 18.0	136.9 ± 18.5	137.6 ± 18.9	0.017
DBP, mmHg	78.6 ± 11.0	78.0 ± 11.1	78.4 ± 11.6	0.675
Overweight/obesity, *n* (%)	671 (67.8)	711 (71.7)	636 (64.3)	0.738
Hypertension, *n* (%)	659 (66.6)	673 (67.8)	668 (67.5)	0.809
Diabetes, *n* (%)	213 (21.5)	430 (43.3)	403 (40.7)	<0.001
Dyslipidemia, *n* (%)	398 (40.2)	474 (47.8)	440 (44.5)	0.041
History of stroke, *n* (%)	101 (10.2)	121 (12.2)	97 (9.8)	0.665
Family history of premature CAD, *n* (%)	87 (8.8)	69 (7.0)	73 (7.4)	0.041
Smoking status, *n* (%)				
Never	503 (50.9)	522 (52.6)	532 (53.8)	0.107
Former	152 (15.4)	145 (14.6)	157 (15.9)	
Current	330 (33.4)	316 (31.9)	298 (30.1)	
Statins use, *n* (%)				
No	551 (55.7)	525 (52.9)	575 (58.1)	0.727
Take statins intermittently	108 (10.9)	71 (7.2)	88 (8.9)	
Take statins continuously Over 1 year, *n* (%)	238 (24.1)	293 (29.5)	223 (22.5)	
FBG, mmol/L	6.4 ± 2.1	7.0 ± 2.3	6.8 ± 2.3	<0.001
TC, mmol/L	3.8 ± 0.9	3.9 ± 0.9	4.0 ± 1.0	<0.001
TG, mmol/L	1.2 (0.9–1.7)	1.4 (1.0–1.9)	1.2 (0.9–1.7)	0.215
HDL-C, mmol/L	1.0 (0.9–1.2)	1.0 (0.9–1.2)	1.0 (0.9–1.2)	0.866
LDL-C, mmol/L	2.2 (1.7–2.7)	2.2 (1.7–2.8)	2.3 (1.8–2.9)	<0.001
Crea, μmol/L	72.0 ± 16.4	71.4 ± 16.9	71.6 ± 18.2	0.610
UA, μmol/L	323.0 ± 84.9	328.9 ± 85.7	329.0 ± 93.2	0.136
BHB, μmol/L	27.9 (21.1–34.6)	55.7 (48.0–66.3)	162.3 (110.5–315.4)	<0.001

Not all data were available for all patients. BHB, β-hydroxybutyrate; CAD, coronary artery disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Crea, creatinine; UA, uric acid. [a]*Data are mean ± SD, median (interquartile range) for continuous variables, or percentage for categorical variables.*

premature CAD) were controlled in the regression models. Results are presented as odds ratios (ORs) and 95% *CI*s. The value of $p < 0.05$ (two-tailed) was considered significant. Statistical analysis was performed using SPSS 25.0 (IBM, Armonk, NY, USA).

RESULTS

Clinical Characteristics

The study population comprised 2,970 patients (62.5% men). Patient characteristics at baseline according to the tertile of the BHB levels are summarized in **Table 1**. Individuals with a higher BHB level also had a higher systolic blood pressure (SBP), higher risk of a family history of premature CAD as well as a higher prevalence of DM and dyslipidemia. Furthermore, with the increase of BHB levels, FBG, TC, and LDL-C significantly increased ($p < 0.001$ for trend). People with a higher BHB level tended to be older (but not significantly so). Whereas, levels of BMI, diastolic blood pressure (DBP), TG, HDL-C, Crea, and UA were similar among the groups. In addition, associations were not found between the BHB level and overweight/obesity, hypertension, history of stroke, smoking status, or statins use.

Correlations of Serum BHB Level With the Traditional CAD Risk Factors

A significant positive correlation was found between the serum BHB level and the level of FBG ($r = 0.067$, $p < 0.001$), TC ($r = 0.070$, $p < 0.001$), and LDL-C ($r = 0.066$, $p = 0.001$). The serum BHB level was significantly positively correlated with age ($r = 0.041$, $p < 0.05$) and SBP ($r = 0.040$, $p < 0.05$). However, an association between the BHB level and level of DBP, TG, HDL-C, Crea, UA, and BMI was not significant (**Table 2**).

TABLE 2 | Spearman's correlation of BHB with traditional CAD risk factors.

	Correlation coefficients	*P*
Age	0.041	0.027
BMI	−0.032	0.087
SBP	0.040	0.028
DBP	−0.011	0.542
FBG	0.067	<0.001
TC	0.070	<0.001
TG	0.019	0.338
HDL-C	0.010	0.596
LDL-C	0.066	0.001
Crea	−0.017	0.377
UA	0.021	0.260

CAD, coronary artery disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Crea, creatinine; UA, uric acid.

Relationship Between the BHB Level and CAD Severity

To analyze the relationship between the BHB level and CAD severity, patients with positive findings upon coronary angiography were classified into subgroups for those with 1–3 and >3 stenosed regions. The BHB level was significantly increased ($p_{trend} = 0.016$) with an increase in the number of stenosed regions. Then, the patients with CAD were divided into subgroups with 1 stenosed or >1 stenosed vessels. As shown in **Figure 1**, no significant change was found for the BHB level among groups. The Gensini score is widely used to evaluate the severity of coronary atherosclerosis, hence the studied patients were divided into different subgroups according to tertile of the Gensini score: the BHB level was associated with the Gensini score ($p_{trend} = 0.05$).

The Multivariable Logistic Regression Analysis of the BHB Level With CAD

Multivariate logistic regression analysis was done to examine the independent relationship between the BHB level and CAD severity. Here, we adopted tertiles of T3/T1 of the Gensini score to represent CAD severity. As shown in **Table 3**, the crude *OR* was increased with increasing BHB levels in Model 1 ($p = 0.004$). After adjustment for age and gender (Model 2), high BHB levels were significantly associated with CAD severity, with *OR* (95% *CI*) of 1.178 (1.045–1.327), $p = 0.007$. In Model 3, traditional risk factors of CAD [age, smoking (former and current smoking), hypertension, DM, and family history of premature CAD] were all positively associated with the Gensini score, whereas being female was a protective factor for CAD, with *OR* of 0.417 (0.308–0.564). An increased level of BHB continued to be independently associated with T3/T1 tertiles of the Gensini score (*OR* = 1.151, 95% *CI*: 1.012–1.309, $p = 0.032$), after adjustment for traditional risk factors, such as age, gender, smoking status, overweight/obesity, hypertension, dyslipidemia, DM, history of stroke, and family history of premature CAD (**Table 3**).

The Stratified Analysis of the BHB Level With CAD

Stratified analysis of the association of the serum BHB level (per 1-SD increment) and the severity of coronary artery lesion was conducted in different groups of age, gender, BMI, smoking status, hypertension, dyslipidemia, DM, history of stroke, and family history of premature CAD. The interaction between the BHB level and these factors on CAD distribution was analyzed. As presented in **Figure 2**, there was a significant association between the BHB level and CAD in women, non-smokers, people with hypertension, DM, or a family history of premature CAD. Moreover, we observed that the association between the BHB level and CAD was modified by gender ($P_{interaction} = 0.015$). The BHB level was associated with CAD in women (*OR*: 1.429, 95% *CI*: 1.138–1.793, $p = 0.002$), but not in men (*OR*: 1.028, 95% *CI*: 0.892–1.1840, $p = 0.704$), after adjustment for age, BMI, smoking status, hypertension, dyslipidemia, DM, history of stroke, and family history of premature CAD. However, an interaction was not observed between the BHB level and age, BMI, smoking status, hypertension, dyslipidemia, DM, history of stroke or family history of premature CAD (**Figure 2**). Then, we further analyzed the association of the BHB level with CAD severity in men and women, respectively, according to the

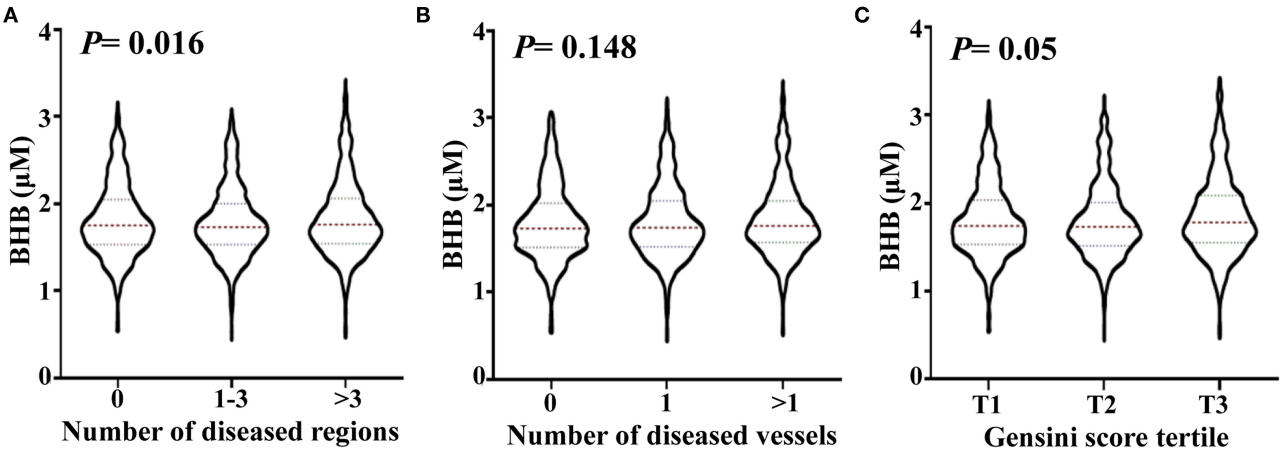

FIGURE 1 | Association of the serum BHB level with the severity of coronary artery lesion. Violin plots of serum BHB concentrations at presentation with the number of stenosed regions **(A)**, the number of stenosed vessels **(B)** and tertile of the Gensini scores **(C)**, showing median (red dashed line) and interquartile ranges (blue dashed line) on a log 10 scale. $p < 0.05$ considered statistically significant.

TABLE 3 | Odds ratios (95% *CI*s) and *p* for the severity of coronary artery lesion.

		P-value	OR (95% confidence intervals)
Model 1[a]	BHB	0.004	1.177 (1.053–1.314)
Model 2[b]	Age	<0.001	1.037 (1.026–1.048)
	Gender	<0.001	0.304 (0.240–0.386)
	BHB	0.007	1.178 (1.045–1.327)
Model 3[c]	Age	<0.001	1.035 (1.023–1.048)
	Gender	<0.001	0.417 (0.308–0.564)
	Smoking status		
	Never	–	–
	Former	0.016	1.689 (1.103–2.587)
	Current	<0.001	2.082 (1.503–2.883)
	Overweight/obesity	0.102	0.802 (0.616–1.045)
	Hypertension	0.020	1.353 (1.048–1.746)
	Dyslipidemia	0.877	0.981 (0.774–1.245)
	Diabetes	<0.001	2.570 (1.979–3.339)
	History of stroke	0.241	1.266 (0.853–1.878)
	Family history of premature CAD	0.011	1.504 (1.097–2.063)
	BHB	0.032	1.151 (1.012–1.309)

[a]*Model 1: Crude risk.* [b]*Model 2: Adjusted for age and gender.* [c]*Model 3: Further adjusted for smoking status, obesity or overweight, hypertension, dyslipidemia, diabetes, stroke, and family history of premature CAD. For gender, smoking status, overweight/obesity, hypertension, dyslipidemia, diabetes, history of stroke, family history of premature CAD, the reference are women, never smoking, body mass index (BMI) <24 kg/m^2, no hypertension, no dyslipidemia, no diabetes, no history of stroke, and no family history of premature CAD, respectively.*

Gensini score. Consistent with this, the BHB level showed a significant association with the Gensini score in women, but not in men (**Figure 3**).

DISCUSSION

In this study, we observed circulating BHB concentration was associated with the traditional risk factors and severity of CAD. An increased BHB level was associated with increased CAD severity, as defined by Gensini score or number of stenosed regions. This association of the BHB level with the Gensini score remained significant after adjustment for the conventional risk factors of CAD by multivariable logistic regression analysis. An increased level of BHB in patients with CAD indicated a more severe state of the disease and more severe systemic metabolic perturbations, especially in women (**Figure 4**). To the best of our knowledge, this study is the first to report that an increased BHB level to be associated with an increased risk of CAD in a Chinese population.

The heart is the most metabolically demanding organ in the body, so impaired metabolism may lead to CAD, thus, the identification and quantification of changes in levels of these metabolites is critical for the diagnosis and treatment of CAD (19). Ketone bodies, such as BHB, AA, and acetone, are "small fuel substrates," derived from increased beta oxidation of free fatty acid. These substances can uniquely substitute and alternate with glucose under conditions of fuel/food deficiency (20). There is considerable evidence that the level of ketone bodies, in particular BHB, is increased in metabolic disorders. The most robust evidence is from a retrospective cohort study which demonstrates that the BHB level is independently and significantly associated with adverse cardiovascular events and therefore could serve as a novel biomarker predicting the survival and risk of cardiovascular events in patients undergoing hemodialysis (16). Furthermore, the circulating level of ketone bodies is increased in patients presenting with ST-segment elevation myocardial infarction and a higher level of ketone bodies after 24 h is associated with functional outcomes. Hence, the increased metabolism of ketones may play a part in the

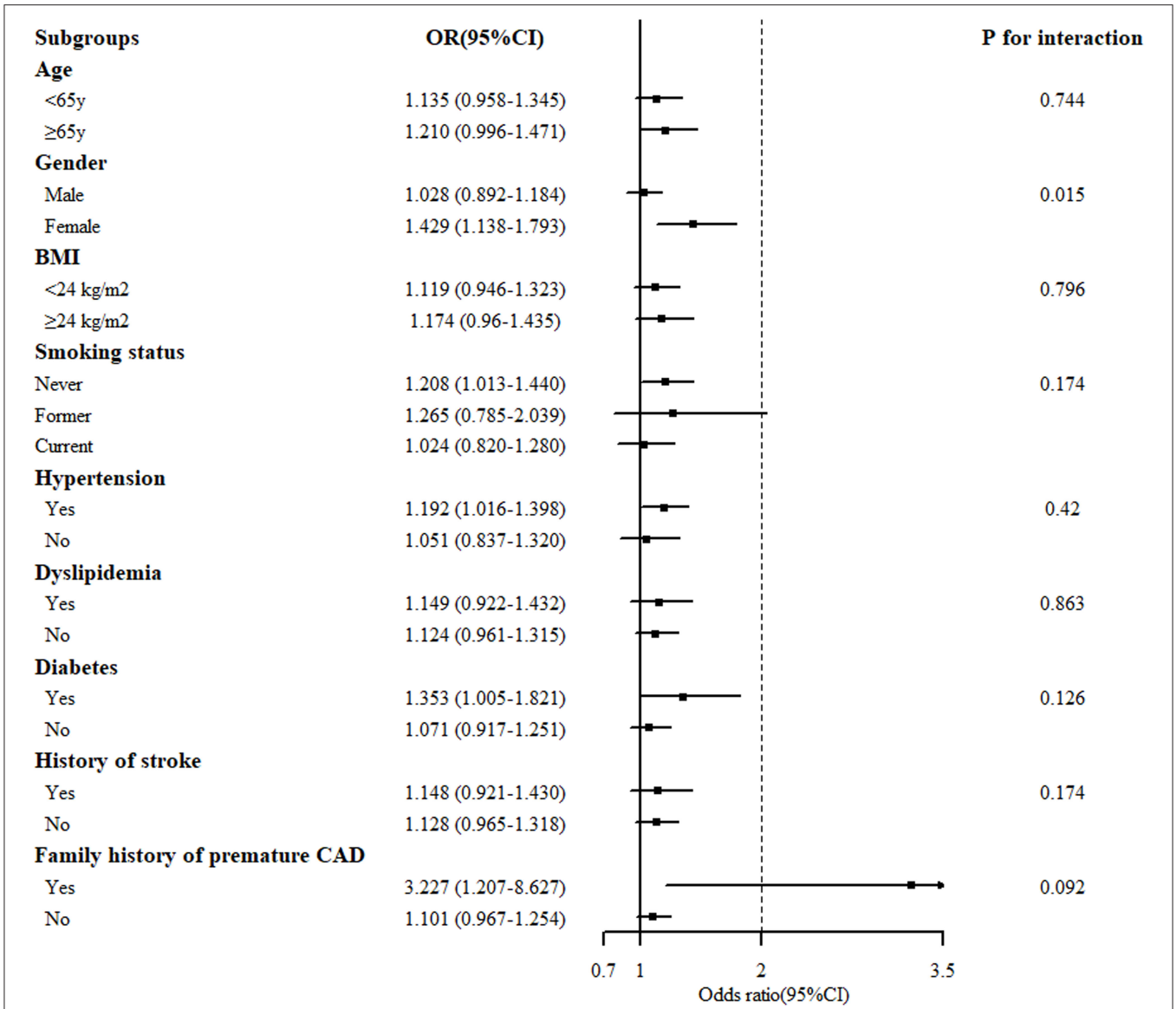

FIGURE 2 | Stratified analysis of the association [odds ratio (*OR*) (95% *CI*)] between the circulating BHB level (per 1-SD increment) and the severity of coronary artery lesion. Values are adjusted for age, gender, smoking status, obesity or overweight, hypertension, dyslipidemia, diabetes, stroke, and family history of premature CAD, stratifying factors excepted. The $p < 0.05$ considered statistically significant.

response to myocardial ischemia (21). Accumulating evidence has shown that the BHB level is increased in patients with heart failure (14, 22), obese and/or T2DM (12, 23). Of importance, with metabolic dysfunction as an inherent feature of the CAD pathophysiology, whether circulating BHB is increased in CAD remains poorly understood. We investigated if the BHB level is associated with CAD in a Chinese Han population.

First we analyzed the association between the circulating BHB concentrations with CAD traditional risk factors. The result revealed a strong association between the BHB level with the concentration of FBG, TC, and LDL-C, as well as a significant association between the BHB concentration and age and SBP. These results were not in line with some of the previous reports (13, 24), possibly on account of different populations studied. High TC and high LDL-C all play a role in CAD (25–28). We discovered a significant association between BHB with TC and LDL-C (**Table 2**). However, multivariable logistic regression analysis revealed dyslipidemia not to be associated with CAD severity. On account of the use of statins therapy, the relationship between the clinical diagnosis of dyslipidemia and CAD was not significant.

Next, we analyzed the relation of BHB with CAD and CAD severity. The study population was first divided into the non-CAD group and CAD group according to coronary angiography. People who developed CAD did not have a significantly higher BHB level in serum compared with that in healthy controls

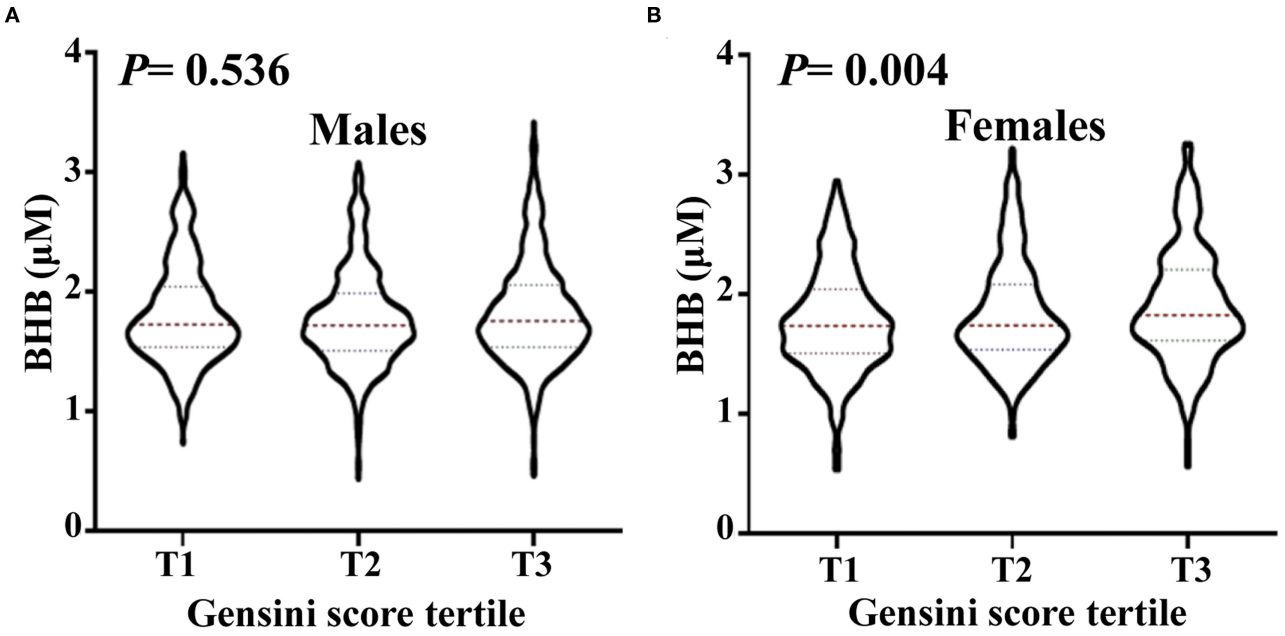

FIGURE 3 | The level of circulating BHB in men and women, respectively, according to Gensini score tertile. Violin plots of serum BHB concentrations in men **(A)** and women **(B)** by Gensini score tertile, showing median (red dashed line) and interquartile ranges (blue dashed line) on a log10 scale. The $p < 0.05$ considered statistically significant.

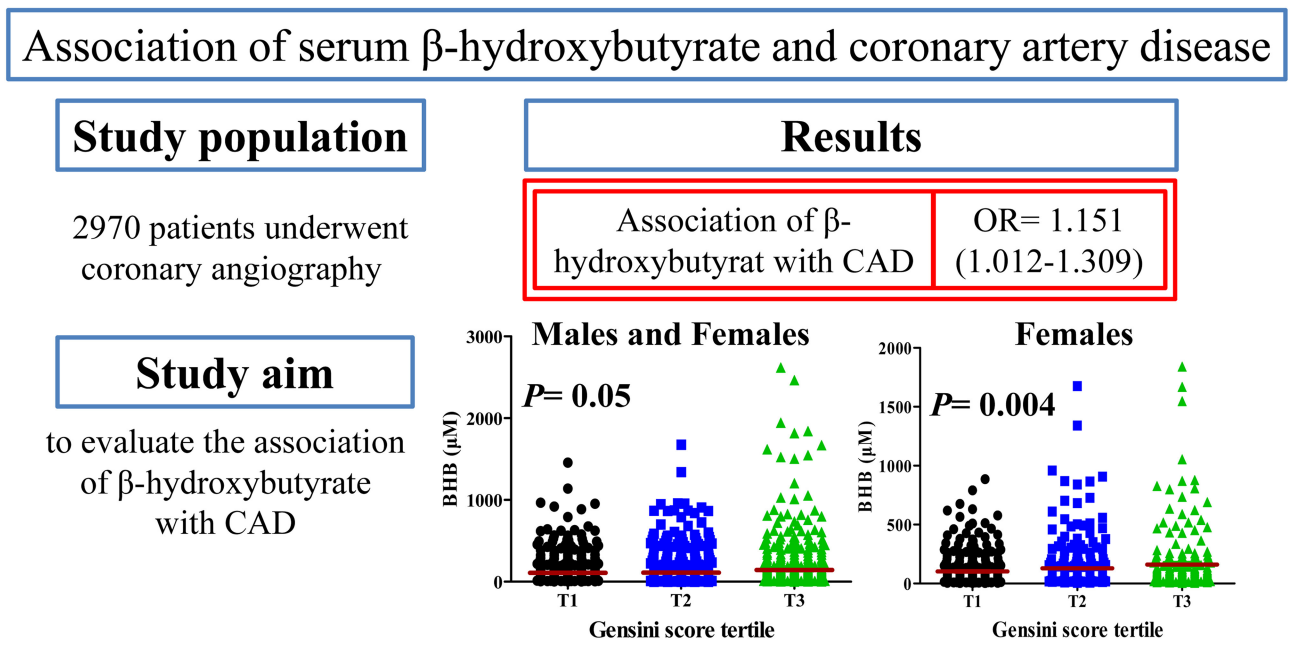

FIGURE 4 | Overview of this study. CAD, coronary artery disease; OR, odds ratio.

(data not shown). In a pilot study undertaken in Japan, Omori et al. found the BHB level to be significantly lower in the CAD group compared with that in the control subjects (in patients with DM), but the study cohorts were quite small (5). To analyze the association between the BHB level and CAD severity, the CAD group was divided into subgroups, based on the number of stenosed vessels or regions: the BHB level was associated with the severity of CAD according to the number

of stenosed regions. Gensini score is widely used for evaluating the coronary atherosclerotic severity with the judgement of the position, number, and stenosis of blood vessels caused by coronary atherosclerosis (17). The CAD patients were divided into 3 groups according to the Gensini score tertile, and the BHB level showed a significant association with the Gensini score tertile (**Figure 1**). Multivariable logistic regression analysis revealed the BHB level to be independently associated with the T3/T1 tertiles of the Gensini score, even after adjustment for other well-known risk factors (**Table 3**).

Additional stratification analysis revealed the BHB level to have a strong association with CAD in women (**Figure 2**). Furthermore, in accordance with this, **Figure 3** revealed that the BHB level was associated with CAD severity in women not men, according to the Gensini score. These results indicated that BHB may be involved in the progress of atherosclerosis especially in women. Actually, cardiac metabolism and the burden of risk factors differ by gender (29, 30), gender may have an influence on both the development and progression of CAD and on the pattern of compositional plaque progression, and women have a higher prevalence of mortality from CAD than men (31). Thus, the role of BHB in the development and progression of CAD in women merits further investigation.

Traditionally, severe ketosis, or ketoacidosis, is known as a harmful event. Ketone bodies are synthesized in the liver from acetyl-coenzyme-A derived primarily from fatty acid β-oxidation and are transported to extrahepatic tissues for terminal oxidation as a source for adenosine triphosphate generation at the expense of glucose oxidation (32). Myocardial ischemia occurs when coronary blood flow is inadequate, and hence, the oxygen supply to the myocardium is not sufficient to meet the oxygen demand. Ischemia elicits disturbances in the balance between energy metabolism and cardiac function (33). It seems that hepatic ketogenesis is activated and cardiac utilization is not enhanced, the concentration of blood ketone bodies is highly increased in patients with ischemic heart disease (29, 33). In addition, people suffering from DM not only have increased production of ketones, their ketone clearance is also decreased significantly, because of low insulin levels, and decreased activities of succinyl-coenzyme-A: 3 oxoacid coenzyme-A-transferase and β-hydroxybutyrate dehydrogenase, which all contribute to an increased level of ketones (34). DM is a major risk factor for CAD, and many patients with DM have CAD, which is frequently associated with insulin resistance; a similar scenario has been noted for patients with CAD with an increased level of BHB (35, 36). A clinical study also supports this concept: patients with CAD have deficits in metabolic fuel extraction, with reduced extraction of fatty acid and ketone compared with that in controls (37). What's more, an increased level of BHB has an effect on upregulating expression of intercellular adhesion molecule-1 in endothelial cells (38), increasing the secretion of tumor necrosis factor-alpha in cultured U937 monocytes (39), and promoting lipid peroxidation levels in patients with DM (40). These phenomena would be implicated in the development of vascular disease and atherosclerosis (34).

However, recent evidence from experimental studies and clinical research has uncovered a protective role for ketones in cardiovascular disease. BHB infusion has been shown to specifically improve working memory performance in patients with T2DM in the absence of changes in global cognition (41). Even in healthy people, exogenous administration of ketones has been shown to improve the cardiac function (42). In addition, data from animal experiments have shown that ketones aid the prevention and treatment of heart failure by reducing inflammation (43), normalizing myocardial adenosine triphosphate production (44), and ameliorating pathologic cardiac remodeling and dysfunction (45). A hepatocyte-macrophage "AA shuttle" has been shown to coordinate the fibrogenic response to hepatic injury *via* mitochondrial metabolism in tissue macrophages after high fat diet-induced fibrosis (46). Furthermore, BHB has been found to protect the heart from ischemia/reperfusion injury (47) and attenuate atherosclerosis (48) in different mice models. Indeed, ketone bodies possess various capabilities and participate in multiple cellular processes (49), especially in cardiovascular disease, further studies to ascertain the action and specific mechanism of BHB in CAD are warranted.

During fast, DM and other pathological conditions, ketone bodies are all increased, the blood levels of BHB can be used to reflect ketotic state of a patient and American Diabetes Association prefers quantitative determination of blood BHB for the diagnosis and follow-up of ketoacidosis (50, 51). In ketone bodies, BHB is the most abundant in mammals. BHB has been found at a higher concentration than that of AA and acetone in subjects of DM and myocardial infarction. In cohort studies, BHB is the ketone body studied most often (21, 34, 52). Therefore, we focused only on BHB in this study, which is one limitation. The serum level of acetone is very low and the potential role of acetone in CAD is poorly known. AA is the central ketone body, and the other ketone bodies are derived from it. An increased level of AA has a role in oxidative modification of LDLs and then causes impaired cholesterol uptake and its deposition in the arterial wall and atherosclerosis in patients with DM (53). AA can also increase interleukin-6 secretion in U937 monocytes (54), upregulate expression of intercellular adhesion molecule-1 (55), facilitate the activity of NADPH oxidase (56), and promote glutathione depletion (57) in human umbilical vein endothelial cells. Those actions aggravate the inflammatory response, monocyte adhesion, and oxidative stress and contribute to the initiation and progression of CAD.

In this study, the control group already had high levels of cardiovascular risk factors and these people did not represent a true healthy control, which may have led to underestimation of the association between the metabolites and CAD and limited the power of this study. This is another limitation of the study.

Third, we did not adequately address that BHB as a biomarker for CAD can be an adaptive response that lack of enough evidence. Additionally, this study was a cross-sectional study, and we only addressed the association of BHB with CAD, this association does not inform causality. Therefore, further

studies are necessary and the findings need to be confirmed in future prospective analysis. This is also a limitation of our study.

CONCLUSION

The circulating level of BHB was independently associated with CAD severity, and a gender-related difference in this association was documented. Further studies are needed to determine the exact role of BHB in CAD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Beijing Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HM, FJ, and XY designed the research and drafted the article. HM, RY, and SW measured BHB in serum, performed the biochemical assays, and processed the data. WZ and XW performed coronary angiography and calculated the Gensini score. HL, JD, and WC calculated the number of stenosed vessels and regions. FJ and XY revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Beijing Natural Science Foundation (7214250), the National Key R&D Program of China (2020YFC2008304), the Non-profit Central Research Institute Fund of the Chinese Academy of Medical Sciences (2019TX310001), and the Beijing Hospital Special Fund Project (BJ-2019-184).

ACKNOWLEDGMENTS

The authors thank Ranran Zhang and Ziyun Li for their excellent technical assistance.

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frontiers in Nutrition

SYSTEMATIC REVIEW
published: 01 March 2022
doi: 10.3389/fnut.2022.812294

Lipid-Lowering Efficacy of the Capsaicin in Patients With Metabolic Syndrome: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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OPEN ACCESS

Edited by:
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Maastricht University, Netherlands

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

Received: 12 November 2021
Accepted: 31 January 2022
Published: 01 March 2022

Citation:
Jiang Z, Qu H, Lin G, Shi D, Chen K and Gao Z (2022) Lipid-Lowering Efficacy of the Capsaicin in Patients With Metabolic Syndrome: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Front. Nutr. 9:812294. doi: 10.3389/fnut.2022.812294

Background: Patients with metabolic syndrome (MetS) have increased cardiovascular risk. Capsaicin (CAP) has been shown to reduce lipids, but efficacy for patients with MetS is unknown.

Methods: A systematic review was performed according to PRISMA guidelines, to compare the effects of CAP against a placebo. Differences in the weight mean difference (WMD) with 95% confidence intervals (95% CI) were then pooled using a random effects model.

Results: Nine randomized controlled trials including 461 patients were identified in the overall analysis. CAP significantly decreased total cholesterol (TC) (WMD = −0.48, 95% CI: −0.63 to −0.34, I^2= 0.00%) and low-density lipoprotein cholesterol (LDL-C) (WMD = −0.23, 95% CI: −0.45 to −0.02, I^2 = 68.27%) among patients with MetS. No significant effects of CAP were found on triglycerides (TG) or high-density lipoprotein cholesterol (HDL-C) (WMD = −0.40, 95% CI: −1.50 to 0.71, I^2 = 98.32%; WMD = −0.08, 95% CI: −0.21 to 0.04, I^2 = 86.06%). Subgroup analyses indicated that sex and intervention period were sources of heterogeneity. The results revealed that CAP decreased TG levels in women (WMD = −0.59, 95% CI: −1.07 to −0.10) and intervention period <12 weeks (WMD = −0.65; 95% CI: −1.10 to −0.20). And there was no potential publication bias according to funnel plot, Begg' test and Egger regression test.

Conclusions: CAP supplementation is a promising approach to decreasing TC and LCL-C levels in patients with MetS. However, short-term (< 12 weeks) use of CAP in women may also reduce TG levels.

Systematic Review Registration: Identifier: CRD42021228032.

Keywords: capsaicin, lipid levels, metabolic syndrome, randomized controlled trials, meta-analysis

INTRODUCTION

Metabolic syndrome (MetS) represents a cluster of metabolic risk factors, including dyslipidemia (raised triglycerides and lowered high-density lipoprotein cholesterol), obesity (especially abdominal obesity), elevated blood pressure, and dysglycemia (1). It is estimated that patients with MetS are twice as likely to develop cardiovascular disease (CVD) in the next 5 to 10 years compared to those without the syndrome, posing a huge burden on global health and the economy (1). The management of each MetS component has been proved to be effective in reducing the incidence of CVD and reducing the risk for major adverse cardiovascular events (MACE) (2).

Dyslipidemia is an important component of the contemporary consensus definition of MetS and also a major risk factor for CVD, which is a leading cause of death worldwide (3–7). Epidemiological data revealed that in addition to decreased levels of high-density lipoprotein cholesterol (HDL-C), elevated levels of low-density lipoprotein cholesterol (LDL-C), triglycerides (TG) and total cholesterol (TC) are also independent predictors of the risk of CVD (8–12). Therefore, lowering blood lipid levels is of great significance in the management of MetS.

Recently there has been rising attention to single dietary components or natural compounds due to the fact they are inexpensive, readily available, and have beneficial effects in the treatment of various diseases such as MetS. For example, studies have shown that daily intake of red yeast rice can reduce LDL-C levels from 15% to 25% (13). In addition, glucomannan (14, 15), probiotics (14, 16), garlic (17), berberine (18), omega-3 (ώ-3) and fatty acids (19), also have a positive effect on improving MetS.

Capsaicin (CAP) (trans-8-methyl-N-vanillyl-6-nonenamide) is the main component in red chili peppers that give chili peppers their spice, which belongs to the Solanaceae family (20, 21). Previous studies have demonstrated that CAP has antioxidant activity (22), analgesic activity (23, 24), and can aid in lowering rates of obesity (25). The lipid-lowering effects of the CAP remain controversial. Numerous studies have shown that CAP can reduce TC, TG, LDL-C and increase HDL-C levels (20, 26, 27). However, a study by Urbina et al. (28) showed that CAP supplementation does not affect serum TG, TC, LDL-C levels, but does decrease HDL-C levels in serum, which is not consistent with evidence reported in previous studies. There have been no systematic reviews or meta-analyses to summarize the available data. Therefore, we carry out a systematic review and meta-analysis to assess the effects of CAP on lipids in patients with MetS.

METHODS

Data Sources and Searches

The meta-analysis protocol was registered on PROSPERO (CRD42021228032, https://www.crd.york.ac.uk/prospero/), and conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (29). PubMed, EMBASE, MEDLINE, and the Cochrane Library were systematically searched from inceptions to February 1, 2021. MeSH terms and free words were used reasonably through the characteristics of literature databases. Detailed search strategies are listed in **Supplementary Material 1**.

Selection Criteria

The selected studies were screened using the PICOS (participants, interventions, comparisons, outcomes, and study design) criteria:

1. Population: patients with MetS were diagnosed according to recognized diagnostic criteria (IFD, WHO, or NCEP-ATP III) (1).
2. Intervention: circulating CAP supplements, dietary CAP or CAP-related supplements.
3. Comparison: no use of CAP or CAP-related supplements categories of exposure.
4. Outcome: lipid parameters (TC, TG, HDL-C and LDL-C).
5. Study design: randomized controlled trials (RCTs).

Data Extraction

Two reviewers independently extracted data using standardized data extraction forms. Any disagreements would be resolved by consensus or consulting the third reviewer. If the information is incomplete or unclear, when necessary, the author was contacted. Data extraction included study design type and participant characteristics (age, sex, and country), intervention and placebo details (sample size, study duration, CAP dose, and controls group used). Outcomes included lipid levels (TC, TG, HDL-C, LDL-C).

Data Synthesis and Analysis

Data for the effect of continuous outcomes were extracted as the weight mean difference (WMD), which represents the mean difference between the intervention and control groups in standard deviation units, with 95% confidence intervals (CIs). Clinical heterogeneity was assessed by I^2 and Chi-squared (χ^2) test at $\alpha = 0.1$. When Cochrane's test showed that $I^2 < 50\%$, there was no statistical heterogeneity among the studies, and therefore a fixed-effect model (with inverse variance method) was used for meta-analysis. If $I^2 \geq 50\%$, there was statistical heterogeneity among the studies, and as such the random effects model (DerSimonian and Laird method) was used to analyze the causes of the heterogeneity. Sensitivity analysis was performed by using the leave-one-out method and/or a subgroup analysis according to that factor. Publication bias was examined using funnel plots, the Begg' test and the Egger regression test. Statistical analysis was conducted using Review Manager, Version 5.3 (Cochrane Collaboration, Oxford, UK) and Stata version 16.0 (Stata Corp., College Station, TX). The protocol for the present meta-analysis was registered on the international prospective register of systematic reviews (PROSPERO, CRD42021228032).

Quality Assessment

The recommendations of the Cochrane Intervention Systems Review Manual (updated September 2009) were used to evaluate

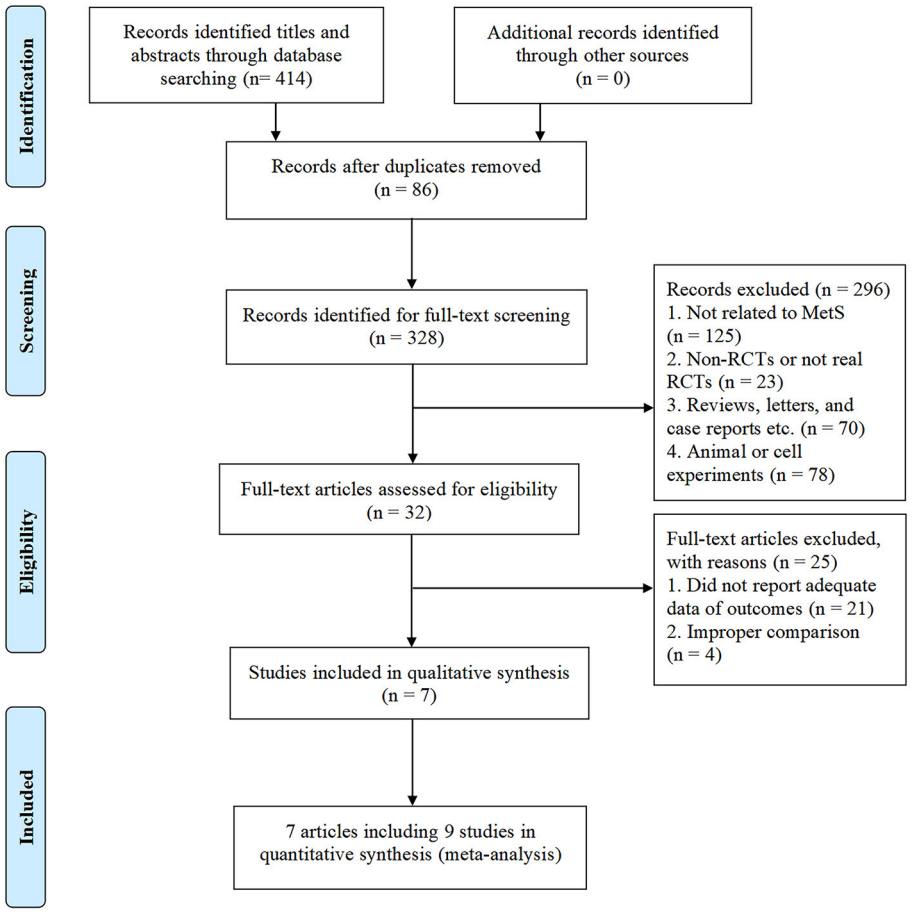

FIGURE 1 | PRISMA 2009 flow diagram for study selection process.

the risk of bias. Studies were stratified as high risk, low risk, or unclear risk. The risk of bias included the following six evaluation criteria: generation of a random sequence, allocation concealment, use of blind method, integrity of result data, and selection of reporting outcome.

RESULTS

Data Sources and Search Results

As shown in **Figure 1**, a total of 414 potentially relevant studies were identified in our initial literature search. We evaluated 328 potentially related articles for eligibility after removing the duplicates of 86 studies from different databases. After screening the titles and abstracts of these studies, we excluded 296 studies for the following reasons: subject was not related to MetS ($n = 125$); study not a RCTs ($n = 23$); reviews, letters, and case reports etc. ($n = 70$); animal or cell experiments (n = 78). Of the retrieved studies, a total of 32 met our inclusion criteria. However, 25 studies were excluded because they did not have sufficient data of outcomes (n = 21) or improper comparison ($n = 4$). Finally, seven studies entered into our meta-analysis, involving 461 patients (227 [49.2%] in the CAP group, 234 [50.8%] in the control group). Three studies (28, 30, 31) were conducted and published in full in the United States, one in Korea (32), one in the Netherlands (33), one in Iran (34), and one in China (35). The average sample size of the trials was 51 participants (ranging from 36 to 87 participants per trial). The course of treatment fluctuated between 4-weeks and 13-weeks. In one of the included trials (28), two different doses of CAP (2 and 4 mg) were administered, hence we considered it as two separate studies. In another trial (30), results were divided by sex despite the same intervention, so we also treated this study as two separate studies. The characteristics of the included trials are shown in **Table 1**.

Quality of Included Studies

The assessment of the risk of bias for all included trials is shown in **Figures 2A,B**. All of the included studies reported randomly assigned participants, two (34, 35) of them described methodological operations for random sequence generation (using a computer-generated list of random numbers), and the remaining studies mentioned they were "random," did not report it in detail. Two studies (28) reported the method of allocation

TABLE 1 | Characteristics of included capsaicin RCTs for MetS.

References	Number of participants (T/C)	Population	Age(years) (T/C)	Intervention group (T/C)	Duration (weeks)	Main outcomes
Arent et al. (30) (United States)	18/18	Overweight men	18–50	METABO (4 capsules/d)/Placebo	8	TC, LDL-C, HDL-C, TG
Arent et al. (30) (United States)	18/18	Overweight women	18–50	METABO (4 capsules/d)/Placebo	8	TC, LDL-C, HDL-C, TG
Cha et al. (32) (Korea)	30/30	Overweight subjects	19–65	KCJ (3,200 mg/d)/Placebo	12	TC, LDL-C, HDL-C, TG
Lejeune et al. (33) (The Netherlands)	40/47	Overweight subjects	18–60	Capsaicin (135 mg/d)/Placebo	13	TG
Lopez et al. (31) (United States)	27/18	Overweight subjects	21–45	METABO (4 capsules/d)/Placebo	8	TC, LDL-C, HDL-C, TG
Taghizadeh et al. (34) (Iran)	25/25	Overweight women	18–50	Capsaicin (25 mg/d)/Placebo	8	TC, LDL-C, HDL-C, TG
Urbina et al. (28) (United States)	27/28	Overweight subjects	18–56	Capsaicinoid (2 mg/d)/Placebo	12	LDL-C, HDL-C, TG
Urbina et al. (28) (United States)	22/28	Overweight subjects	18–56	Capsaicinoid (4 mg/d)/Placebo	12	LDL-C, HDL-C, TG
Yuan et al. (35) (China)	20/22	Women with gestational diabetes mellitus	(31.1 ± 4.4)/ (29.8 ± 4.5)	Capsaicin (5 mg/d)/Placebo	4	TC, LDL-C, HDL-C, TG

RCTs, randomized controlled trials; MetS, metabolic syndrome; T/C, treatment/control; METABO, consisted of raspberry ketone, caffeine, capsaicin, garlic organosulfur compounds, gingerols, shogaols, Citrus aurantium and related alkaloids, B vitamins, and chromium; KCJ, a fermented soybean-based red pepper paste, containing powdered red pepper 11.9g; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglycerides.

concealment (by using numbered bottles). All trials were double-blind, of these, six studies (28, 30, 34, 35) described the specific blinding of participants and personnel, and detailed the blinding of the outcome assessment. Clinical trial registration and pre-published protocol were reported by the authors of eight studies (28, 30–32, 34, 35), but it was not feasible to effectively assess whether there was a risk of selective reporting bias.

Effects of CAP on Lipid Levels

Total Cholesterol

Six studies (30–32, 34, 35) (138 patients in the CAP supplementation group vs. 131 patients in the placebo group) evaluated the effects of CAP supplementation on TC levels among patients with MetS. The overall results of the random-effect model exhibited a favorable effect on reducing TC levels following CAP supplementation (WMD = −0.48; 95% CI: −0.63 to −0.34; $P = 0.00$; $I^2 = 0.00\%$) (**Figure 3A**). The results of leave-one-out sensitivity analysis support the robustness of our findings (**Figure 4A**). Inter-group heterogeneity changed after subgroup analysis based on race, sex, dose and duration of CAP supplementation, but there was no significant difference in TC levels before and after the subgroup analysis (**Figure 5**).

Triglycerides

Nine studies (28, 30–35) (227 patients in the CAP supplementation group vs. 234 patients in the placebo group) compared the effects of CAP and placebo on TG levels in patients with MetS. The results showed that CAP had no significant effect on TG levels compared with placebo (WMD = −0.40; 95% CI: −1.50 to 0.71; $P = 0.48$; $I^2 = 98.32\%$) (**Figure 3B**). Sensitivity analysis showed that the results did not change before and after sensitivity analysis (**Figure 4B**).

Figure 5 summarizes the subgroup analysis results of the effects of CAP on TG levels in patients with MetS. TG levels in women were significantly decreased after CAP supplementation (WMD = −0.59; 95% CI: −1.07 to −0.10). Furthermore, serum TG levels decreased after CAP supplementation for <12 weeks (WMD = −0.65; 95% CI: −1.10 to −0.20).

High-Density Lipoprotein Cholesterol

Eight studies (28, 30–32, 34, 35) (187 patients in the CAP supplementation group vs. 187 patients in the placebo group) reported the effects of CAP on serum HDL-C. The random effects model showed that the pooled mean effect size was not significant (WMD = −0.08; 95% CI: −0.21 to 0.04; $P = 0.20$), with significant heterogeneity (I^2= 86.06%) (**Figure 3C**). The results of the sensitivity analysis were not altered after excluding the individual trials (**Figure 4C**). The heterogeneity changed with differences in race, gender, intervention time groups and dose, but there were no significant differences before and after subgroup analysis (**Figure 5**).

Low-Density Lipoprotein Cholesterol

Eight studies (28, 30–32, 34, 35) indicated beneficial results from taking CAP supplementation ($n = 187$), as seen by a reduction in serum LDL-C levels, compared to that with placebo group ($n = 187$) (WMD = −0.23; 95% CI: −0.45 to −0.02, $P = 0.03$; $I^2 = 68.27\%$) using the random-effect model (**Figure 3D**). Sensitivity analysis showed no significant change in the overall estimate of effect size after the elimination of individual trials (**Figure 4D**). The subgroup analysis of the LDL-C levels showed no significant differences within subgroups based on the dose of CAP, duration of CAP use, race or gender (**Figure 5**).

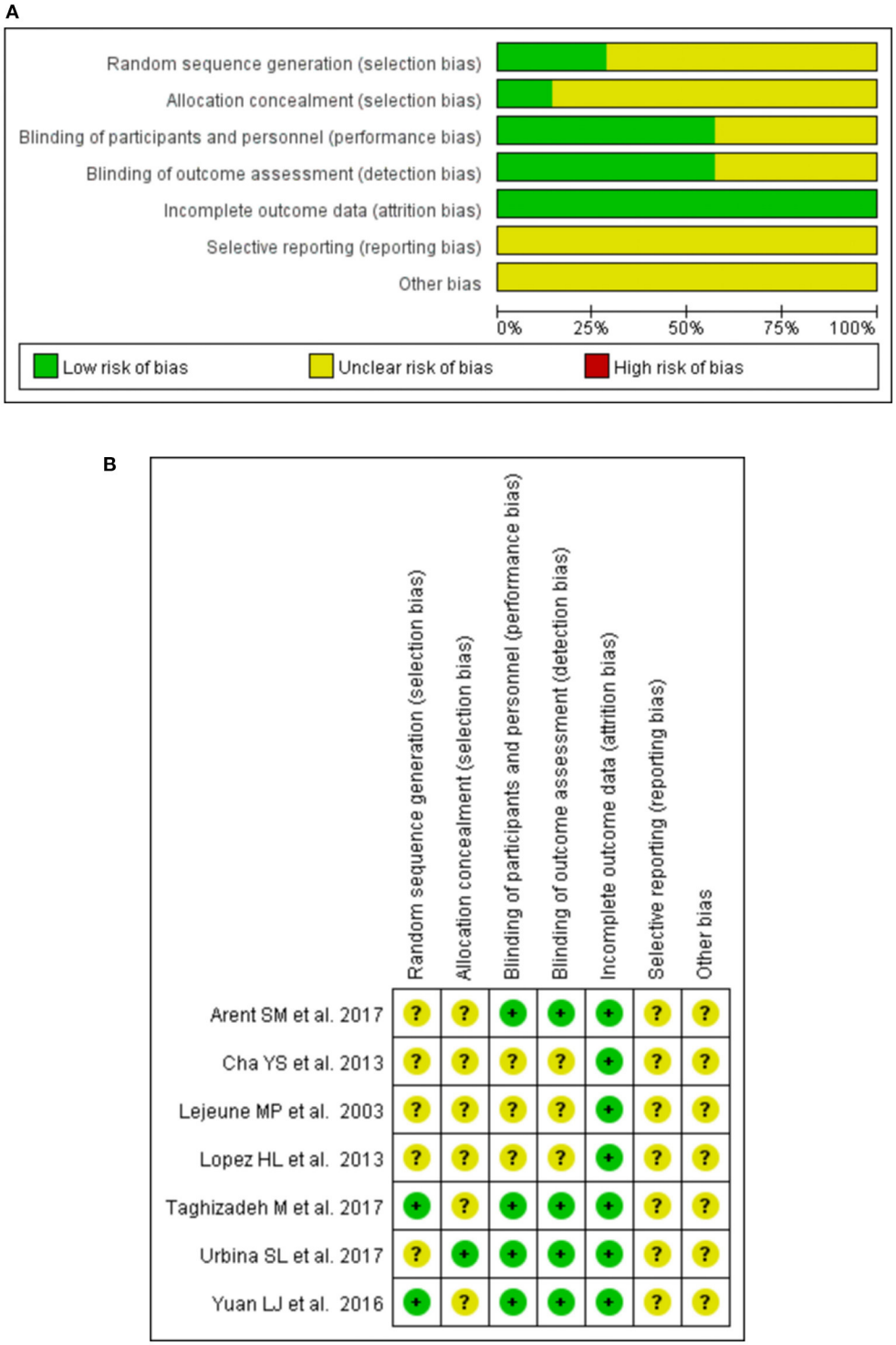

FIGURE 2 | Graph of bias risk assessment for included studies by the Cochrane Collaboration's tool. **(A)** Risk of bias graph. **(B)** Risk of bias summary.

Publication Bias

A funnel plot, Begg' test and Egger regression test were used to evaluate the effects of CAP on TC levels, and no publication bias was found (Egger regression test, coefficient, −0.42; 95% CI, −1.72 to 0.89; $P = 0.43$). For the effects of CAP on TG and HDL-C levels, there was no evidence of publication bias according to

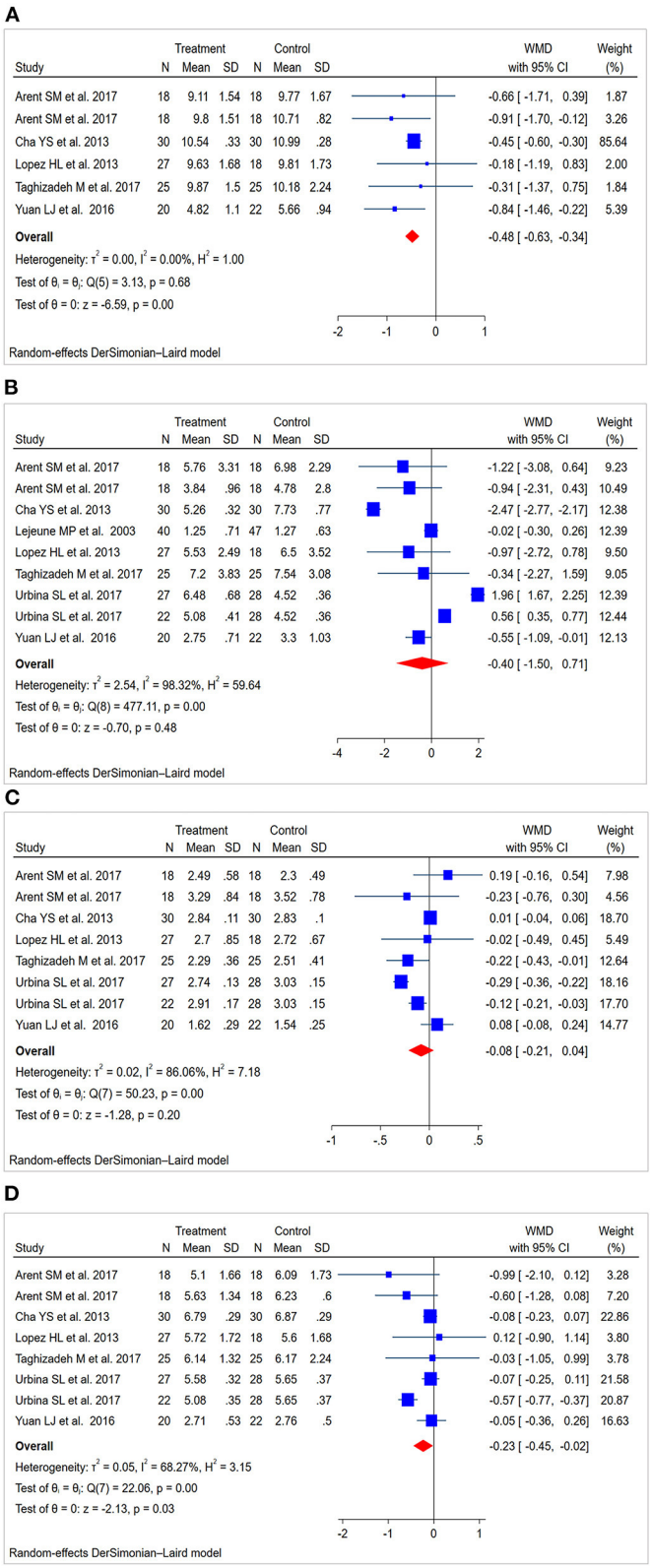

FIGURE 3 | Forest plot for lipid levels: capsaicin vs. placebo (random-effect model). **(A)** Funnel plot for total cholesterol (TC). **(B)** Funnel plot for triglycerides (TG). **(C)** Funnel plot for high-density lipoprotein cholesterol (HDL-C). **(D)** Funnel plot for low-density lipoprotein cholesterol (LDL-C).

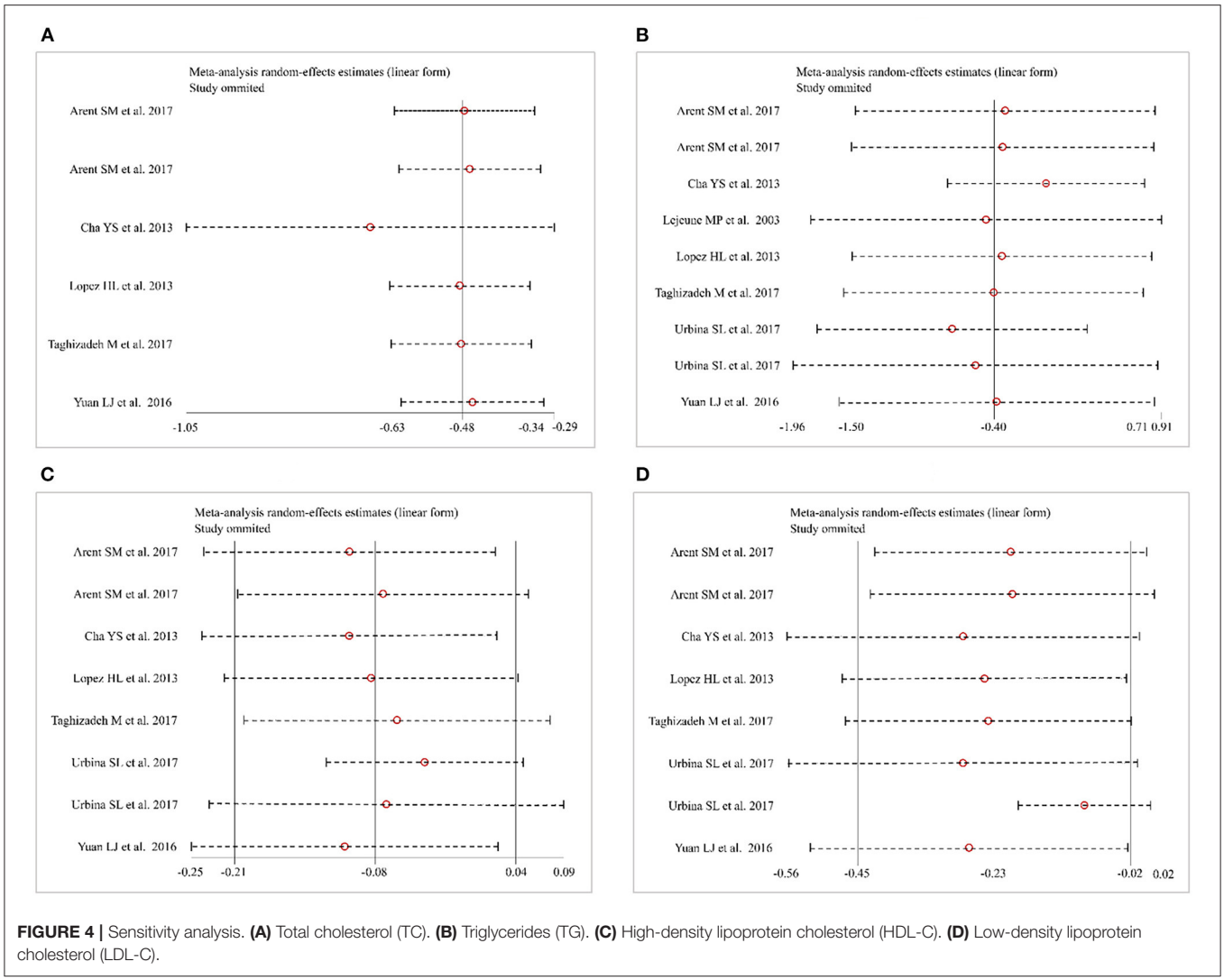

FIGURE 4 | Sensitivity analysis. **(A)** Total cholesterol (TC). **(B)** Triglycerides (TG). **(C)** High-density lipoprotein cholesterol (HDL-C). **(D)** Low-density lipoprotein cholesterol (LDL-C).

the results of a funnel plot, Begg' test and Egger regression test (coefficient, −2.15; 95% CI, −13.08 to 8.79; $P = 0.66$; coefficient, −0.07; 95% CI, −4.22 to 4.08; $P = 0.97$). Lastly, no potential publication bias for LDL-C was identified according to funnel plot, Begg' test and Egger regression test (coefficient, −0.59; 95% CI, −3.39 to 2.21; $P = 0.62$) (**Figure 6**).

DISCUSSION

To the best of our knowledge, this is the first systematic review and meta-analyses of RCTs evaluating the effects of CAP supplementation on lipid levels among patients with MetS. A total of nine studies (involving 461 patients) were included in this meta-analysis. The main finding of our meta-analysis was that CAP supplementation may have beneficial therapeutic effects in reducing TC and LDL-C levels. No significant effects of CAP were found regarding TG and HDL-C levels. However, subgroup analyses revealed that CAP reduced TG levels in women and at <12 weeks of the intervention.

Many small studies have reported that CAP might decrease lipid levels among patients with MetS. This effect has also been proposed CAP as one of the agents treating dyslipidemia. In most instances, however, the studies regarding CAP and lipid levels had methodological limitations (mainly owing to small numbers of patients included), leaving this hypothesis unproven. Our meta-analysis pooled all the RCTs regarding the effects of CAP on lipid levels, and the results showed that CAP supplementation may be beneficial in reducing TC, and LDL-C. Overall, CAP may be a complementary approach in the patients with dyslipidemia who cannot be treated with statins or other LDL-C-lowering therapies.

It is essential to actively manage risk factors for MetS, such as dyslipidemia. Studies have shown that lowering atherogenic cholesterol levels can effectively reduce morbidity and mortality of CVD (36–38). The ATP-III guidelines emphasized that LDL-C reduction is the primary target of lipid management in MetS, and low HDL-C and TG are secondary targets (39). Large LDL-C reductions, such as 2 to 3 mmol/L (77.4–116.1 mg/dL), can reduce the relative risk of CVD by 40–50% (37). The

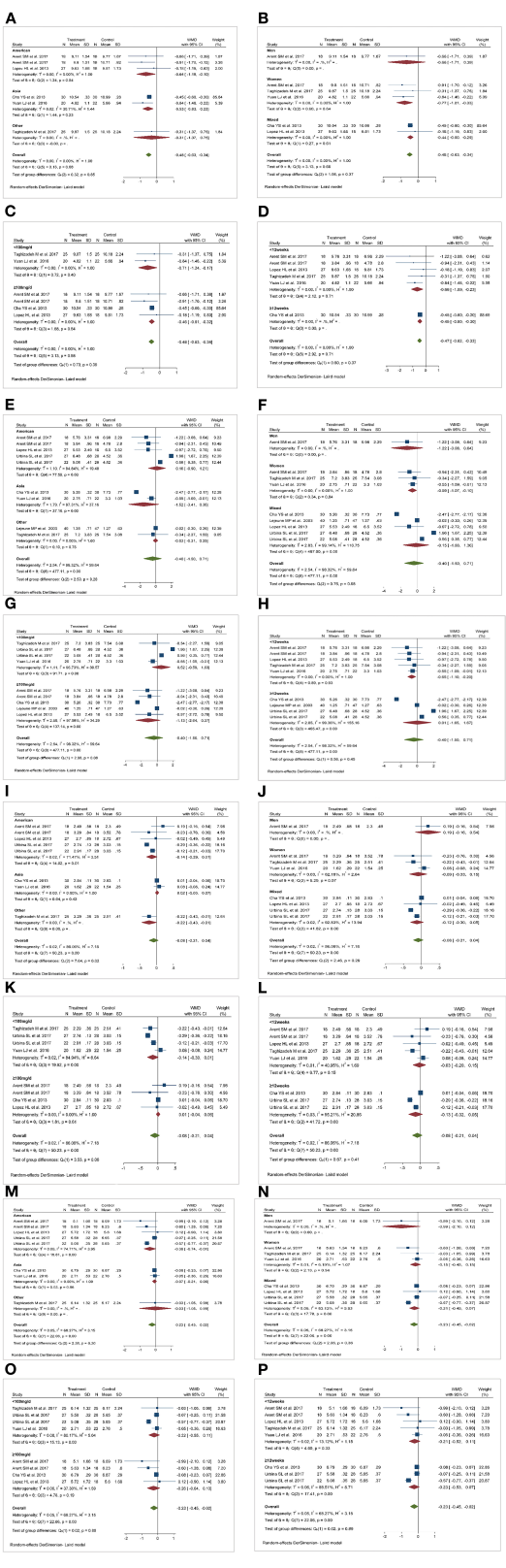

FIGURE 5 | Results of subgroup analysis. **(A,E,I,M)** Effect of capsaicin (CAP) on total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol *(Continued)*

FIGURE 5 | (HDL-C), and low density lipoprotein cholesterol (LDL-C) in different races. **(B,F,J,N)** Effect of CAP on TC, TG, HDL-C and LDL-C in different genders. **(C,G,K,O)** Effect of dose of CAP on TC, TG, HDL-C and LDL-C. **(D,H,L,P)** Effect of duration of CAP intervention on TC, TG, HDL-C and LDL-C. WMD, weighted mean difference.

availability and use of lipid-lowering medication, such as statin therapy and ezetimibe, or proprotein convertase subtilisin-kexin type 9 (PCSK9) inhibitors, significantly reduces lipid levels. In turn, reducing the number of patients with hyperlipidemia and therefore the risk of an acute cardiovascular event (40, 41). Statin therapies also generally reduced the risk of an acute cardiovascular event by 25 to 45%, which was noted over 5 years of follow-ups (6). However, despite the existence of effective treatments and well-established treatment guidelines, lipid abnormalities are still very common in adults, with an estimated 53% (105.3 M) of U.S. adults having at least one lipid abnormality, 27% (53.5 million) having high LDL-C, 23% (46.4 million) having low HDL, and 30% (58.9 million) with high TG (42). A clinical guideline for the management of dyslipidemia conducted by Downs and O'Malley showed that 10 to 20% of patients using statins experienced muscle-related symptoms (43). Hereby, our meta-analysis showed that CAP can improve dyslipidemia and has the advantages of a lower price and easy availability. For these reasons, CAP supplementation as an adjunct nutritional therapy for the treatment of dyslipidemia in MetS patients is easy to implement and may lead to better compliance in patients with MetS.

There are several mechanisms which could potentially explain the effects of CAP on lipid levels. It has been shown that CAP plays a role in countering the detrimental effects of a high-fat diet, such as glucose intolerance and/or hypercholesterolemia. It does this primarily by increasing the expression of metabolically important thermogenic genes, including uncoupling protein 1 (UCP-1), bone morphogenetic protein 8b (BMP 8b), Sirtuin1 (SIRT-1), peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α), and positively regulated domain containing zinc finger protein 16 (PRDM-16) (44). CAP activates its receptor transient receptor vanilloid subtype 1 (TRPV1), which can activate sympathetically-mediated brown adipose tissue (BAT) thermogenesis and reduce body fat (45). In addition, CAP inhibits the expression of peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT-enhancer-binding protein-α (C/EBP-α) and leptin; but induces up-regulation of adiponectin at the protein level. Therefore, it can effectively induce apoptosis of 3T3-L1 pre-adipocytes and adipocytes; and inhibit adipogenesis *in vitro* (46).

The meta-analysis has a number of limitations, of which heterogeneity across the included studies is the most important. We conducted a sensitivity and subgroup analysis to determine the factors (race, gender, dose, and duration, etc.) that might cause large heterogeneity and thus to explore the source of heterogeneity. Heterogeneity changed after analysis, but the overall results were stable and reliable. In clinical practice,

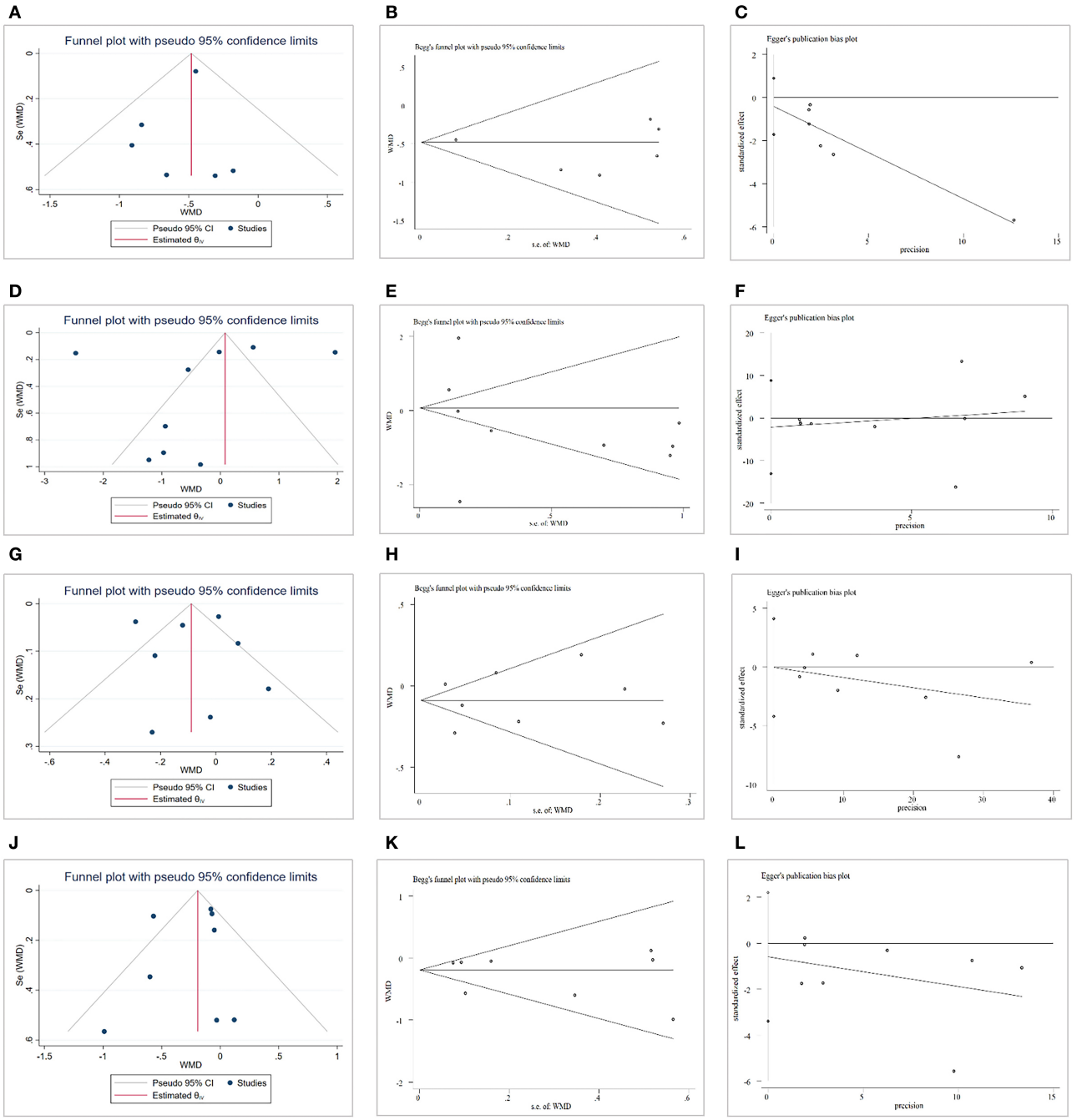

FIGURE 6 | Publication bias. **(A)** Funnel plot for total cholesterol (TC). **(B)** Begg' test for TC. **(C)** Egger test for TC. **(D)** Funnel plot for triglycerides (TG). **(E)** Begg' test for TG. **(F)** Egger test for TG. **(G)** Funnel plot for high-density lipoprotein cholesterol (HDL-C). **(H)** Begg' test for HDL-C. **(I)** Egger test for HDL-C. **(J)** Funnel plot for low-density lipoprotein cholesterol (LDL-C). **(K)** Begg' test for LDL-C. **(L)** Egger test for LDL-C.

disparate formulations and delivery routes of CAP may affect the results, which should be noted in future research. Second, this meta-analysis was limited by the small number of studies and the small size of existing RCTs. Therefore, additional studies are needed to confirm our findings and to expand our understanding of CAP.

In conclusion, the findings of this meta-analysis demonstrated that CAP supplementation is effective in improving lipid levels and should be considered in the prevention and treatment of MetS. Large-scale, high-quality, and precise RCTs are needed to further demonstrate the effects of CAP on lipid levels, and we will follow up on this study.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ZJ and HQ were involved in the conception and design. ZJ, HQ, GL, and ZG were involved in literature retrieval, data collection, extraction and analysis. KC and DS were involved in systematic review and meta-analysis. KC and ZG are responsible for the final approval of the version to be published. All authors revised and approved the final version of the manuscript.

FUNDING

This work was financially supported by the National Science and Technology Major Project of the Ministry of Science and Technology of China (2019ZX09201005-002-006), Beijing Traditional Chinese Medicine Science and Technology Development Fund Project (JJ-2020-79), and the Capital Health Research and Development of Special (2018–1–4171).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.812294/full#supplementary-material

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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 Nutrition

ORIGINAL RESEARCH
published: 11 April 2022
doi: 10.3389/fnut.2022.848983

Maternal High-Fructose Intake Activates Myogenic Program in Fetal Brown Fat and Predisposes Offspring to Diet-Induced Metabolic Dysfunctions in Adulthood

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Excess dietary fructose intake is a major public health concern due to its deleterious effect to cause various metabolic and cardiovascular diseases. However, little is known about the effects of high-fructose consumption during pregnancy on offspring metabolic health in adulthood. Here, we show that maternal consumption of 20% (w/v) fructose water during pregnancy does not alter the metabolic balance of offspring with a chow diet, but predisposes them to obesity, fatty liver, and insulin resistance when challenged by a high-fat diet. Mechanistically, diet-induced brown fat reprogramming and global energy expenditure in offspring of fructose-fed dams are impaired. RNA-seq analysis of the fetal brown fat tissue reveals that the myogenic pathway is predominantly upregulated in the fructose-treated group. Meanwhile, circulating fructose level is found to be significantly elevated in both fructose-fed dams and their fetuses. Importantly fructose gavage also acutely activates the myogenic program in mice brown fat. Together, our data suggest that maternal high-fructose intake impairs fetal brown fat development, resultantly attenuates diet-induced thermogenesis and causes metabolic disorders in adult offspring probably through inducing myogenic signature in brown fat at the fetal stage.

Keywords: maternal high-fructose intake, offspring, brown fat, myogenic pathway, metabolic dysfunctions

OPEN ACCESS

Edited by:
Ellen E. Blaak, Maastricht University, Netherlands

Reviewed by:
David A. Fields, University of Oklahoma Health Sciences Center, United States
Tongxing Song, Huazhong Agricultural University, China

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

Received: *05 January 2022*
Accepted: *28 February 2022*
Published: *11 April 2022*

Citation:
Wang P, Wu T, Fu Q, Liao Q, Li Y, Huang T, Li Y, Zhou L and Song Z (2022) Maternal High-Fructose Intake Activates Myogenic Program in Fetal Brown Fat and Predisposes Offspring to Diet-Induced Metabolic Dysfunctions in Adulthood. Front. Nutr. 9:848983. doi: 10.3389/fnut.2022.848983

INTRODUCTION

The global epidemic of obesity and type 2 diabetes mellitus (T2DM) remains a major public health issue. Numerous studies have demonstrated that the nutritional environment that an individual is exposed to before birth is a critical determinant of their risk of developing obesity and metabolic diseases in their later life (1, 2). Exposure to unbalanced maternal nutrition, particularly overnutrition, is associated with altered development of key physiological systems, which predisposes them to adult onset of non-communicable diseases, such as obesity, T2DM, fatty liver, and cardiometabolic disease (1, 3–5).

Among the specific nutritional factors, excess fructose consumption is increasingly considered as a major contributor to the emerging epidemics of obesity and the associated metabolic diseases (6–9). Fructose is a simple sugar found naturally in honey, fruit, and some vegetables. Over the last decade, fructose consumption has increased significantly and has become the most typical sugar

consumed by man, owing to its use as a sweetener in processed foods and soft drinks in the form of sucrose or high-fructose corn syrup (9). The increasing prevalence of soft-drinks consumption results in a higher incidence of pregnant women having excess fructose intake. However, so far the consequence and mechanism of maternal high-fructose intake on offspring metabolic health in later life are still unclear, albeit existing of few animal studies (10–14).

In adult obesity or obesity "programmed" by early-life insults, adipose tissue dysfunction is considered to be an important contributor to the metabolic alterations in humans and rodents (15). There are mainly two types of adipose tissues, namely, white adipose tissue (WAT) and brown adipose tissue (BAT) (16), which have distinct characteristics and functions. WAT is responsible for energy storage in the form of triglyceride (TG), while BAT dissipates energy in the form of heat because of the presence of abundant mitochondria and uncoupling protein 1 (UCP1) (16). Brown adipocytes derive from a common pool of precursors with skeletal muscle during early development, with the divergence occurring between embryonic day (E) 9.5 and E12.5 in mice (17). Disturbance of the divergence by genetic or nutritional manipulations leads to abnormal BAT development and results in offspring obesity and metabolic disorders in later life (18–21). However, up to date, there is a paucity of data examining the impacts of increased fructose intake during pregnancy and subsequent effects on fetal BAT development and offspring energy metabolism later on.

Therefore, this study was designed to reveal the impacts and potential mechanism of maternal high-fructose intake on offspring metabolic health, with an emphasis on fetal BAT development and its associated energy metabolism in adulthood. We have found that maternal high-fructose intake during pregnancy promotes a myogenic program in fetal BAT, resultantly impairs high-fat diet (HFD)-induced BAT thermogenesis and exacerbates HFD-induced obesity and metabolic disorders in adult offspring.

MATERIALS AND METHODS

Animal Studies

All animal studies were according to protocols approved by the Animal Ethics Committee of Guangxi University (GXU2021-123). Twelve-week-old C57BL/6J mice were purchased from the Guangxi Medical University (Nanning, China) and housed under controlled light and temperature conditions (12-h light/dark cycle; 22 ± 2°C). After 1 week's adaption, the mice were mated for one night. Success in mating was confirmed by the presence of a vaginal plug, and then pregnant mice were randomly assigned to either sterilized tap water or 20% (w/v) fructose (Sigma, F3510) water until delivery. At E18.5, a subset of pregnant mice was killed and the BAT of male fetuses was collected for further analysis. Fetal sex was identified by PCR (22). The remaining female mice were allowed to give birth. On the day of birth, litter sizes were balanced to five pups. All pups were weaned on postnatal day 21 and separated into two groups at 7 weeks old, one group was fed a regulated chow (10% kcal energy from fat) and the other group was fed a HFD (60% kcal energy from fat). To avoid confounding sexual effects, only male offspring were used in this study.

Glucose and Insulin Tolerance Test

A glucose tolerance test (GTT) was performed on 15-week-old mice after a fast for 15 h followed by an intraperitoneal injection of glucose (1.5 g/kg D-glucose). One week later, an insulin tolerance test (ITT) was performed after a fast for 5 h followed by an intraperitoneal injection of insulin (1 U/kg insulin). The blood glucose level was measured by tail bleeding at 0, 15, 30, 60, 90, and 120 min after glucose or insulin injection using a glucose meter.

Body Composition and Indirect Calorimetry Analysis

The fat mass and lean mass of the mice were analyzed once a month through NMR (Niumag QMR23-060H-I, Suzhou, China) following the instruction of the manufacturer. At 16–17 weeks of age, indirect calorimetry measurements were performed using the Promethion Metabolic Cage System (Sable Systems International, Las Vegas, NV, United States). Mice were acclimatized for 24 h in the Promethion system before the measurement was started. Instrument control and data acquisition were performed according to the instructions of the manufacturer. Raw data were processed using ExeData software (Sable Systems).

Measurement of Plasma and Hepatic Parameters

Triglyceride (TG) and total cholesterol (TC) content in plasma and liver tissues were measured by TG assay kit (A110-1-1, Nanjing Jiancheng Bioengineering Institute, China) and TC assay kit (A111-1-1, Nanjing Jiancheng Bioengineering Institute, China) according to the instructions of the manufacturer. For tissue samples, protein concentrations were measured by using the BCA protein quantitative assay kit (Beyotime Biotechnology, Shanghai, China), and the TG and TC data were expressed as μmol/g protein. Fructose level in plasma was determined by fructose assay kit (G0530W, Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China) by following the instruction of the manual and the optical density was evaluated at 450 nm by using the continuous spectrum microplate plate reader (Epoch) (Bio Tek Instruments, Inc.).

H&E Staining and Oil Red O Staining of Mouse Samples

Livers and adipose tissues were dissected and fixed in tissue-fixing liquid overnight at 4°C for the paraffin section and frozen section. The paraffin sections were stained with H&E and the frozen sections were stained with Oil Red O. Sample sectioning and staining were performed at the Wuhan Servicebio Technology Co., Ltd. (Wuhan, Hubei, China). Images were collected by light microscope (Biological microscope ML31, MSHOT, Guangzhou, China) and cell area was analyzed by ImageJ software.

RNA Extraction and Quantitative Real-Time PCR

RNA extraction from BAT tissues was performed by re-suspending 10–20 mg of frozen tissues in 1 ml Trizol (Life Technologies, United States), and lysed by using a tissue lyser (Qiagen, Germany) for 3 min at 30 Hz. Then, total RNA was isolated following Trizol manufacturer's instructions. The purity and concentration of the total RNA were determined by Tecan infinite M200 Pro (Grödig, Austria). cDNA was synthesized from 1 μg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, United States). Real-time qPCR was performed using a 2× RealStar Green Fast Mixture (GenStar, Beijing, China). All data were normalized to the *Tbp* level and analyzed following the $2^{-\Delta\Delta Ct}$ method. The sequences of primers used are listed in **Supplementary Table 1**.

RNA-Seq Analysis

Purified total RNA samples from E18.5 fetal BAT were sent to the Guangzhou Gene *Denovo* Biotech Co. Ltd. (Guangzhou, China) for library construction and sequencing by using the Illumina novoseq 6000 platform. The raw RNA-seq data were submitted to the Gene Expression Omnibus data repository[1] with the login number GSE193031. The procedure of data analysis is as follows: first, the high-quality sequences, which were filtered by Fastp (v0.23.2), were mapped to the mouse reference genome (mm10) by Bowtie2 (v2.0.0.5) and Tophat (v2.0.0). Then, the mapping readings were assembled and GTF files were generated by StringTie (v2.2.0). The GTF files were then merged into one file by Cuffmerge, from which the counts of each gene were extracted. Differentially expressed genes were analyzed based on the gene counts by using a RNA-seq processing tool DESeq2 and selected by p-value < 0.05 and fold change (FC) >1.3, and visualized using R tools ggplot2. For Gene Set Enrichment Analysis (GSEA), the normalized gene expression data were ranked according to the log2 FC and were visualized by ClusterProfiler (R package, version 3.18.1).

Statistical Analysis

The results are expressed as mean ± SD. Significance was estimated by unpaired Student's t-test (for two groups) or one-way ANOVA (for multiple groups). A probability of $p < 0.05$ was considered to be statistically significant. The statistical analysis and figures were prepared using GraphPad Prism 8.0.

RESULTS

High-Fructose Intake During Pregnancy Does Not Alter Body Weight but Causes Slight Lipid Metabolic Disorders in Female Mice

In this study, C57/BL6J mice were used as an animal model to evaluate the effects of maternal high-fructose consumption in gestation on offspring metabolic health. First, we observed that 20% (w/v) fructose water administration significantly increased the water consumption, but remarkably decreased the food intake, by dams during the course of gestation (**Figures 1A,B**). However, intriguingly, total energy consumption analysis showed that the two groups of animals consumed equal amounts of energy (**Figure 1C**). Consistent with the energy intake, no difference was seen in the bodyweight and even in the postpartum weight between the two groups (**Figures 1D,E**). Impressively, the dams with high-fructose feeding showed slight fatty liver and lipid droplet-enlarged BAT, while leaving WAT less affected at 18.5 days of gestation (**Figures 1F,G**). Meanwhile, the mice also exhibited higher plasma TG and TC levels (**Figures 1H,I**). These data indicate that short-term high-fructose feeding has already disturbed maternal metabolic homeostasis. Next, given fructose is the leading factor to the metabolic phenotypes, thus we measured the circulating fructose level in dams. The result showed that plasma fructose level in fructose-fed dams was about 1.6-folds higher than that in the control mice (**Figure 1J**). By contrast, the blood glucose level was not altered by maternal fructose feeding (**Figure 1K**). Collectively, these data suggest that maternal high-fructose consumption causes gestational fatty liver and dyslipidemia but not obesity.

Maternal High-Fructose Intake During Pregnancy Predisposes Adult Offspring to High-Fat Diet-Induced Obesity, Fatty Liver, and Insulin Resistance

Next, to evaluate the effects of maternal high-fructose consumption on offspring metabolic health, offspring were placed on a chow diet (CD) or a HFD. On regular chow, offspring from dams with high-fructose intake (henceforth referred to as HF offspring) did not display any notable difference in metabolic phenotypes compared with the controls except the glucose tolerance (**Supplementary Figure 1**), indicating maternal high-fructose consumption has limited effects on offspring metabolic health under CD condition. However, when under HFD exposure, the HF offspring exhibited obvious metabolic disorders compared with the controls. First, the HF offspring gained more body weight, which was mainly due to increased fat mass but not lean mass (**Figures 2A–C**), indicating the HF offspring were prone to develop obesity than the controls on a HFD. Specifically, the weight of inguinal adipose tissue (iWAT) and liver was significantly higher in HF offspring, while interestingly, the weight of epididymal adipose tissue (eWAT) was similar between the two groups (**Figure 2D**). In agreement with the tissue weight, histological analysis showed that the cell size of iWAT, but not eWAT, was larger in HF offspring than in the controls (**Figures 2E,F**). Besides, the liver of HF offspring had accumulated more lipid droplets than the controls, which was further evidenced by Oil Red O staining and TG measurement (**Figures 2G,H**), suggesting the HF offspring developed more serious fatty liver. Furthermore, the HF offspring had high levels of plasma TG and TC (**Figures 2I,J**). In addition, we evaluated the glucose tolerance and insulin sensitivity of the offspring. As expected, the HF offspring showed significantly higher blood glucose concentrations than controls (**Figures 2K,L**).

[1] http://www.ncbi.nlm.nih.gov/projects/geo/

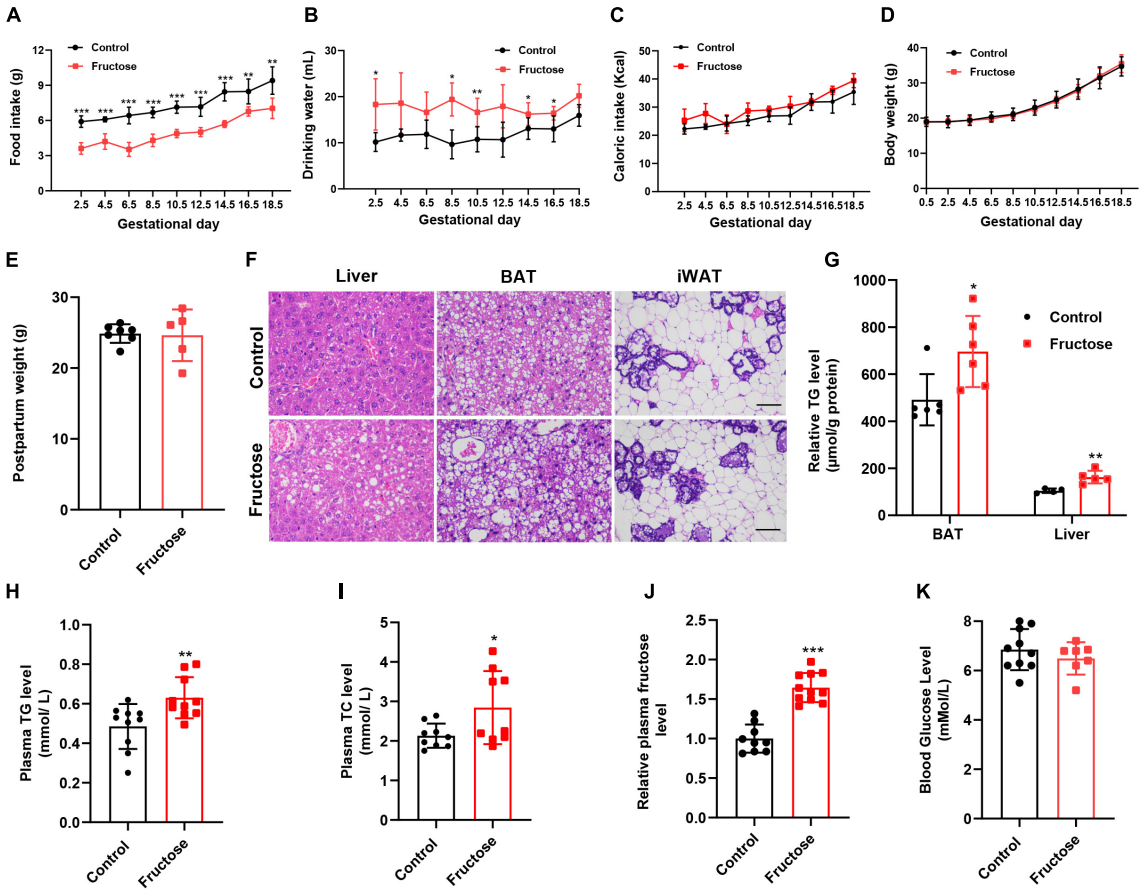

FIGURE 1 | High-fructose intake during pregnancy does not alter body weight but causes slight lipid metabolic disorders in female mice. **(A–C)** Daily food intake, drinking water consumption, and total caloric intake of the control pregnant mice and the high-fructose-fed pregnant mice during the period of gestation (n = 5–6). **(D)** Bodyweight of the control pregnant mice and the high-fructose-fed pregnant mice during the period of gestation (n = 7–10). **(E)** Postpartum weight of the control pregnant mice and the high-fructose-fed pregnant mice (n = 5–7). **(F–K)** All data were from control pregnant mice and the high-fructose-fed pregnant mice at day 18.5 of pregnancy. Mice fasted for 4–6 h before sacrifice. **(F)** H&E staining of paraffin sections of liver, brown fat tissue (BAT), and inguinal white fat tissue (iWAT). Scale bar, 50 μm for liver and BAT, 100 μm for iWAT. **(G)** Quantification of triglyceride content in liver and BAT (n = 4–6). **(H,I)** Plasma concentrations of triglyceride (TG) and total cholesterol (TC) (n = 9–10). **(J)** Relative fasting plasma fructose level (n = 11). **(K)** Fasting blood glucose level (n = 7–10). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Similarly, the HF offspring exhibited impaired responses to insulin during an ITT (**Figures 2M,N**). Taken together, these data strongly suggest that maternal high-fructose consumption during pregnancy predisposes adult offspring to HFD-induced obesity, fatty liver, and insulin resistance.

High-Fat Diet-Induced Energy Expenditure Is Impaired in Adult Offspring of Dams Fed With High Fructose

Next, to investigate the mechanisms of HF offspring predisposing to HFD-induced metabolic disorders than the controls, we performed an indirect calorimetry assay as disturbed energy metabolism plays a key role in the development of metabolic dysfunction (19, 23). First, we found when on a CD there was no difference between HF offspring and controls in terms of energy intake, energy expenditure, or physical activities (**Supplementary Figure 2**). However, when under HFD challenge, HF offspring exhibited less oxygen consumption, carbon dioxide release, and heat production compared with the controls, with the exception of food intake and physical activities (**Figure 3**). Thus, these findings suggest that maternal high-fructose consumption results in impairment of diet-induced energy expenditure in HF offspring.

High-Fat Diet-Induced Thermogenic Program of Brown Fat Is Attenuated in Adult Offspring of Dams Fed With High Fructose

As brown fat is highly involved in the regulation of thermogenesis, glucose metabolism, and insulin sensitivity (24, 25), we then investigated whether the BAT function of offspring was adversely affected by maternal high-fructose feeding. First, we examined the mass of the BAT depot, and

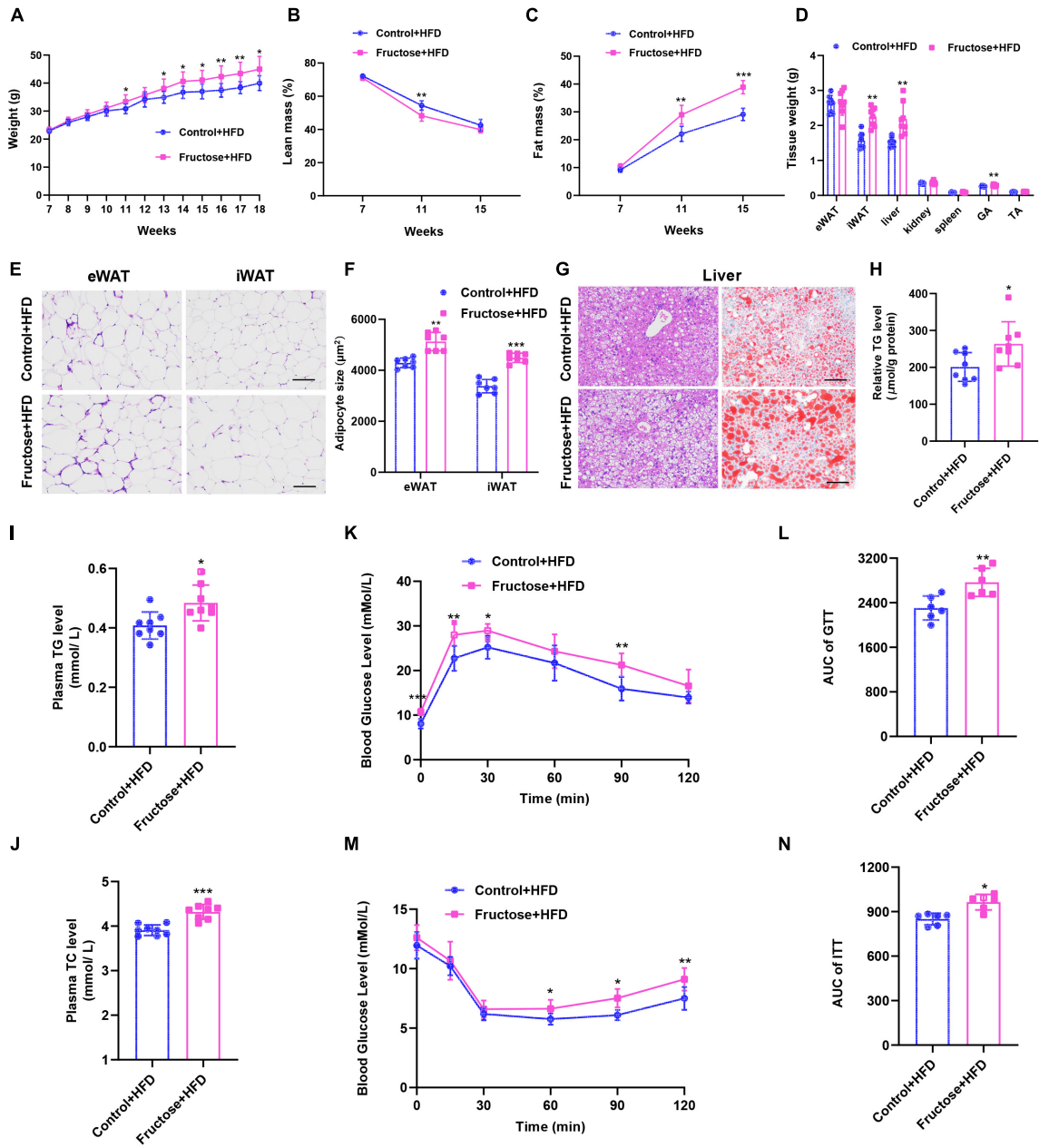

FIGURE 2 | Maternal high-fructose intake during pregnancy predisposes adult offspring to HFD-induced obesity, fatty liver, and insulin resistance. All data were from the HFD-fed control offspring and HF offspring. **(A)** Bodyweight curve of the offspring. **(B,C)** Relative body composition of the offspring. **(D)** Tissue weights of the offspring. **(E)** H&E staining of paraffin sections of inguinal white fat (iWAT) and epididymal white fat (eWAT). Scale bar, 100 μm. **(F)** Quantification of average adipocyte area from images depicted in **(E)**. **(G)** H&E staining of paraffin sections of liver and Oil Red O staining of frozen section of the liver. Scale bar, 100 μm. **(H)** Quantification of triglyceride content in liver ($n = 8$). **(I,J)** Plasma concentrations of triglyceride (TG) and total cholesterol (TC) ($n = 8$). **(K)** Blood glucose concentrations during glucose tolerance test ($n = 6$). **(L)** Quantification of the area under the curve of **(K)** ($n = 6$). **(M)** Blood glucose concentrations during insulin tolerance test ($n = 6$). **(N)** Quantification of the area under the curve of **(M)** ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

found the BAT weight of HF offspring was similar to the controls under CD condition; however, it significantly increased when under HFD feeding (**Figure 4A**). Moreover, we found that this increase in BAT weight was mainly due to the increased lipid content as showed by H&E staining and TG-level measurement (**Figures 4B,C**). Since the higher TG content in BAT is usually associated with its lower thermogenic activities (26), we then analyzed the expression of a panel of BAT marker genes in offspring BAT. On the CD, we did not notice any difference in mRNA levels of the genes, namely, BAT-selective genes, pan-adipocyte genes, and WAT-selective genes (**Figure 4D**). However, when under HFD condition, the mRNA levels of BAT-selective

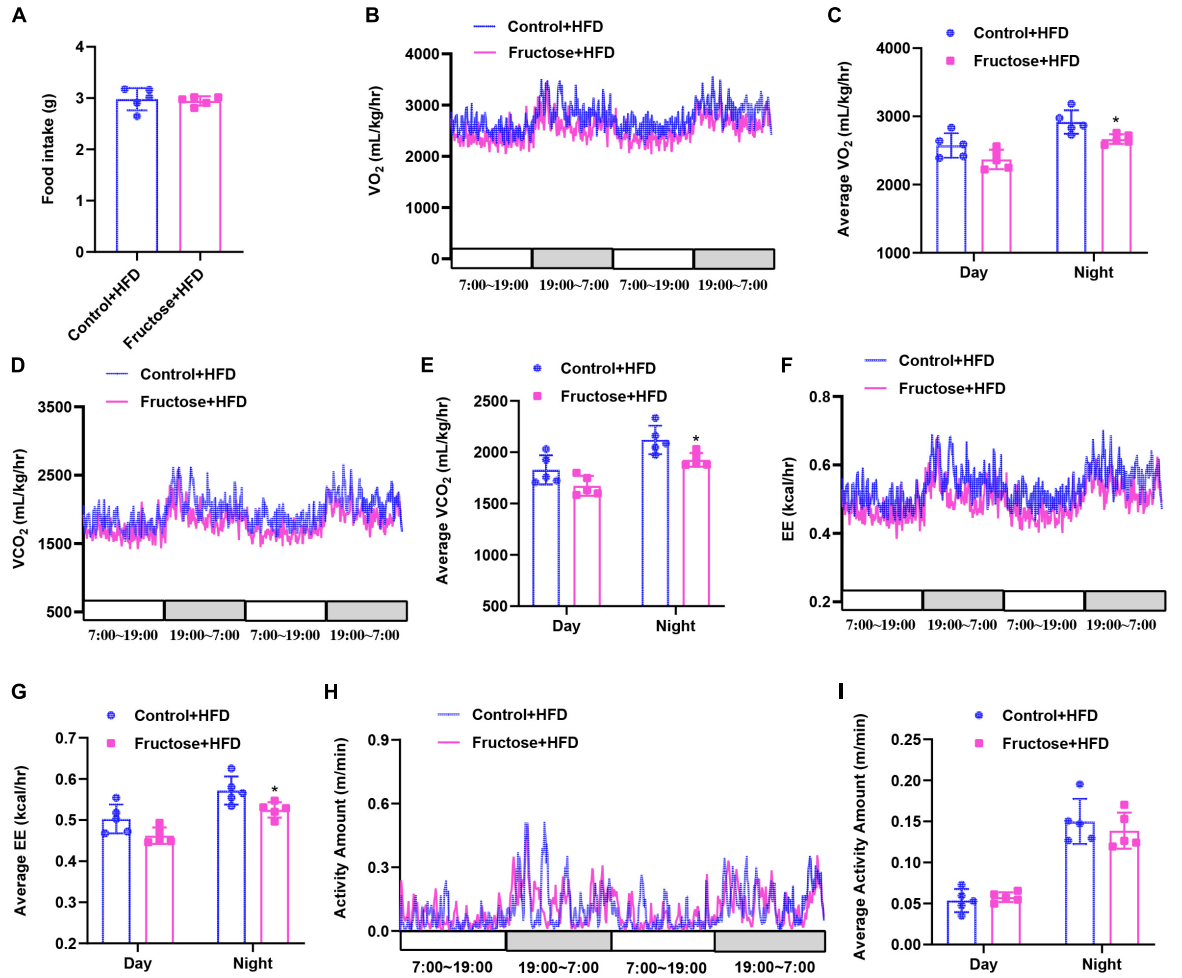

FIGURE 3 | High-fat diet-induced energy expenditure is impaired in adult offspring of dams fed with high fructose. All data were from the 17-week-old HFD-fed control offspring and HF offspring. **(A)** Daily food intake of the mice ($n = 5$). Indirect calorimetry analysis of day and night oxygen consumption (VO_2, **B,C**), carbon dioxide production (VCO_2, **D,E**), heat production **(F,G)**, and physical activity **(H,I)** of the mice ($n = 5$). *$p < 0.05$.

genes were significantly lower in HF offspring than the controls, whereas the mRNA levels of common adipogenic genes and WAT-specific genes were significantly upregulated in BAT of HF offspring compared with the controls (**Figure 4E**). Collectively, these data indicate that maternal high-fructose feeding blunts HFD-induced BAT reprogramming and thermogenic function in offspring, which could be responsible for the suppression of energy expenditure in HF offspring.

Maternal High-Fructose Intake During Pregnancy Induces Myogenic Signature in Fetal Brown Fat

Next, to investigate how maternal high-fructose feeding causes adult offspring BAT dysfunction, we switched our focus on the fetal stage since the fructose was only administrated to the dams in the gestation period. Thus, fetuses and their BAT at E18.5 days were isolated and analyzed. No difference was observed in total fetus weight (**Figure 5A**), but a significant increase was seen in the fetal BAT weight at the end of gestation (**Figure 5B**). Microscopic examination detected the emergence of a lot of adipocytes containing single lipid droplets only in fetal BAT from fructose-fed dams but not from the control dams (**Figure 5C**). Consistently, TG level was also significantly increased in fetal BAT of fructose-fed dams (**Figure 5D**). These data suggest that the fetal BAT from fructose-fed dams has lipid metabolic disorder. Next, to identify genes or pathways that contribute to this phenotype, we, therefore, analyzed the global gene expression profiles of the fetal BAT, and found a total of 2,464 genes that were differentially expressed, with 1,708 upregulated genes and 756 downregulated genes (**Figure 5E**). As expected, key lipogenic transcriptional factor ChREBP and its target genes *Fasn*, *Acc1*, and *Acly* were significantly upregulated (**Figure 5F**). But, unexpectedly, GSEA revealed that the muscle development pathway was at the top of the significantly upregulated pathways, whereas genes responsible for the mitochondrial respiratory chain were significantly blunted (**Figures 5G–I**). In support of the GSEA

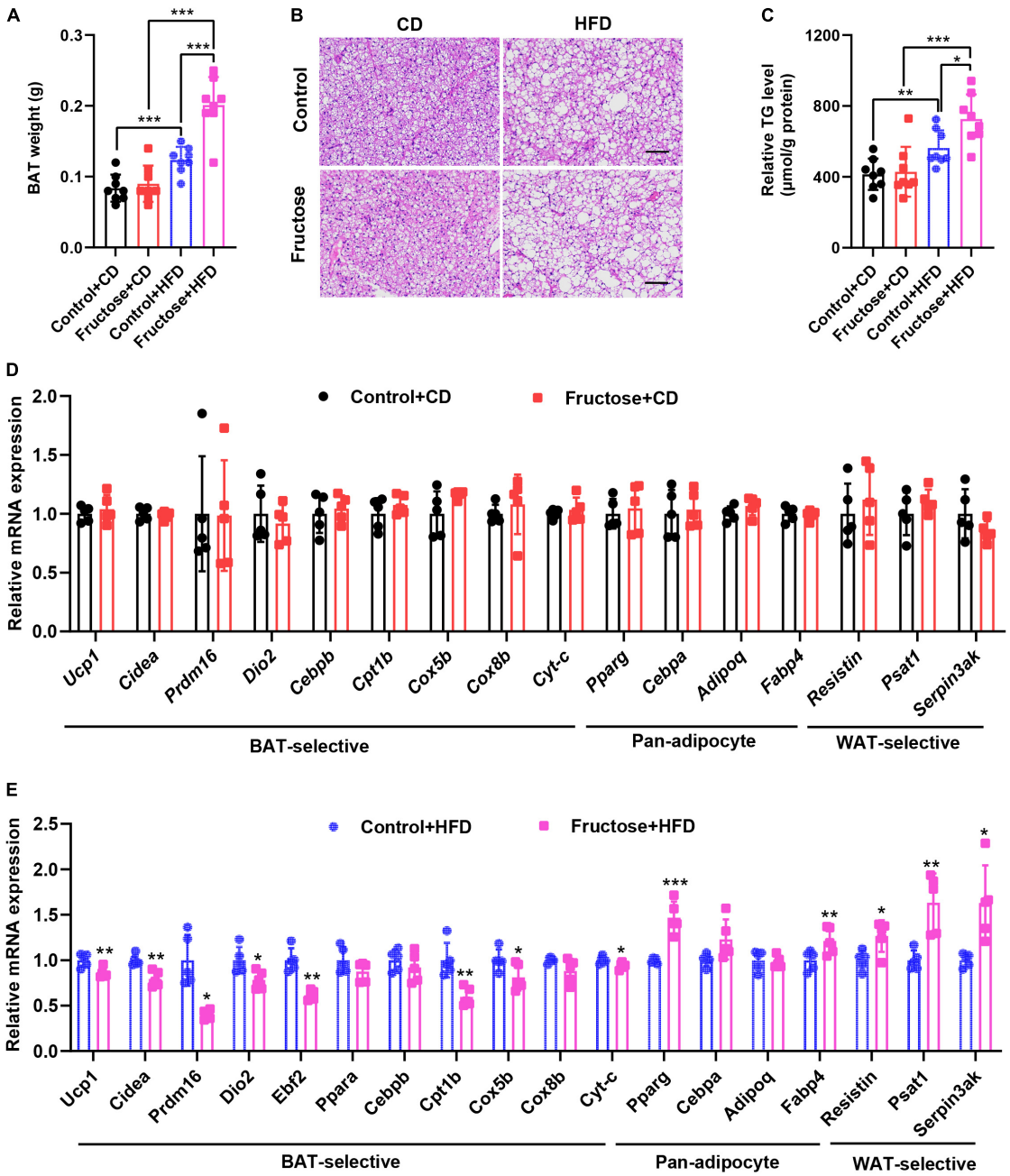

FIGURE 4 | High-fat diet-induced thermogenic program of brown fat is attenuated in adult offspring of dams fed with high fructose. **(A)** The weight of BAT tissue in CD-fed or HFD-fed control offspring or HF offspring. **(B)** H&E staining of paraffin sections of BAT. Scale bar, 100 μm. **(C)** Quantification of triglyceride content in BAT ($n = 8$). **(D)** qRT-PCR analysis of BAT-selective genes, pan-adipocyte genes, and WAT-selective genes in BAT from CD-fed control offspring or HF offspring ($n = 5$). **(E)** qRT-PCR analysis of BAT-selective genes, pan-adipocyte genes, and WAT-selective genes in BAT from HFD-fed control offspring or HF offspring ($n = 5$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

result, quantitative real-time (qRT)-PCR analysis revealed that the expression levels of myogenic signature genes *Myod*, *Myog*, *Mef2c*, *Myh1*, *Myh4*, and *Myh7* and pro-myogenic genes *Igf2* and *Igf2bp3* were significantly upregulated (**Figure 5J**), whereas the common adipogenic genes *Pparg*, *Cebpa*, *Adipoq*, and *Fabp4* were downregulated (**Figure 5K**), interestingly leaving the BAT marker genes *Ucp1*, *Cidea*, *Prdm16*, and *Cebpb* not affected (**Figure 5L**). Because it has already known that the myogenic pathway is usually inhibited during BAT development and abnormal activation of this pathway leads to BAT dysfunction and metabolic disorders (18, 20, 21, 27), thus our findings suggest that the induction of myogenic signature in fetal BAT partially

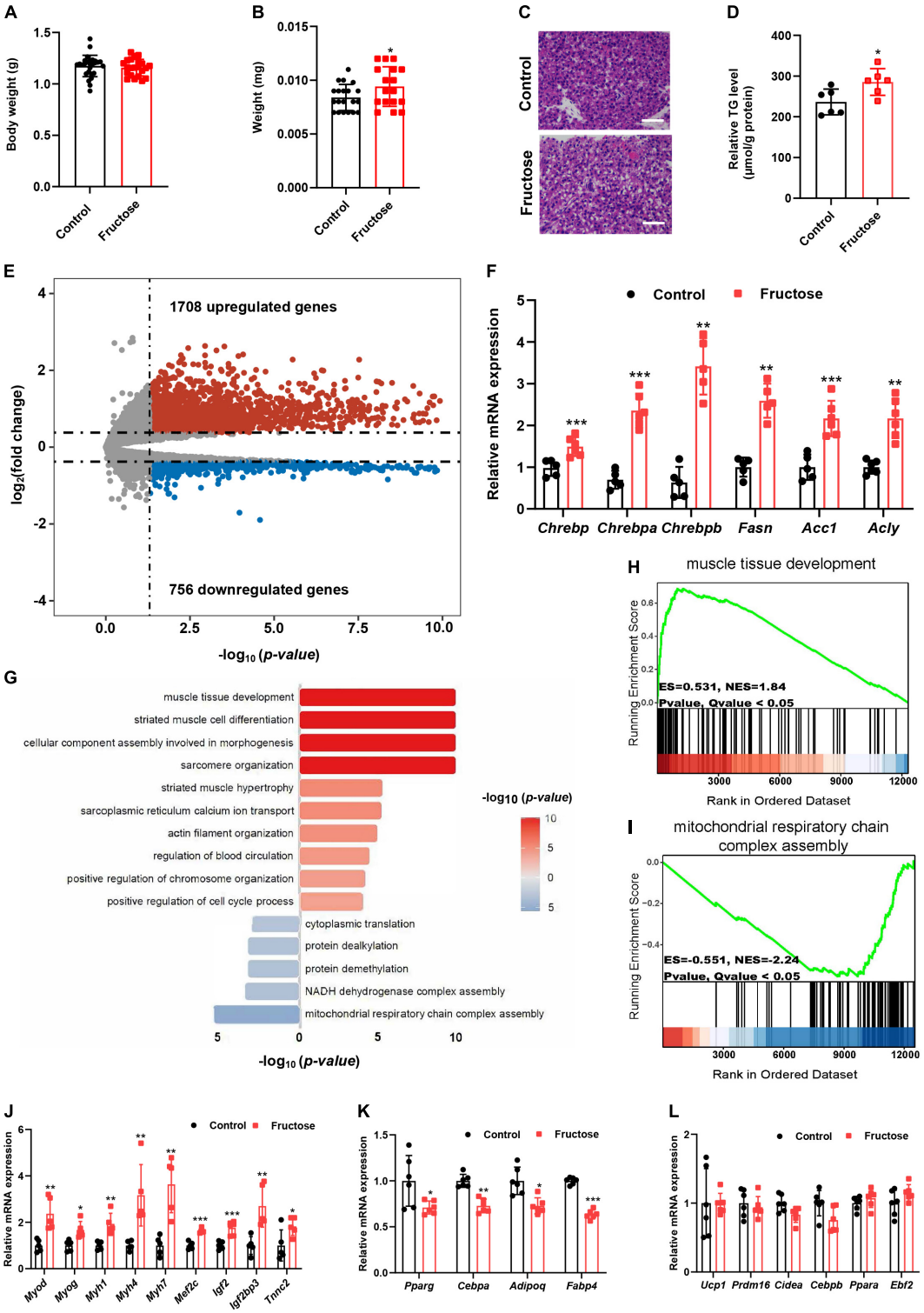

FIGURE 5 | Maternal high-fructose intake during pregnancy induces a myogenic signature in fetal brown fat. All data were collected from fetuses at E18.5. **(A)** Bodyweight of fetuses ($n = 22$–27). **(B)** Fetal BAT weight ($n = 17$–20). **(C)** H&E staining of paraffin sections of fetal BAT. Scale bar, 50 μm. **(D)** Quantification of triglyceride content in fetal BAT ($n = 6$). **(E)** Volcano plot comparison of genes in fetal BAT regulated by maternal high-fructose versus the control ($n = 2$ per group). **(F)** qRT-PCR analysis the levels of *Chrebp* and its target genes in fetal BAT ($n = 5$–6). **(G)** Gene Set Enrichment Analysis (GSEA) of differentially expressed genes. **(H,I)** GSEA analysis of the most significant upregulated and downregulated pathways. **(J–L)** qRT-PCR analysis of myogenic genes, pan-adipocyte genes, and brown fat selective genes in fetal BAT ($n = 5$–6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

results in the impairment of the BAT development and function in HF offspring.

Brown Adipose Tissue Myogenic Programming Can Be Acutely Induced by Fructose Administration

Next, to further reveal how maternal high-fructose feeding activates fetal BAT myogenic gene program, we first measured the circulating fructose and glucose levels in fetal blood at E18.5. Interestingly, the glucose level was comparable between the two groups (**Figure 6A**); however, fetal HF offspring showed about 1.5-folds higher plasma fructose levels than the controls (**Figure 6B**). Moreover, the expression of the fructose transporter gene *Glut5* in BAT was also strikingly upregulated in fetal HF offspring (**Figure 6C**). These data suggest that maternal-derived fructose may directly play a regulatory role in fetal BAT development. Then, to test whether blood fructose elevation is sufficient to induce myogenic signature in BAT, we fasted male mice for 24 h and then acutely fed mice with fructose or water through gavage. About 6 h later, mice BAT were isolated and analyzed by qRT-PCR. As expected, the acute high-fructose feeding significantly upregulated the levels of *Chrebp* and its target genes (**Figure 6D**). Strikingly, it also activated the expression of myogenic genes, such as *Myh1, Myh4,* and *Myh7* (**Figure 6E**). Of note, interestingly, the thermogenic gene was differentially regulated by the acute high-fructose challenge. Specifically, mRNA levels of *Dio2* and *Pgc1a* were dramatically elevated but *Ebf2*, *Ppara*, and *Cebpb* were significantly downregulated (**Figure 6F**). Overall, these data indicate that dietary fructose could activate BAT myogenic gene program. Taken together, these data support that maternal high-fructose feeding induces fetal BAT myogenic signature probably through a fructose-to-BAT mechanism.

DISCUSSION

In this study, we explored the effects and potential mechanisms underlying maternal high-fructose intake on offspring metabolic health. We found maternal high-fructose feeding predisposed adult offspring to HFD-induced obesity, fatty liver, and insulin resistance. Mechanistically, maternal-derived fructose hampered the fetal BAT development partially *via* inducing a myogenic signature in fetal BAT and resultantly impaired HFD-induced thermogenesis in adult mice (**Figure 7**). Thus, this study provides evidence for a pregnant woman to control their daily fructose intake. Also, this study reveals a previously uncovered myogenic pathway that probably mediates the detrimental effects of excess fructose consumption on metabolism.

Maternal nutrition plays a pivotal role in determining descendant metabolic health (3); therefore, it is important to clarify the effects of primary nutrients in our daily diet on the health of the offspring, to provide dietary guidance for the pregnant women to protect the health of offspring. Fructose is widely used as a food and drink sweetener, and excess consumption of fructose is considered as a major contributor to the global obesity epidemic (6). However, whether maternal high-fructose intake is also responsible for obesity prevalence remains largely unknown. In this study, we found that offspring delivered from dams fed with high-fructose water were prone to HFD-induced obesity, fatty liver, and insulin resistance (**Figure 2**). To our knowledge, this is the first report comprehensively describing the effects of maternal high-fructose feeding on adult offspring metabolic health. Interestingly, we noticed that the adverse metabolic consequences caused by maternal high-fructose feeding to adult offspring are similar to that caused by maternal HFD feeding (28, 29), thus our study highlights the importance of the diet intake control of fructose besides fat, especially during the period of pregnancy.

Then, we explored the underlying mechanism from the angle of energy metabolism and found HF offspring showed impaired energy expenditure only on a HFD but not on a CD (**Figure 3** and **Supplementary Figure 2**). This finding not only explains why offspring with a CD do not develop obesity but also indicates that the heat-production organ, namely, BAT, is probably involved in this adverse process. Indeed, histological and molecular examination confirmed that the function of BAT is disrupted in HFD-fed HF offspring (**Figures 3**, **4**). Although we are unable to exclude the contributions of other organs, BAT dysfunction is definitely a major player in the development of obesity and metabolic disorders in HF offspring.

The fetal BAT development has a profound effect on adult BAT function and global energy homeostasis in later life (30, 31). Studies showed that maternal nutrition influences the metabolic health of the adult offspring partially by affecting fetal BAT development. For instance, maternal HFD results in offspring metabolic disorders *via* inhibiting fetal BAT development (28, 32), whereas maternal resveratrol or n-3 PUAF supplementation ameliorates HFD-induced obesity through enhancing offspring BAT development (33, 34). However, to date, whether maternal high-fructose feeding also adversely affects fetal BAT development is still unknown. To this end, we closely examined fetal BAT morphology and gene expression profiles (**Figure 5**). As expected, maternal high-fructose feeding increased fetal BAT lipid deposition and elevated expression of genes in the *de novo* lipogenesis pathway (**Figures 5C,D,F**). That is probably because plasma fructose level in fetuses is significantly increased after dams fed with a high-fructose drink (**Figure 6B**), and it is already known that fructose-derived intermediate metabolites can activate the transcriptional activity of ChREBP, a major lipogenic transcription factor in adipose tissue (35). Of note, it was reported recently that overexpression of *Chrebpb*, a constitutively active isoform of ChREBP (36), exclusively in BAT increased the lipid droplet size and impaired BAT thermogenic function (26). Therefore, activating the ChREBP-mediated pathway in fetal BAT may also partially contribute to the adverse metabolic phenotypes observed in HF offspring. However, unexpectedly, the GSEA result clearly displayed that the most predominantly upregulated pathway in fetal BAT was muscle tissue development but not lipogenesis (**Figure 5G**). Because the myogenic pathway is usually inhibited during BAT determination (17), and alleviating this inhibition always leads to BAT developmental delay and

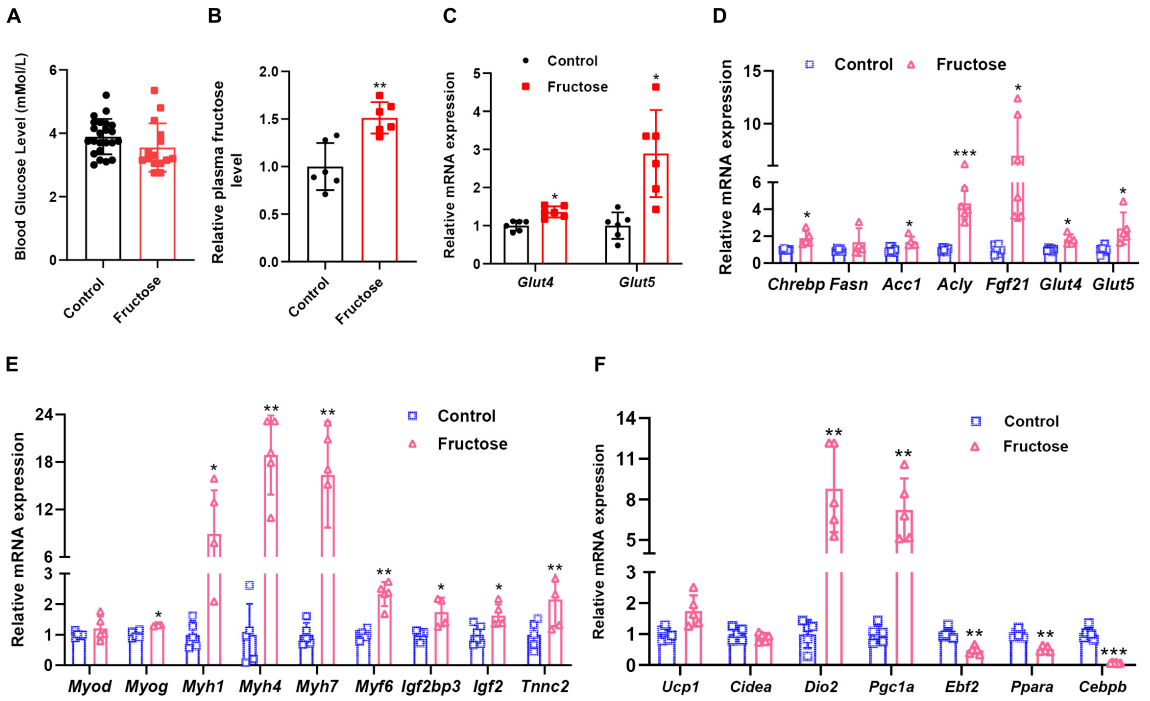

FIGURE 6 | Brown adipose tissue myogenic programming can be acutely induced by fructose administration. **(A)** The blood glucose level in fetuses at E18.5 ($n = 15$–23). **(B)** The relative level of plasma fructose in fetuses at E18.5 ($n = 6$). **(C)** qRT-PCR analysis of *Glut4* and *Glut5* expression in BAT of fetuses at E18.5 ($n = 6$). qRT-PCR analysis of lipogenic genes **(D)**, myogenic genes **(E)**, and thermogenic genes **(F)** in BAT of mice gavaged with fructose or water ($n = 4$–6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

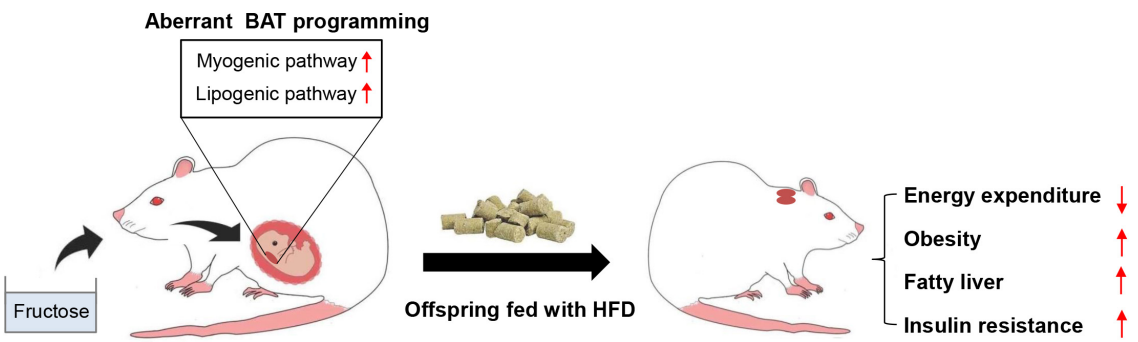

FIGURE 7 | A model depicting the effects of maternal high-fructose feeding on offspring BAT development and metabolic health. Maternal high-fructose feeding during gestation reprograms brown fat lineage specification, especially exhibiting activated myogenic pathway and lipogenic pathway, which probably results in the impairment of HFD-induced energy expenditure and the predisposition of HFD-induced obesity, fatty liver, and insulin resistance in adult offspring. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

metabolic disorders in later life (18, 20, 21, 27), thus fructose-induced activation of myogenic programming in fetal BAT could be a leading contributor to the BAT dysfunction and later metabolic disorders.

Currently, we do not know exactly how maternal-derived fructose activates fetal BAT myogenic programming, however, based on the finding that fructose solution gavage acutely activates BAT lipogenic and myogenic pathways (**Figures 6D,E**), one possibility is that circulating fructose can directly act on the BAT and regulate target genes expression. Of note, fructose can be rapidly catalyzed by Ketohexokinase (KHK) to generate fructose-1-phosphate (F-1-P) once entering cells through transporter GLUT5 (37), thus it could be the intermediate metabolites of fructose but not fructose itself that exerts the regulatory function. Therefore, as the major organs for fructose metabolism, the liver and small intestine may be also involved in the regulation of fetal BAT myogenic programming. To date, although there are no reports regarding fructose intermediate metabolites in regulating myogenesis, we noticed that one recent study showed that myoblast cellular level of fructose-1,6-bisphosphate (F-1-6-P),

a glucose intermediate metabolite, maintains the protein stability of MyoD (38), a key myogenic factor. Since F-1-P has a similar structure with F-1-6-P, it is possible that F-1-P may also regulate BAT MyoD protein stability in the same way with F-1-6-P, but more studies are required to test this possibility in the future.

CONCLUSION

In summary, we have demonstrated that maternal high-fructose exposure induces myogenic signature in fetal BAT and impairs fetal BAT development and HFD-induced energy expenditure, which predisposes adult offspring to obesogenic diet-induced obesity and metabolic disorders. This study highlights the importance of limiting the intake of fructose-enriched diets in pregnancy to protect offspring metabolic health in later life.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE193031.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Guangxi University.

AUTHOR CONTRIBUTIONS

ZS conceived, designed, and supervised the study. PW and TW performed most of the experiments and data analyses. ZS and PW wrote the manuscript. QF, QL, and YaL participated in the experiments. TH, YiL, and LZ provided scientific advice and discussion. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Youth Science Foundation of National Natural Science Foundation of China (No. 82100913) and the Natural Science Foundation of Guangxi Province (No. 2020GXNSFAA297043).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.848983/full#supplementary-material

Supplementary Figure 1 | The effects of maternal high-fructose intake during pregnancy on adult offspring fed with a chow diet (CD). All data were from the CD-fed control offspring and HF offspring. **(A)** Bodyweight curve of the offspring. **(B,C)** Relative body composition of the offspring. **(D)** Tissue weights of the offspring. **(E)** H&E staining of paraffin sections of inguinal white fat (iWAT), epididymal white fat (eWAT), and liver. Scale bar, 100 μm. **(F)** Quantification of average adipocyte area from images depicted in **(E)**. **(G)** Quantification of triglyceride (TG) content in liver ($n = 8$). **(H,I)** Plasma concentrations of TG and total cholesterol (TC) ($n = 8$). **(J)** Blood glucose concentrations during glucose tolerance test ($n = 6$). **(K)** Quantification of the area under the curve of **(J)**. **(L)** Blood glucose concentrations during insulin tolerance test ($n = 6$). **(M)** Quantification of the area under the curve of **(L)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

Supplementary Figure 2 | Maternal high-fructose intake during pregnancy does not affect energy expenditure in adult offspring with a chow diet. All data were from the 17-week-old CD-fed control offspring and HF offspring. **(A)** Daily food intake of the mice ($n = 5$). Indirect calorimetry analysis of day and night oxygen consumption (VO_2, **B,C**), carbon dioxide production (VCO_2, **D,E**), heat production **(F,G)**, and physical activity **(H,I)** of the mice ($n = 5$).

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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.

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ORIGINAL RESEARCH
published: 15 April 2022
doi: 10.3389/fnut.2022.873249

Pre-diagnosis Dietary One-Carbon Metabolism Micronutrients Consumption and Ovarian Cancer Survival: A Prospective Cohort Study

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Background and Aims: Epidemiological evidence on the relation between one-carbon metabolism (OCM) micronutrients intake and ovarian cancer (OC) survival are limited and conflicting. We evaluated the aforementioned associations in a prospective cohort-the Ovarian Cancer Follow-Up Study.

Methods: A total of 635 newly diagnosed OC patients aged 18–79 y were enrolled in the present study. Dietary intake related to one-carbon metabolism, including methionine, vitamins B2, B3, B6, B9, B12, choline, and betaine, was assessed using a validated 111-item food frequency questionnaire. Deaths were ascertained until March 31, 2021, *via* medical records and active follow-up. Cox proportional hazards regression model was used to evaluate the hazard ratios (HRs) and 95% confidence intervals (CIs) for these aforementioned associations.

Results: During a median follow-up of 37.2 months (interquartile: 24.7–50.2 months), 114 deaths were identified. We observed an improved survival with the highest compared with the lowest tertile of dietary vitamin B6 (HR = 0.52, 95%CI: 0.32–0.84, P-trend <0.05) and choline intake (HR = 0.50, 95%CI: 0.30–0.83, P-trend <0.05). No significant associations with OC survival were observed for dietary vitamins B2, B3, B9, B12, methionine, and betaine intake. We also observed a curvilinear association between vitamin B6 intake and OC survival (P non-linear <0.05).

Conclusion: Our study suggests that pre-diagnosis higher intake of vitamin B6 and choline may improve OC survival. Further clarification of these associations is warranted.

Keywords: cohort, diet, one-carbon metabolism, ovarian cancer, survival

OPEN ACCESS

Edited by:
Ellen E. Blaak, Maastricht University, Netherlands

Reviewed by:
Cecilie Kyrø, Danish Cancer Society Research Center (DCRC), Denmark
Isis Trujillo-Gonzalez, University of North Carolina at Chapel Hill, United States

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

Received: *10 February 2022*
Accepted: *23 March 2022*
Published: *15 April 2022*

Citation:
Xu H-L, Gong T-T, Liu F-H, Wei Y-F, Chen H-Y, Yan S, Zhao Y-H, Gao S, Jiao Y-S and Wu Q-J (2022) Pre-diagnosis Dietary One-Carbon Metabolism Micronutrients Consumption and Ovarian Cancer Survival: A Prospective Cohort Study. Front. Nutr. 9:873249. doi: 10.3389/fnut.2022.873249

INTRODUCTION

Ovarian Cancer (OC) is one of the most common gynecologic cancers with a high mortality rate (1). In 2020, there were 3,13,959 new cases and 2,07,252 deaths of OC worldwide (2). The number of cases and deaths in China is 55,342 and 37,519 (3). Given the poor prognosis of this disease (4) and limited population-level strategies for early detection and long-term treatment success (5), knowledge of modifiable risk factors for prevention and improved prognosis is important.

During the past decades, there has been increasing epidemiological evidence of the relationship between inadequate intake of micronutrients and the appearance of tumor processes (6). The one-carbon metabolism (OCM) cycle is known to support multiple physiological processes essential for human development (7, 8), such as biosynthesis (purines and thymidine), amino acid homeostasis (glycine, serine, and methionine), epigenetic maintenance, and redox defense. Recent genomics and metabolomics approaches have also highlighted the distinctive aspects of OCM in cancer development and prognosis (9, 10). Therefore, micronutrients implicated in OCM —vitamins B2 (riboflavin), B3 (niacin), B6, B9 (folate), B12, methionine, choline, and betaine— deserve special attention.

One-carbon metabolism micronutrients are carriers or methyl-group donors (e.g., folates, choline, betaine, methionine) or cofactors of enzymes involved in the transfer reactions of these groups to DNA (namely vitamins B2, B6, and B12) (11). A dietary imbalance or deficiency in those micronutrients may disrupt DNA methylation or induce the disincorporation of nucleotide synthesis, which could lead to carcinogenesis (12–14). Although several observational studies have reported associations between individual OCM micronutrients and OC risk, the results of these studies are conflicting (14–24). For example, several case-control studies suggested null association between dietary folate intake and OC risk (15–19), whereas results from prospective studies suggested a modest inverse association (14, 20–22). Findings of methionine and vitamin B6 are also inconsistent. In the New England Case-Control Study, significant inverse associations between dietary methionine and vitamin B6 intake and OC risk were observed (23). However, the aforementioned associations were non-significant in the Nurses' Health Study (14). Only one study previously investigated the relationship between dietary choline and betaine intake and the risk of OC and showed no association (24). Of note, recent, only two studies have investigated the relationship between individual OCM micronutrients and OC survival. For example, Dixon et al. (25) found no evidence that pre-diagnostic folate, vitamins B2, B6, and B12, methionine, betaine, or choline intake was associated with OC survival based on 1270 OC patients from Australia. However, Zhang et al. (26) observed that high folate intake was significantly associated with a lower risk of OC death based on 215 OC patients.

Notably, given these controversial results as well as the current lack of prospective evidence regarding the impact of dietary OCM micronutrients on OC survival, we present results from a prospective cohort, the Ovarian Cancer Follow-Up Study (OOPS), to clarify the associations of pre-diagnosis dietary consumption of OCM micronutrients with the survival of OC.

MATERIALS AND METHODS

Study Design and Participants

The ovarian cancer follow-up study (OOPS) is a prospective longitudinal cohort study of patients newly diagnosed with OC to investigate the risk and prognostic factors for cancer-related outcomes. Complete details of the study design are available elsewhere (27–30). The OOPS was approved by the Institutional

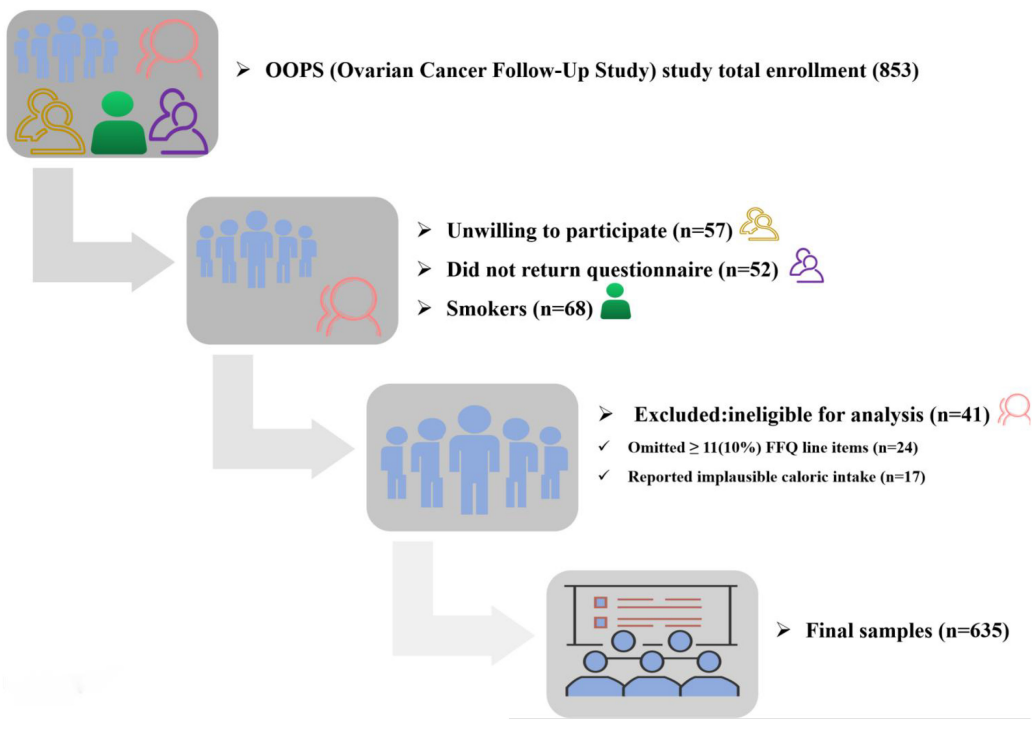

FIGURE 1 | Flow of participants through study.

Review Board of the Ethics Committee of Shengjing Hospital of China Medical University, Shenyang, China, and informed consent was obtained from all patients.

Between 2015 and 2020, 853 OC patients with 18–75 years of age were recruited at the Shengjing Hospital of China Medical University. Among them, 796 women (93%) consented to participate and 744 (87%) women returned the completed study questionnaire. For quality assurance of research, we excluded OC patients for: implausible caloric intake (<500 or >3500 calories per day; n = 17), 11 (10%) or more food items blank (n = 24). In addition, considering that smoking lowers serum folate and inhibits the one-carbon response (31, 32), smokers (n = 68) were likewise excluded. Finally, a total of 635 women were eligible for the analysis. Details are shown in the flow chart of the study participants (**Figure 1**).

Data Collection

During the enrollment period, the participants were interviewed in-person by skilled interviewers with relevant medical knowledge. Information on socio-demographic characteristics including education, monthly household income, the levels of physical activity, cigarette smoking, medical and reproductive history, exogenous hormone use, anthropometric measures as well as alcohol consumption habits were gathered using lifestyle questionnaires as detailed elsewhere (27–30). Furthermore, clinical characteristics were extracted from the electronic medical records, including age at diagnosis, histological type (serious and non-serious), histopathologic grade (well, moderate, and poorly differentiated), International Federation of Gynecology and Obstetrics (FIGO) stage (I, II, III, IV, and unknown), residual lesions (none, <1, and ≥1 cm), and comorbidities (hypertension, coronary heart disease, diabetes, and so on) (yes and no).

Dietary Exposure Assessment

Pre-diagnosis dietary intake was assessed at recruitment with a 111-item food frequency questionnaire (FFQ), which was previously validated (27, 28). The reproducibility coefficients (Spearman correlation coefficients and intraclass correlation coefficients) were above 0.5 for most food groups, and the correlation coefficients (Spearman correlation coefficients) were between 0.3 and 0.7 for most food groups between the FFQ and weighed dietary records. During the in-person interview, newly diagnosed OC patients reported their usual frequency of consumption of each food item in the 12 months before diagnosis. The frequency of intake ranged from almost none to twice a day or more. Nutrient contents of the food items were determined through a linkage of the FFQ responses to 2018 Chinese Food Composition Tables (33). Intakes of the following OCM micronutrients from food sources were available for analysis: vitamins B2 (riboflavin), B3 (niacin), B6, B9 (folate), B12, methionine, choline, and betaine. OCM micronutrients are thought to influence disease risk by donating methyl groups for methylation reactions (7, 34), we also calculated a "methyl-donor index" as a composite measure of dietary methyl intake by standardizing the nutrient intake levels on the log-scale [(nutrient value – mean)/standard deviation] then summed across all eight micronutrients, as described previously (35, 36). Intakes of OCM micronutrients from food sources were available for analysis. Each nutrient and methyl-donor index were adjusted for total energy intake based on the residual method (37).

TABLE 1 | Baseline characteristics of ovarian cancer patients (n = 635).

Characteristics	All patients
No. of patients/deaths	635/114
Mean (SD) age at diagnosis (years)	53.76 (9.30)
Mean (SD) follow-up time (months)	32.33 (16.37)
Mean (SD) body mass index (kg/m^2)	23.29 (3.61)
Mean (SD) physical activity (MET h/d)	15.65 (11.35)
Ever alcohol drinking	126 (19.84)
Ever tea drinking	192 (30.24)
Ever menopause	457 (71.97)
Parity	
≤1	473 (74.49)
≥2	162 (25.51)
Educational level	
Junior secondary or below	343 (54.02)
Senior high school/technical secondary school	127 (20.00)
Junior college/university or above	165 (25.98)
Income per month (Yuan)	
<5000	381 (60.00)
5000 to <10000	174 (27.40)
≥10000	80 (12.60)
Mean (SD) total energy intake (kcal/d)	1461.54 (555.10)
Mean (SD) carbohydrate intake (kcal/d)	913.00 (315.71)
Mean (SD) vegetable intake (kcal/d)	52.97 (29.78)
Mean (SD) fruit intake (kcal/d)	118.94 (92.57)
Mean (SD) meat intake (kcal/d)	72.02 (61.96)
Mean (SD) methionine intake (mg/d) *	1077.48 (256.85)
Mean (SD) vitamins B_2 (riboflavin) intake (mg/d) *	0.89 (0.20)
Mean (SD) vitamins B_3 (niacin) intake (mg/d) *	13.59 (2.50)
Mean (SD) vitamins B_6 intake (mg/d) *	0.44 (0.12)
Mean (SD) vitamins B_9 (folate) intake (μg/d) *	214.78 (73.88)
Mean (SD) vitamins B_{12} intake (μg/d) *	0.14 (0.20)
Mean (SD) choline intake (mg/d) *	279.17 (73.33)
Mean (SD) betaine intake (mg/d) *	57.16 (39.64)

MET, metabolic equivalents of task; SD, standard deviation.
**Energy adjustment by residual method.*
Values are numbers (percentages) unless stated otherwise.

Cohort Follow-Up and Outcome Ascertainment

The OOPS participants were followed up until the occurrence of mortality from any cause or the last follow-up (March 31, 2021). Data on vital status were obtained by active follow-up and annual linkage to the Vital Statistics Unit in the Liaoning Centers for Disease Control and Prevention.

Statistical Analysis

We calculated descriptive statistics of general and dietary characteristics. The Kaplan–Meier technique was used to plot crude survival curves and estimate the crude overall survival (OS) probabilities. Adjusted hazard ratios (HRs) and

TABLE 2 | Adjusted hazard ratio (HR) and 95% confidence intervals (CIs) for the association between dietary one-carbon metabolism micronutrients intake and total mortality of ovarian cancer (n = 635)*.

Characteristics	Tertiles of energy-adjusted intake **			*P* trend †	Continuous ‡
	I	II	III		
Methionine (Range, mg/d)	<979.81	979.81–1103.40	≥1103.40		
Deaths, N (% of total deaths)	38 (33.33)	44 (38.60)	32 (28.07)		
Model 1	1.00 (Ref)	1.07 (0.69–1.66)	0.75 (0.46–1.20)	0.19	0.92 (0.79–1.06)
Model 2	1.00 (Ref)	1.15 (0.73–1.81)	0.84 (0.52–1.38)	0.44	0.93 (0.80–1.07)
Model 3	1.00 (Ref)	1.23 (0.77–1.96)	0.85 (0.51–1.40)	0.44	0.93 (0.80–1.07)
Vitamins B_2 (riboflavin) (Range, mg/d)	<0.82	0.82–0.96	≥0.96		
Deaths, N (% of total deaths)	40 (35.09)	38 (33.33)	36 (31.58)		
Model 1	1.00 (Ref)	0.93 (0.60–1.45)	0.86 (0.54–1.35)	0.50	0.90 (0.73–1.11)
Model 2	1.00 (Ref)	0.88 (0.56–1.40)	0.88 (0.55–1.40)	0.60	0.91 (0.74–1.11)
Model 3	1.00 (Ref)	0.96 (0.60–1.56)	0.93 (0.58–1.51)	0.71	0.93 (0.76–1.14)
Vitamins B_3 (niacin) (Range, mg/d)	<12.52	12.52–14.85	≥14.85		
Deaths, N (% of total deaths)	40 (35.09)	40 (35.09)	34 (29.82)		
Model 1	1.00 (Ref)	1.04 (0.67–1.62)	0.77 (0.48–1.22)	0.31	0.89 (0.69–1.15)
Model 2	1.00 (Ref)	0.96 (0.61–1.51)	0.70 (0.42–1.15)	0.18	0.88 (0.67–1.17)
Model 3	1.00 (Ref)	0.99 (0.63–1.56)	0.77 (0.46–1.28)	0.35	0.93 (0.70–1.24)
Vitamins B_6 (Range, mg/d)	<0.39	0.39–0.48	≥0.48		
Deaths, N (% of total deaths)	48 (42.11)	34 (29.82)	32 (28.07)		
Model 1	1.00 (Ref)	0.59 (0.38–0.92)	0.61 (0.39–0.95)	<0.05	0.75 (0.58–0.97)
Model 2	1.00 (Ref)	0.52 (0.33–0.81)	0.54 (0.34–0.86)	<0.05	0.73 (0.57–0.95)
Model 3	1.00 (Ref)	0.48 (0.30–0.76)	0.52 (0.32–0.84)	<0.05	0.70 (0.53–0.92)
Vitamins B_9 (folate) (Range, μg/d)	<184.77	184.77–233.25	≥233.25		
Deaths, N (% of total deaths)	39 (34.21)	40 (35.09)	35 (30.70)		
Model 1	1.00 (Ref)	0.97 (0.62–1.50)	0.78 (0.49–1.23)	0.26	0.85 (0.70–1.04)
Model 2	1.00 (Ref)	0.87 (0.56–1.38)	0.72 (0.45–1.12)	0.18	0.82 (0.67–1.00)
Model 3	1.00 (Ref)	0.78 (0.49–1.25)	0.75 (0.47–1.20)	0.24	0.81 (0.65–1.00)
Vitamins B_{12} (Range, μg/d)	<0.05	0.05–0.14	≥0.14		
Deaths, N (% of total deaths)	34 (29.82)	42 (36.85)	38 (33.33)		
Model 1	1.00 (Ref)	1.23 (0.78–1.93)	1.04 (0.65–1.65)	0.99	1.02 (0.91–1.16)
Model 2	1.00 (Ref)	1.34 (0.81–2.19)	1.03 (0.64–1.66)	0.83	1.00 (0.89–1.14)
Model 3	1.00 (Ref)	1.42 (0.86–2.35)	1.02 (0.63–1.65)	0.75	1.02 (0.90–1.17)
Choline (Range, mg/d)	<245.60	245.60–310.02	≥310.02		
Deaths, N (% of total deaths)	46 (40.35)	41 (35.96)	27 (23.69)		
Model 1	1.00 (Ref)	0.82 (0.54–1.26)	0.54 (0.33–0.87)	<0.05	0.78 (0.61–0.98)
Model 2	1.00 (Ref)	0.67 (0.43–1.04)	0.51 (0.31–0.84)	<0.05	0.76 (0.60–0.97)
Model 3	1.00 (Ref)	0.67 (0.42–1.06)	0.50 (0.30–0.83)	<0.05	0.79 (0.61–1.01)
Betaine (Range, mg/d)	<41.35	41.35–61.55	≥61.55		
Deaths, N (% of total deaths)	34 (29.82)	40 (35.09)	40 (35.09)		
Model 1	1.00 (Ref)	1.09 (0.69–1.73)	1.12 (0.71–1.78)	0.64	1.01 (0.86–1.20)
Model 2	1.00 (Ref)	1.22 (0.75–1.97)	1.21 (0.76–1.94)	0.47	1.01 (0.87–1.20)
Model 3	1.00 (Ref)	1.15 (0.70–1.86)	1.12 (0.70–1.80)	0.69	0.98 (0.83–1.16)

CI, confidence interval; HR, hazard ratio; Ref, reference.

**HR and 95% CI were calculated with the use of the Cox proportional hazards regression model.*

***Adjusted for energy by the residual method.*

†*Test for trend based on variables containing the median value for each tertile.*

‡ *Continuous intakes were calculated by per unit increase.*

Model 1 adjusted for age at diagnosis and body mass index.

Model 2 adjusted for age at diagnosis, total energy, body mass index, alcohol drinking, diet change, education, income, physical activity, menopausal status, parity, multivitamin use, multimineral use, red meat, and methyl-donor index.

Model 3 adjusted for age at diagnosis, total energy, body mass index, alcohol drinking, diet change, education, income, physical activity, menopausal status, parity, multivitamin use, multimineral use, red meat, methyl-donor index, comorbidities, FIGO stage, histological type, histopathologic grade, and residual lesions.

corresponding 95% confidence intervals (CIs) were derived from Cox proportional hazards regression model where the entry time was the date at which the OC patients enrolled in the OOPS and the exit time was the date when the participant died or was censored due to loss to follow-up or end of study follow-up on March 31, 2021, whenever occurred first. The proportional hazards assumption was tested through including an interaction term between dietary OCM nutrients and the logarithm of time,

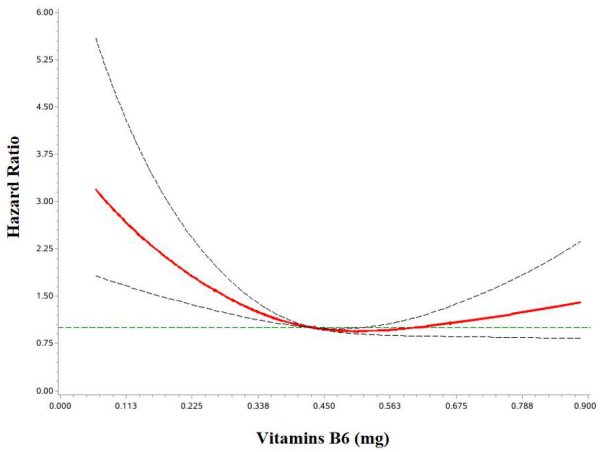

FIGURE 2 | HR and 95%CIs of overall survival among OC patients by vitamin B6. The association was adjusted for age at diagnosis, body mass index, total energy, alcohol drinking, diet change, education, income, physical activity, menopausal status, parity, multivitamin use, multimineral use, red meat, methyl-donor index, comorbidities, FIGO stage, histological type, histopathologic grade, and residual lesions.

and no violations were found (all $p > 0.05$). The HR and 95%CI for each tertile were calculated using the first tertile as a reference. The linear trend of the association between dietary OCM nutrients intake and OC survival was assessed by assigning the median value of each tertile and treating it as continuous in a model. Continuous intakes were also calculated by per unit increase. We calculated age at diagnosis (continuous, years) and body mass index (BMI) (continuous, kg/m2)-adjusted HRs (Model 1). Model 2 was additionally adjusted for dietary changes (yes or no), alcohol drinking status (yes or no), education (junior secondary or below, senior high school/technical secondary school, and junior college/university or above), income (<5000, 5000–10000 or >10000 RMB), physical activity (continuous, MET-hours/day), menopausal status (yes or no), parity (≤1 or ≥2), multivitamin use (yes or no), multimineral use (yes or no), red meat intake (continuous, g/day), methyl-donor index (<5.22, 5.22–8.42, ≥8.42) and total energy. Model 3 was adjusted further for comorbidities (yes or no), FIGO stage (I-II, III-IV, or unknown), histological type (serious or non-serious), histopathologic grade (well, moderate, or poorly differentiated), residual lesions (none, <1, or ≥1 cm) to minimize the impact of clinical characteristics on survival. In our study, dietary change and parity were collected using a self-administered questionnaire. A restricted cubic spline model with three knots (i.e., 10, 50, and 90th percentiles) was also performed to test for non-linear relationships (38).

Stratified analyses were conducted by alcohol drinking (no and yes), age at diagnosis (<50 and ≥50 years), menopausal status (no and yes), FIGO stage (I-II and III-IV), residual lesions (no and yes), histological type (serious and non-serious), and BMI (<24 and ≥24 kg/m). Interactions were tested by using likelihood-ratio tests.

We conducted several sensitivity analyses to test the robustness of the primary findings. First, we restricted the study sample to participants among people who had not taken vitamin supplements. Second, we mutually adjusted for all of the dietary OCM nutrients to evaluate whether the associations were independent of each other (36). In addition, the data were analyzed in quartiles and compared with recommended intake (RI). Analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, United States). Two-sided *P*-values less than 0.05 were considered statistically significant.

RESULTS

During the median follow-up of 37.2 months (interquartile: 24.7–50.2 months), 114 deaths from all causes were recorded among all 635 patients. **Table 1** summarizes the basic characteristics of OC patients. Later-stage disease and greater residual disease were statistically significantly associated with worse survival in this cohort (**Supplementary Table 1**).

Multivariable-adjusted HRs and 95%CIs for associations between dietary intakes of OCM micronutrients and OC survival are shown in **Table 2**. Higher dietary vitamin B6 intake was associated with lower mortality of OC (HR Tertile 3 vs. Tertile 1 = 0.52; 95%CI = 0.32–0.84; P trend <0.05). Additionally, dietary choline intake was associated with a decreased OC survival (HR Tertile 3 vs. Tertile 1 = 0.50; 95% CI = 0.30–0.83; P trend <0.05) (**Supplementary Figure 1**). However, we failed to observe significant associations for OC mortality with the intake of vitamins B2, B3, B9, B12, methionine, and betaine. Of note, we observed a curvilinear association between vitamin B6 intake and OC survival (P non-linear <0.05) (**Figure 2** and **Supplementary Figure 2**).

No significant interactions were found in the subgroup analyses stratified by demographic and clinical characteristics (**Table 3**). The direction of these results was mainly consistent with the main findings but not all of them showed statistical significance. The inverse associations between vitamin B6 and choline intake and OC mortality seemed slightly stronger in patients with an age greater than 50 years, menopausal status, serious patients, and no residual lesion patients (**Supplementary Tables 2–4**). In addition, the inverse association between vitamin B6 intake and OC mortality was slightly stronger in OC patients with BMI ≥24, alcohol drinkers, and stage III–IV, while the protective effect of choline was stronger in those with BMI <24, non-drinkers and stage I-II patients.

In sensitivity analysis that excluded women who had taken vitamin supplements, the results were equivalent to the original analysis (**Supplementary Table 5**). Furthermore, the association for dietary vitamin B6 and choline intake remained significant in the most elaborate model that included all eight micronutrients (**Supplementary Table 6**). We have also carried out analyses in quartile comparison and the results are consistent with those of tertiles (**Supplementary Table 7**). Vitamin B2, B3, B9, and choline did not show an association with OC survival when grouped according to RI (**Supplementary Table 8**).

TABLE 3 | Subgroup analyses for adjusted hazard ratio (HR) and 95% confidence intervals (CIs) for the association between dietary vitamin B_6 and choline intake and total mortality of ovarian cancer (n = 635).

Characteristics	Tertiles of energy-adjusted intake*							
	Vitamins B_6				Choline			
	I	II	III	$P_{interaction}$ **	I	II	III	$P_{interaction}$ **
Age at diagnosis (years)				0.32				0.75
≤50	1.00 (Ref)	0.31 (0.12–0.83)	0.84 (0.34–2.08)		1.00 (Ref)	0.67 (0.27–1.65)	0.81 (0.31–2.15)	
>50	1.00 (Ref)	0.49 (0.27–0.89)	0.39 (0.21–0.74)		1.00 (Ref)	0.55 (0.31–0.98)	0.40 (0.21–0.75)	
Menopausal status				0.45				0.38
No	1.00 (Ref)	0.17 (0.05–0.55)	0.55 (0.18–1.71)		1.00 (Ref)	0.65 (0.24–1.74)	0.49 (0.13–1.86)	
Yes	1.00 (Ref)	0.56 (0.32–1.00)	0.51 (0.29–0.91)		1.00 (Ref)	0.57 (0.33–1.00)	0.52 (0.29–0.95)	
Body mass index (kg/m^2)				0.83				0.09
<24	1.00 (Ref)	0.77 (0.40–1.45)	0.74 (0.38–1.42)		1.00 (Ref)	0.50 (0.26–0.96)	0.59 (0.31–1.15)	
≥24	1.00 (Ref)	0.19 (0.09–0.42)	0.25 (0.10–0.63)		1.00 (Ref)	0.81 (0.38–0.1.72)	0.42 (0.17–1.05)	
Alcohol drinking				0.42				0.80
No	1.00 (Ref)	0.54 (0.31–0.93)	0.60 (0.35–1.03)		1.00 (Ref)	0.62 (0.36–1.07)	0.44 (0.25–0.79)	
Yes	1.00 (Ref)	0.15 (0.04–0.54)	0.10 (0.02–0.46)		1.00 (Ref)	0.90 (0.29–2.77)	0.69 (0.17–2.85)	
Histological type				0.75				0.99
Serous	1.00 (Ref)	0.45 (0.26–0.79)	0.46 (0.25–0.84)		1.00 (Ref)	0.71 (0.40–1.25)	0.51 (0.27–0.95)	
Non-serous	1.00 (Ref)	0.45 (0.16–1.24)	0.59 (0.23–1.56)		1.00 (Ref)	0.47 (0.16–1.35)	0.40 (0.13–1.23)	
FIGO stage				0.89				0.96
I–II	1.00 (Ref)	0.53 (0.22–1.28)	0.47 (0.19–1.14)		1.00 (Ref)	0.23 (0.09–0.60)	0.21 (0.08–0.56)	
III–IV	1.00 (Ref)	0.35 (0.20–0.64)	0.47 (0.25–0.88)		1.00 (Ref)	0.73 (0.39–1.37)	0.52 (0.27–1.03)	
Residual lesions				0.48				0.25
No	1.00 (Ref)	0.52 (0.29–0.93)	0.48 (0.26–0.89)		1.00 (Ref)	0.50 (0.29–0.89)	0.43 (0.23–0.82)	
Yes	1.00 (Ref)	0.28 (0.11–0.73)	0.55 (0.22–1.35)		1.00 (Ref)	1.21 (0.52–2.82)	0.79 (0.29–2.15)	

CI, confidence interval; HR, hazard ratio; Ref, reference.

**Adjusted for energy by the residual method.*

***Test for interaction based on strata and dietary vitamin B6 and Choline intake. HR and 95% CI were calculated with the use of the Cox proportional hazards regression model with adjustment for age at diagnosis, body mass index, total energy, alcohol drinking, diet change, education, income, physical activity, menopausal status, parity, multivitamin use, multimineral use, red meat, methyl-donor index, comorbidities, FIGO stage, histological type, histopathologic grade, and residual lesions.*

DISCUSSION

The present study is one of the limited studies evaluating the role of pre-diagnosis dietary OCM nutrients in the survival of OC. This paper highlights the inverse, statistically significant relationship between pre-diagnostic dietary vitamin B6 and choline intake levels and OC survival. Null associations were observed for vitamins B2, B3, B9, B12, methionine, and betaine. Further, the relationship between vitamin B6 intake and OC survival was curvilinear.

To date, only two observational studies (25, 26) examined the association between pre-diagnosis OCM nutrients and OC survival. One study suggested null associations between OCM nutrients intake and OC survival, another study indicated that folate intake was significantly associated with a lower risk of OC death. However, our findings were partly inconsistent with both of them. Possible explanations for the discrepancy might be attributed to the different demographic and clinical characteristics of OC patients, FFQ measurements, dietary habits, sample size and follow-up periods. For example, compared with these two previous studies, we included a moderate sample size (635 vs. 1270 and 215) and had a shorter follow-up period (3.1 years vs. 10 and 4.02 years). In addition, more advanced FIGO stage III-IV (71.02% vs. 48.35%) and diagnostic age >50 (81.8% vs. 63.46%) were included in the study by Dixon et al. than us (25). Furthermore, Zhang et al. failed to adjust for key confounding factors including FIGO stage, tumor grade, and presence of residual disease (26). Dietary habits in different countries and different regions of the same country may also provide insights into these contradictions.

Our study suggested that higher intake of vitamin B6 and choline was associated with better OC survival among post-menopausal women. Our previous study suggested that pre-diagnosis cruciferous vegetables intake was only associated better survival of OC in postmenopausal patients when stratified by menopausal status (28). These results are consistent with previous studies that have shown a stronger effect of some dietary nutrients (such as alpha-carotene, h-cryptoxanthin) in reducing OC incidence in postmenopausal rather than in premenopausal women (39, 40). Studies have demonstrated that dietary nutrients intake can alter circulating levels of estrogen and other sex hormones (41). The possible mechanism might partially lie in that vitamin B6 and choline affect the ovarian synthesis of sex hormones or the alteration of other menstrual cycle characteristics (42). Vitamin B6 and choline may be effective only at low sex hormone concentrations which was shown in

postmenopausal women. Further studies are recommended to assess dietary OCM nutrients intake and OC survival separately in pre- and post-menopausal women.

A protective effect of vitamin B6 on OC survival is biologically plausible given vitamin B6's role as a cofactor for enzymes involved in the DNA synthesis and methylation pathways of OCM (43). A diet low in vitamin B6 results in a decreased production of the methyl donor, methylene-tetrahydrofolate (44) and eventually leads to chromosome breaks and thus involvement in tumor progression (45). In addition, laboratory studies have demonstrated that vitamin B6 is effective at scavenging free radicals which if not properly controlled can promote carcinogenesis (19), so vitamin B6 may influence OC through its antioxidant properties. The mean dietary intake of choline in our study was 279.17 mg/day, which is relatively lower than its recommended intake (RI = 400 mg/d). Choline is a methyl-group donor involved in OCM cycle, abnormal choline metabolism is emerging as a metabolic hallmark that is associated with oncogenesis and tumor progression (46, 47). Humans ingest approximately 50 mmol of methyl groups per day, and 60% of them are derived from choline. Animals fed diets deficient in methyl donors (choline) have hypomethylated DNA (48). These changes occur not only in global methylation, but also in the methylation of specific genes (49, 50), which can easily be influenced by changes in human diet. This proven scientific insight promises to enhance our understanding of how choline affects the prognosis of OC.

In our study, we found that the mean dietary intake of vitamin B2, B6, B9, B12, and choline was less than the Dietary Nutrient Reference Intakes for Chinese Residents. This phenomenon is also observed in several previous studies (19, 51–53). For example, the mean dietary vitamin B9 intake of OC patients were below the recommended intake (346 ug/d < RI = 400 ug/d) in a case-control study from the United States (19). A hospital-based case-control study from Hong Kong, China also showed that the mean dietary vitamin B6 (0.79 ug/d < RI = 1.6 ug/d) and B9 (207.4 ug/d < RI = 400 ug/d) intakes were relatively lower in breast cancer cases (52). However, these phenomena were not common in the general population (22, 54).

Currently little is known about the possible relation between vitamin-B12 and cancer risk. However, since vitamin-B12 has a key role in one-carbon metabolism and cells require one-carbon units for DNA synthesis, methylation as well as redox and reductive metabolism, vitamin-B12 may influence pathways enhancing the proliferation of cancer cells (55). However, we found a non-significant association between dietary vitamin B12 intake and OC survival. This might be partly attributed to the low intake of dietary vitamin B12 in OC patients (mean value is 0.14 μg/d). According to the ChineseDRIs, the value should be 2.4 ug/d. In addition, compared to several previous studies (25, 51, 53, 56), dietary vitamin B12 intake was relatively lower in our study. This difference might be attributed to the different study participants and dietary habit. Furthermore, coupled with the difficulty of accurately measuring vitamin B12 (57), this may lead to greater changes and weaken these links.

Strengths of our study include data that were prospectively collected in high-quality population-based registers, reducing bias in ascertainment of the exposure and outcomes. Another strength of this study is one of the limited studies to assess the association between pre-diagnosis OCM nutrients intake and OC survival. Also, the collection of numerous clinical and lifestyle covariates related to OC survival allowed for statistical adjustment of these factors to limit potential confounding. A reproducible and validated FFQ also enabled us to achieve a comprehensive dietary intake assessment of OCM nutrients.

Potential limitations of our study should also be considered. First, information on dietary intake and other covariates were self-reported, and therefore, non-differential misclassification of these variables resulting from recall and reporting biases was possible. However, we used a highly reproducible validated FFQ and selected highly trained and skilled researchers to collect information. Second, the current study was the single assessment of diet, which eliminated the possibility to examine dietary changes during follow-up. Whereas, we have adjusted for dietary change as a confounder. Third, because regular use of any type of supplement was rare in our cohort (<10%), we were only able to assess food sources of nutrients. Meanwhile, the finding of the sensitivity analysis in the population not taking B vitamin supplements was consistent with the main analysis. Fourth, we did not determine the internal levels of these nutrients in the body, and the relatively less precise assessment by the FFQ may have attenuated any associations. Lastly, we could not evaluate the association between OCM micronutrients and OC specific mortality because the data for the cause of death were not available. Thus, our findings should be interpreted cautiously and need to be confirmed by future studies.

In summary, this prospective cohort study demonstrated inverse associations between pre-diagnostic dietary vitamin B6 and choline and OC survival. Future large prospective cohorts should validate our findings and would improve our understanding of the role of OCM micronutrients intake in the prognosis of OC.

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 The Institutional Review Board of the Ethics Committee of Shengjing Hospital of China Medical University, Shenyang, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

H-LX, T-TG, and Q-JW conceived the study. T-TG, Y-HZ, SG, Y-SJ, and Q-JW contributed to the design. H-LX, T-TG, SY, and SG collected the data.

H-LX, F-HL, Y-FW, SY, and Q-JW cleaned the data and checked the discrepancy. H-LX, F-HL, Y-FW, and H-YC analyzed the data. H-LX, T-TG, F-HL, Y-FW, H-YC, and Q-JW interpreted the data. All authors interpreted the data, read the manuscript, and approved the final vision.

FUNDING

This work was supported by the Natural Science Foundation of China (Nos. 82073647 and 81602918 to Q-JW and No. 82103914 to T-TG), LiaoNing Revitalization Talents Program (No. XLYC1907102 to Q-JW), and 345 Talent Project of Shengjing Hospital of China Medical University (No. M0268 to Q-JW and No. M0952 to T-TG).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.873249/full#supplementary-material

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frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 26 April 2022
doi: 10.3389/fnut.2022.838617

Determination of the Effects of Duodenal Infusion Soy Protein Hydrolysate on Hepatic Glucose and Lipid Metabolism in Pigs Through Multi-Omics Analysis

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OPEN ACCESS

Edited by:
Ellen E. Blaak,
Maastricht University, Netherlands

Reviewed by:
Zhaolai Dai,
China Agricultural University, China
Qingbiao Xu,
Huazhong Agricultural University, China

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

Received: *18 December 2021*
Accepted: *01 April 2022*
Published: *26 April 2022*

Citation:
Li Z, Ding L, Zhu W and Hang S (2022) Determination of the Effects of Duodenal Infusion Soy Protein Hydrolysate on Hepatic Glucose and Lipid Metabolism in Pigs Through Multi-Omics Analysis. Front. Nutr. 9:838617. doi: 10.3389/fnut.2022.838617

High animal protein intake increases hepatic lipid deposition and the risk of diabetes. However, the effects of high plant protein (HPP) intake on glycaemic responses and hepatic lipid metabolism in healthy people, as well as the underlying mechanisms, remain unclear. The current study explored the metabolomic and transcriptomic responses in the livers of pigs to assess the effects of HPP intake on host glucose and lipid metabolism. Sixteen pigs were infused with sterile saline or soy protein hydrolysate (SPH; 70 g/day) through a duodenal fistula twice daily during a 15 days experimental period. Hepatic metabolomic and transcriptomic analyses were performed, and the serum and hepatic biochemical parameters were measured. The results revealed that SPH infusion decreased serum glucose, hepatic triglyceride (TG), total cholesterol and low-density lipoprotein cholesterol levels, while it increased serum urea and eight hepatic amino acid levels ($P < 0.05$). Hepatic metabolomics displayed that SPH treatment produced seven different metabolites, four of which were related to lipid metabolism and one was related to glucose metabolism. In particular, lower ($P < 0.05$) glycocholic acid and glucose 1-phosphate levels and higher ($P < 0.05$) phosphatidylethanolamine (PE), arachidonic acid, prostaglandin F2α, l-carnitine and indole-3 acetic acid levels were observed following SPH infusion. A further metabolic pathway enrichment analysis found that these differential metabolites were mainly enriched in pathways related to lipid and glucose metabolism. Hepatic transcriptomics also demonstrated that multiple genes related to glucose and lipid metabolism were affected by SPH ($P < 0.05$). Together, SPH infusion reduced the hepatic TG levels by accelerating fatty acid β-oxidation and inhibiting TG synthesis. In addition, SPH infusion reduced the serum glucose levels by promoting hepatic glucose uptake and glycolysis. This study's result demonstrated that HPP intake regulated glycaemic responses and hepatic lipid metabolism in pigs without increasing the risk of hepatic lipid deposition and hyperglycaemia.

Keywords: soy protein hydrolysate, high plant protein, metabolome, transcriptome, liver, pigs

INTRODUCTION

The intake of high-protein (HP) diet as a daily dietary practice or as part of a lifestyle pattern has recently been increasing. HP diet is related to weight loss and induces several positive metabolic effects, such as control and correction of certain risk factors related to the metabolic syndrome (1). However, there is scientific controversy about the safety of HP diet consumption. Some studies have reported that high animal protein intake increases hepatic lipid deposition and the risk of diabetes in healthy animals (2, 3). Compared to plant protein, high animal protein intake is often accompanied by the intake of animal fat and cholesterol. Several comparison experiments have proved that plant protein has a more prominent metabolic protection effect than animal protein (4, 5). However, the effect of high plant protein (HPP) intake on glycaemic responses and hepatic lipid metabolism in healthy animals, as well as its underlying mechanisms, remain unclear.

Soy protein is a high-quality protein derived from soybeans and provides an abundant source of dietary protein for humans and animals. For a long time, the associated health benefits of soy protein have received considerable attention, especially in regulating the host metabolism. In this study, soy protein was selected and hydrolysed into soy protein hydrolysate (SPH), which mimics the dietary proteins digested in the luminal chyme. The small intestine is a key site for dietary protein digestion and metabolites to be absorbed and transported into the liver. In this study, a duodenal fistula technique, which allows nutrients to directly act on the small intestine, was used. A pig was chosen as the model animal because its physiological structure is very similar to that of humans (6). Previous studies have investigated the effects of free amino acids and milk on hepatic amino acid absorption and metabolism using duodenal fistula pigs (7, 8).

Liver is a key organ for lipid metabolism. Hepatic lipid levels can be affected by various potential pathways, including lipid uptake and export, fatty acid β-oxidation, and lipid synthesis (9). Therefore, the mechanisms through which nutrients regulate hepatic lipid metabolism are complicated. Transcriptome analysis using RNA-sequencing (RNA-seq) can provide an unbiased overview of alterations that can occur at the molecular level (10). Metabolomics can analyse numerous metabolites from biological samples and provide an overview of metabolic changes related to dietary intake (11). Combined transcriptomic and metabolomic analyses in liver are highly valuable for comprehending the mechanisms through which HPP intake regulates the host lipid metabolism. In addition, serum glucose levels are closely related to hepatic glucose metabolism, and hepatic omics analysis facilitates the illustration of the mechanism of HPP intake for regulating glycaemic responses.

In this study, SPH was infused in the duodenum to evaluate the effects of HPP intake on host glucose and lipid metabolism and reveal the underlying mechanisms by which HPP diet regulates the serum glucose and hepatic lipid levels in a fistula pig model, through transcriptome and metabolome analyses.

MATERIALS AND METHODS

SPH Preparation

The SPH was prepared following a previous method with slight modification (12). Briefly, the soy protein isolate (SPI, protein content 90%; Yuanye Biotechnology, Shanghai, China) was suspended in distilled water (10%, w/v) and adjusted to pH 2.0 using 2 N HCl. Then, the porcine pepsin (800–1000 U/mg protein; Yuanye Biotechnology) was added to hydrolyse SPI at an enzyme-to-substrate ratio of 1:100 (w/w). After 1 h of constant agitation at 37°C, the reaction was terminated by adjusting the solution pH to 7.0 with 2 N NaOH. The obtained hydrolysate was frozen at −20°C and lyophilised in a FreeZone 4.5 l Freeze Dry System (Labconco Co., Kansas City, MO, USA) for further use.

Animals and Experimental Procedures

The Animal Welfare and Health Committee of Nanjing Agricultural University approved the experimental design and procedures. Sixteen castrated pigs (Duroc × Landrace × Large White, aged 50 days) with an initial weight of 14.5 ± 0.2 kg were obtained from a local commercial pig farm in Nanjing, China. All pigs were housed in individual metabolic cages under a controlled temperature of 25 ± 2°C and given unlimited access to water and feed. After a week of acclimatization, the pigs were made to fast for 12 h before installing a simple T-cannula (8.2 cm length, 10 cm width and 1.5 cm internal diameter) in the duodenum (just posterior to the pancreatic and bile duct) (13). After the surgery, all pigs were hypodermically injected with ceftriaxone sodium and treated with iodine tincture in the wound and adjacent skin for 1 week (twice a day) to avoid potential infection. After they fully recovered from the duodenal fistula surgery over a 2-week recuperation period, a 2-week short-term experiment of SPH on the secretion of an intestinal satiety hormone was performed (14). After a week of recovery, all pigs were randomly allocated to the control (CON, $n = 8$) group and SPH group ($n = 8$) with no differences in body weight (35.2 ± 0.3 kg) and feed intake. The entire experimental period was 15 days, during which the pigs in the CON and SPH groups were infused with 10 ml sterile saline and 10 ml SPH solution (70 g/day), respectively, through a duodenal fistula at 8:00 a.m. and 5:00 p.m. each day. The SPH solution was adjusted to pH 5.0, which is close to the native pH of porcine duodenum (15). The basal diet in the experiment was designed based on the National Research Council (NRC; 2012; **Supplementary Table S1**). The feed consumption of each pig was recorded every day to calculate average feed intake. In addition, the body weights of all pigs were recorded on days 1 and 16 to determine average weight gain.

Sample Collection

All pigs were slaughtered after they fasted for 12 h on day 16. Blood samples (10 ml) were obtained from their jugular vein and centrifuged for 15 min (4°C, 3000 × g) to collect serum, which was then stored at −20°C. The livers of all pigs were obtained and immediately frozen in liquid nitrogen. The serum and liver tissues were thawed on ice before use.

Biochemical Indicator Analysis

The serum biochemical indicators were determined using an Olympus AU2700 biochemical analyser (Olympus Optical Co., Ltd. Tokyo, Japan), including aspartate aminotransferase (AST), alanine aminotransferase, total protein, albumin, globulin, albumin/globulin (A/G), urea, glucose, total cholesterol (T-CHO), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). Hepatic TG (Cat No. A110-1-1), T-CHO (Cat No. A111-1-1), HDL-C (Cat No. A112-1-1) and LDL-C (Cat No. A113-1-1) levels were analyzed using commercial biochemical assay kits following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institution, Nanjing, China).

Amino Acid Analysis

First, 100 mg of the liver samples were put in a 2 ml sterile centrifuge tube containing 0.5 ml of 0.5 mm silica/zirconium beads (BioSpec, Cat No. 11079105z) and 500 μl 0.9% saline solution. Homogenization was performed on FastPrep®-24 Instrument (MP Biochemicals, LLC, CA, USA) using 4–5 cycles at 6 m/s for 30–40 s with 2 min incubation time on ice between cycles. Then, the mixture was centrifuged (3000 × g, 4°C, 15 min) to obtain supernatants. Next, a 200 μl liver supernatant was collected and mixed with 1 ml 5% sulfosalicylic acid solution, and then maintained at 4°C for 30 min. Subsequently, the mixture was centrifuged for 20 min (4°C, 20,000 × g) and filtered with a filter (0.22 μm) for further analysis. Hepatic amino acid concentrations were measured using an LA8080 automatic amino acid analyser (Hitachi, Tokyo, Japan) following the method proposed by Kim et al. (16).

Hepatic Metabolomic Profiles

Based on a previously described method (17), liver tissues were prepared from the CON and SPH groups for liquid chromatograph–mass spectrometer (LC–MS) analysis. The tissue samples (∼50 mg) were homogenized in pre-cooled 50% methanol buffer (120 μl) using a high-throughput TissueLyser (Xinzhi Biotechnology Co., Ltd. Ningbo, China). The mixture was maintained on ice for 10 min and then centrifuged (4000 × g, 4°C, 20 min) to collect supernatants for subsequent analysis. All chromatographic separations were analyzed using an ultra-performance liquid chromatography (UPLC) system (Ultimate 3000; Dionex, CA, USA) equipped with an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.7 μm, Waters, MA, USA). The SIMCA-P software (version 13.0; Umetrics AB, Umea, Sweden) was used to establish the partial least-squares discriminant analysis (PLS-DA) model. The metabolites based on variable importance in the projection (VIP) value > 1.0 and *P*-value < 0.05 were considered as the differential metabolites between the CON and SPH groups. The metabolic pathways and metabolite set enrichment analysis were conducted using the MetaboAnalyst (version 5.0) tool.

Transcriptome Sequencing Analyses

Total RNAs were isolated from the liver tissues using a TRIzol Reagent (Invitrogen, CA, USA, Cat No. 15596018) following the manufacturer's procedure. Then, Bioanalyzer 2100 and RNA 6000 Nano Lab Chip Kit (Agilent, CA, USA, Cat No. 5067-1511) were used to verify the RNA quantity and purity with RNA integrity number >7.0. High-quality RNA samples were collected for subsequent analysis. Under high-temperature conditions, divalent cations were used to fragment the purified mRNA into small fragments. The mRNA fragments were reverse transcribed to establish the cDNA library following the protocol for the mRNA-seq sample preparation kit (Illumina, San Diego, CA, USA, Cat No. RS-122-2101). Then, paired-end sequencing was performed on the Illumina HiSeq 4000 platform (LC Sciences, Santiago, CA, USA) at Shanghai Biozeron Biotechnology Co., Ltd. (Shanghai, China). To screen differential expression genes (DEGs) between the CON and SPH groups, the expression level for each gene was quantified following the fragments per kilobase of exon per million mapped reads method. We selected differential genes based on fold changes (FCs) ≥1.5 and *P*-values < 0.05. We used the ggplot2 package to draw a visual volcano map between different objects with the R software. GOATOOLS (https://github.com/tanghaibao/Goatools) and Kobas (http://kobas.cbi.pku.edu.cn/home.do) were used for gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, respectively.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA of liver tissues was extracted from the CON and SPH groups with the RNApure Total RNA Kit (Vazyme, Nanjing, China, Cat No. RC112-01). The concentration of the extracted RNA was determined using a Nano-Drop spectrophotometer (ThermoFisher Scientific, Wilmington, DC, USA) and adjusted to 500 ng/μl. The Roche SYBR Green PCR Kit (Vazyme, Nanjing, China, Cat No. Q221-01) was used to perform the polymerase chain reaction (PCR) in a final volume of 20 μl. Quantitative real-time PCR (qRT-PCR) was performed using the primers purchased from Invitrogen Life Technologies (Invitrogen, Shanghai, China), whose sequences are presented in **Supplementary Table S2**. For subsequent analysis, the average threshold cycle (C_t) was calculated according to the triplicate qRT-PCR values of each cDNA. The formula $2^{-\Delta\Delta Ct}$ was used to analyse the relative expression of the gene.

Statistical Analysis

Biochemical indices, amino acid concentrations and qRT-PCR data were presented as mean ± standard error of the mean (SEM). All data were compared in SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and graphs were drawn using GraphPad Prism 8.0.2 (La Jolla, CA, USA). Data were analyzed using a Student's *t*-test, and significant differences were observed when $P < 0.05$.

RESULTS

Growth Performance and Protein Levels

The average daily feed intake, average daily gain, and ratio of feed to gain were not markedly different between the CON and SPH pigs ($P > 0.05$; **Supplementary Table S3**). The protein levels of the CON group (18.0% protein) and the SPH group

TABLE 1 | Serum and hepatic parameters in the CON and SPH groups.

Items	CON	SPH	*P*-value
Serum parameters			
AST (U/l)	40.43 ± 1.53	45.38 ± 5.01	0.372
ALT (U/l)	61.14 ± 2.55	69.88 ± 3.83	0.089
TP (g/l)	67.94 ± 2.20	69.75 ± 3.08	0.243
ALB (g/l)	37.37 ± 1.16	36.68 ± 1.05	0.288
GLOB (g/l)	30.57 ± 1.90	33.07 ± 2.16	0.084
A/G	1.27 ± 0.05	1.10 ± 0.04	0.027
Urea (mmol/l)	5.04 ± 0.29	6.47 ± 0.56	0.045
Glucose (mmol/l)	7.19 ± 0.37	6.06 ± 0.29	0.034
T-CHO (mmol/l)	2.38 ± 0.13	2.23 ± 0.16	0.789
TG (mmol/l)	0.55 ± 0.03	0.45 ± 0.02	0.018
HDL-C (mmol/l)	1.15 ± 0.07	1.11 ± 0.07	0.644
LDL-C (mmol/l)	1.39 ± 0.05	1.39 ± 0.11	0.953
Hepatic parameters			
TG (mmol/gprot)	0.15 ± 0.02	0.11 ± 0.01	0.047
T-CHO (mmol/gprot)	0.04 ± 0.01	0.01 ± 0.00	0.026
LDL-C (mmol/gprot)	2.08 ± 0.30	1.19 ± 0.21	0.030
HDL-C (mmol/gprot)	9.60 ± 1.65	10.12 ± 1.25	0.808

Values are mean ± SEM (n = 8). P < 0.05 was considered statistically significant. AST, aspartate transaminase; ALT, alanine aminotransferase; TP, total protein; ALB, albumin; GLOB, globulin; ALP, alkaline phosphatase; A/G, albumin/globulin; T-CHO, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

TABLE 2 | Hepatic amino acid levels in the CON and SPH groups.

Items	CON	SPH	*P*-value
Leucine	2.80 ± 0.07	3.24 ± 0.38	0.361
Valine	2.07 ± 0.08	2.77 ± 0.17	0.007
Isoleucine	1.14 ± 0.04	1.47 ± 0.09	0.013
Tryptophan	1.32 ± 0.05	1.55 ± 0.06	0.027
Tyrosine	1.56 ± 0.05	1.87 ± 0.08	0.011
Serine	2.34 ± 0.65	4.50 ± 0.55	0.027
Lysine	2.53 ± 0.11	3.24 ± 0.18	0.010
Aspartic acid	0.61 ± 0.16	1.05 ± 0.11	0.049
Glutamate	7.62 ± 0.13	8.47 ± 0.97	0.486
Methionine	0.80 ± 0.03	1.09 ± 0.07	0.005
Proline	2.84 ± 0.09	3.76 ± 0.24	0.011
Alanine	6.56 ± 0.23	6.88 ± 0.72	0.718
Cysteine	0.22 ± 0.00	0.22 ± 0.01	0.957
Phenylalanine	1.60 ± 0.03	1.89 ± 0.13	0.078
Histidine	1.22 ± 0.06	1.32 ± 0.05	0.189
Arginine	0.28 ± 0.04	0.44 ± 0.13	0.279
Glycine	9.89 ± 0.36	10.46 ± 1.09	0.680

Values are mean ± SEM (n = 8); P < 0.05 was considered statistically significant.

(21.4% protein) were calculated based on the dietary composition (**Supplementary Table S1**) and average daily feed intake.

Serum and Hepatic Biochemical Parameters

Table 1 demonstrates that lower ($P < 0.05$) serum TG, glucose and A/G levels and higher ($P < 0.05$) serum urea levels were observed in the SPH group. The levels of other serum parameters did not differ between the CON and SPH groups ($P > 0.05$). The hepatic TG, T-CHO and LDL-C levels decreased following SPH infusion ($P < 0.05$), whereas the HDL-C levels remained unaffected ($P > 0.05$).

Hepatic Amino Acid Concentrations

The concentration of amino acids in the liver, including valine, isoleucine, tyrosine, serine, lysine, aspartic acid, methionine and proline, were higher in the SPH group than the CON group ($P < 0.05$), whereas other amino acid concentrations did not show the difference between the two groups ($P > 0.05$; **Table 2**).

Metabolome Profiles in Liver

Metabonomic analysis was conducted using LC–MS to understand the hepatic metabolic changes that occurred after SPH treatment. The results of the PLS-DA analysis showed that samples in the SPH group were significantly discriminated from those in the CON group (**Figure 1A**). The differential metabolites between the two groups are presented in **Table 3**. SPH treatment significantly altered seven metabolites, of which four were involved in lipid metabolism, one was related to glucose metabolism and the other two were produced by amino acid metabolism. In particular, SPH infusion increased the levels of phosphatidylethanolamine (PE), arachidonic acid, prostaglandin F2α, L-carnitine and indole-3 acetic acid and decreased the levels of glycocholic acid and glucose 1-phosphate. The results of the metabolic pathway enrichment analysis revealed that these differential metabolites were mainly enriched in pathways related to lipid and glucose metabolism, including arachidonic acid metabolism, beta oxidation of very-long-chain fatty acids, alpha linolenic acid and linoleic acid metabolism, glycolysis and gluconeogenesis (**Figure 1B**).

Hepatic Transcriptomics Analysis

To clarify the molecular processes of SPH infusion, RNA-seq was performed to analyse hepatic gene expression profiles. Following SPH treatment, 452 DEGs (352 up-regulated and 100 down-regulated) were identified from 17,521 genes (**Figure 2A**). The genes related to lipid metabolism, including elongation of very-long chain fatty acid protein 2 (*ELOVL2*), peroxisome proliferator-activated receptor alpha (*PPARα*), long-chain acyl-CoA synthetase 6 (*ACSL6*), ethanolaminephosphotransferase 1 (*EPT1*), diacylglycerol O-acyltransferase 1 (*DGAT1*), diacylglycerol O-acyltransferase 2 (*DGAT2*), hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*) and 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), were up-regulated by SPH treatment. Amino acid metabolism-related genes, including amino acid transporters solute carrier family 38 member 1 (*SLC38A1*) and solute carrier family 7 member 1 (*SLC7A1*), were up-regulated in the SPH group. The glucose transport gene glucose transporter type 2 (*GLUT2*) and glycolysis genes, including 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*PFKFB3*), hexokinase domain component

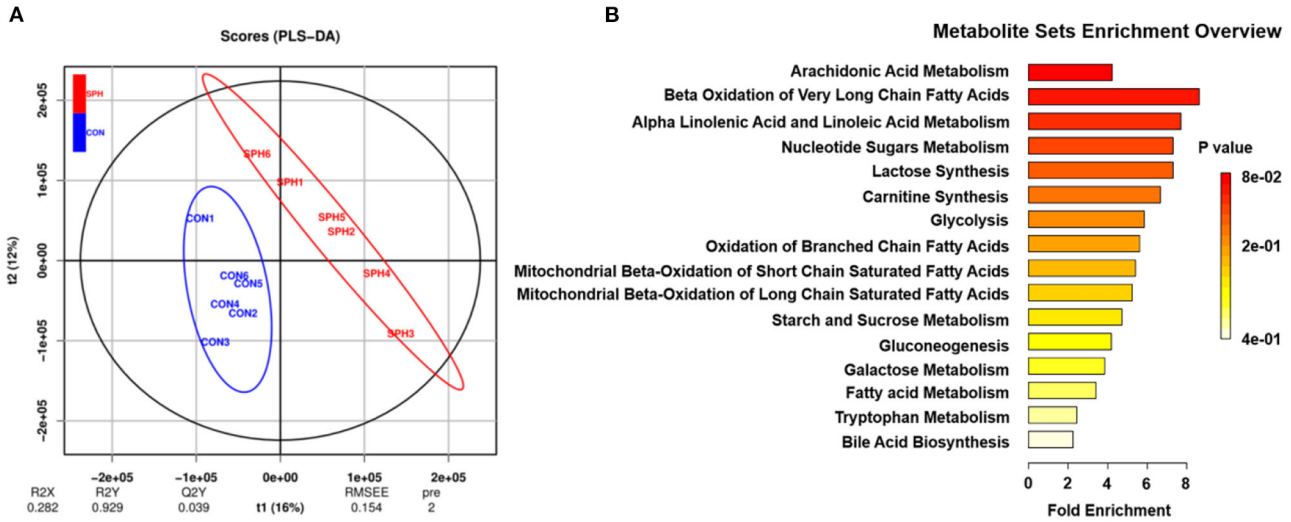

FIGURE 1 | Liver metabolome of the CON and SPH groups. **(A)** PLS-DA of metabolites (n = 6). t1 explains 16% of the variation; t2 explains 12% of the variation. **(B)** Metabolite set enrichment overview map of the differential metabolites (VIP > 1.0 and $P < 0.05$) screened. The color of the rectangle indicates the *P*-value and its length indicates the pathway impact values. Longer length and darker colors indicate greater pathway enrichment and higher pathway impact values, respectively.

TABLE 3 | Differential metabolites in the liver between the CON and SPH groups.

Metabolites	Metabolic subpathway	log_2(FC)	*P*-value	VIP
l-carnitine	Carnitine synthesis	0.56	0.02	1.26
Glucose 1-phosphate	Glycolysis/gluconeogenesis	−0.75	0.02	1.77
Prostaglandin F2α	Arachidonic acid metabolism	0.54	0.03	1.10
Phosphatidylethanolamine	Glycerophospholipid metabolism	0.94	0.03	1.73
Arachidonic acid	Arachidonic acid metabolism	0.69	0.04	1.31
Glycocholic acid	Bile acid biosynthesis	−0.91	0.04	2.56
Indole-3-acetic acid	Tryptophan metabolism	0.56	0.05	1.12

log_2(FC), the logarithmic transformation of fold change for the relative abundance of metabolites in SPH over CON. The P-value was obtained from the Wilcoxon–Mann–Whitney test and a value of <0.05. VIP was obtained from the partial least-squares discriminant analysis model with a threshold of 1.0.

1 (*HKDC1*) and lactate dehydrogenase B (*LDHB*), were up-regulated by SPH infusion (**Table 4**). Furthermore, GO enrichment analysis and KEGG pathway enrichment analysis were conducted on DEGs.

The result of the GO enrichment analysis is presented in **Figure 2B**. At the biological process level, several GO terms in the SPH group were involved in lipid metabolism, such as the response to lipid, fatty acid metabolic process, unsaturated fatty acid metabolic process and arachidonic acid metabolic process. Moreover, the GO term of the carbohydrate metabolic process was enriched in the SPH group. At the cellular component level, most genes were significantly enriched in the extracellular region and extracellular space. At the molecular function level, most genes were represented in the protein-containing complex binding and peptidase activity.

When the DEGs were portrayed to KEGG pathways, SPH treatment significantly changed 15 pathways (**Figure 2C**). Several pathways were related to lipid metabolism, including fat digestion and absorption, arachidonic acid metabolism, glycerolipid metabolism and steroid hormone biosynthesis. In addition, SPH treatment significantly affected lysine biosynthesis, glucagon signaling pathway and other pathways. Both GO and KEGG analyses significantly changed the pathways related to lipid and carbohydrate metabolism.

Confirmation of Sequencing Results Obtained by qRT-PCR

Quantitative real-time PCR (qRT-PCR) analysis was performed, focusing on the most prominent pathways, to validate the transcriptome analysis result (**Figure 3**). As the SPH treatment strongly altered hepatic lipid metabolism, the following six lipid metabolism-related genes were verified: *PPARα*, *EPT1*, *ELOVL2*, *HMGCR*, *DGAT1* and *DGAT2*. In addition, *PFKFB3* involved in glycolysis and *GLUT2* involved in glucose uptake were also determined. These genes were consistent with the expression pattern observed in the transcriptomic analysis, which confirmed the reliability of the transcriptome data.

DISCUSSION

Dietary protein is mainly digested and absorbed in the small intestine, and its metabolites can be transported to the liver through blood circulation for a direct effect on hepatic metabolism (18). In this study, SPH increased eight amino acid levels in the liver, including lysine, methionine and serine, and increased the expression of genes related

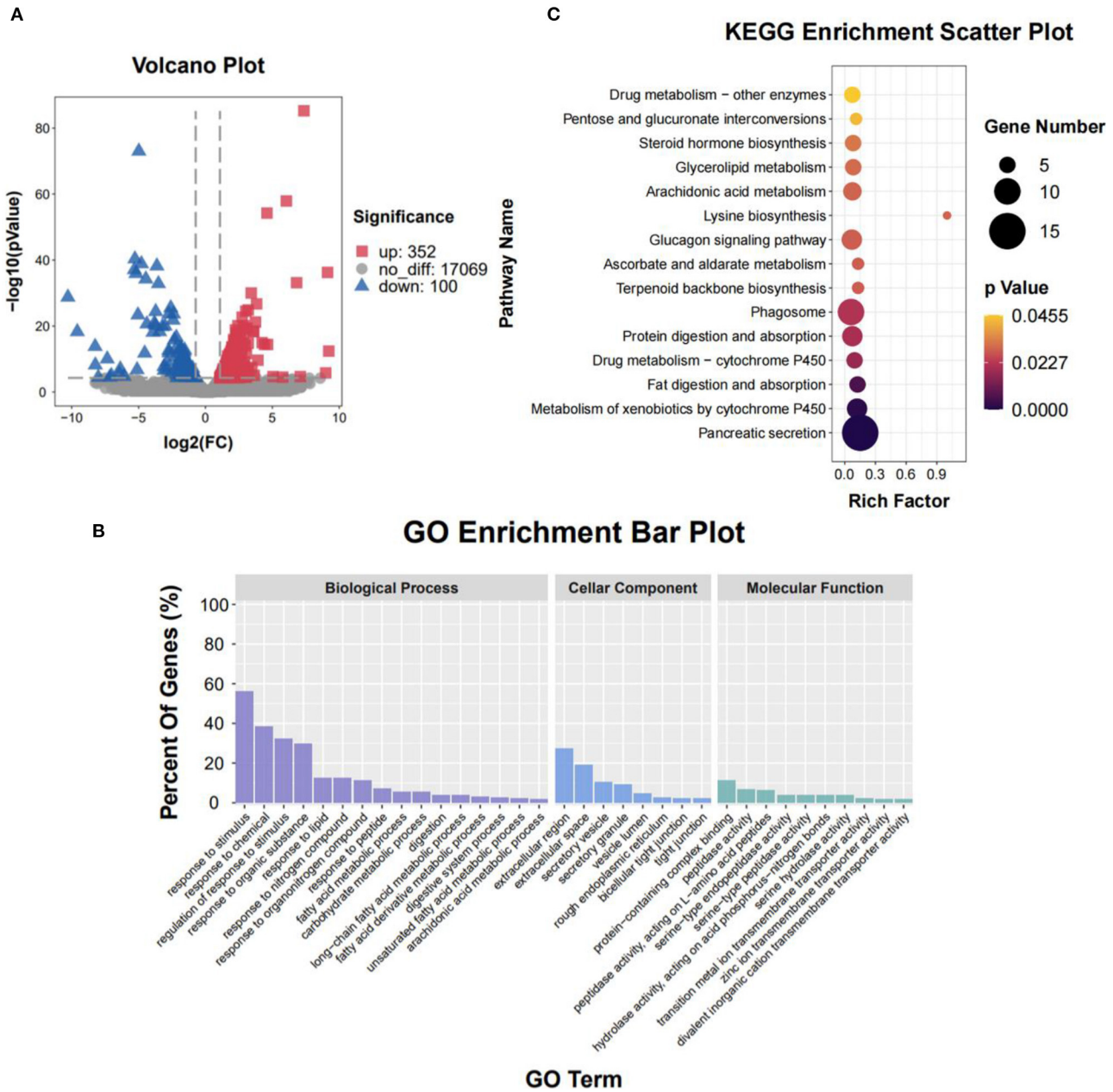

FIGURE 2 | Liver transcriptome of the CON and SPH groups. **(A)** Volcano plot of distribution trends for differential expression genes (DEGs). Down-regulation and up-regulation are presented with blue and red dots, respectively ($n = 3$). **(B)** Gene ontology (GO) pathway enrichment analysis of DEGs. Each annotated sequence is divided into at least one GO term of the following: biological process, cellular component or molecular function. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs. The size of the circles indicates the number of genes enriched in the pathway, and its color indicates the *P*-value.

to amino acid transport, including *SLC38A1* and *SLC7A1*, which are involved in the transport of neutral and cationic amino acids, respectively (19). The amino acids absorbed into the liver participate in multiple metabolic pathways, and excess amino acids produce urea through the urea cycle. In this study, SPH infusion also increased serum urea production. Previous studies indicated that rats and pigs fed an HP diet increased amino acid uptake and activated the hepatic urea cycle and increased serum urea production (2, 20).

Previous studies have demonstrated that HP diet decreases postprandial blood glucose levels and improves glucose tolerance in individuals with type 2 diabetes (21, 22). However, Sluijs et al. (3) revealed that high animal protein (meat, milk and casein)

TABLE 4 | Most relevant genes affected by SPH infusion in the liver.

Metabolic pathway	Gene name	Gene symbol	log_2(FC)	*P*-value
Carbohydrate metabolism	Glucose transporter type 2	*GLUT 2*	1.33	3.72E-07
	Hexokinase domain component 1	*HKDC1*	1.03	8.33E-04
	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3	*PFKFB3*	1.68	1.35E-11
	Lactate dehydrogenase B	*LDHB*	4.59	4.77E-55
	Fructose-1,6-bisphosphatase isozyme 2	*FBP2*	−0.90	2.22E-05
Amino acid metabolism	Amino acid transporters solute carrier family 38 member 1	*SLC38A1*	0.92	1.89E-03
	Solute carrier family 7 member 1	*SLC7A1*	0.95	1.62E-03
Lipid metabolism	Elongation of very-long chain fatty acid protein 2	*ELOVL2*	1.14	3.88E-05
	Peroxisome proliferator-activated receptor alpha	*PPARα*	2.32	8.33E-18
	Long-chain acyl-CoA synthetase 6	*ACSL6*	4.19	2.55E-03
	Hydroxy-3-methylglutaryl-CoA synthase 1	*HMGCS1*	0.99	3.38E-04
	3-hydroxy-3-methylglutaryl-CoA reductase	*HMGCR*	1.65	1.94E-10
	Ethanolaminephosphotransferase 1	*EPT1*	2.94	5.26E-20
	Diacylglycerol O-acyltransferase 1	*DGAT1*	−0.89	1.19E-05
	Diacylglycerol O-acyltransferase 2	*DGAT2*	−1.70	1.17E-05

log_2(FC), the logarithmic transformation of fold change for expression levels of genes in SPH over CON. The gene expressions are significantly affected when FC > 1.5 and P < 0.05.

intake increases the risk of diabetes in healthy people, which is a metabolic disease characterized by hyperglycaemia. In this study, SPH infusion reduced the serum glucose levels, indicating that HPP intake does not promote the occurrence of hyperglycaemia. The metabolite profile showed that SPH infusion reduced the levels of hepatic glucose 1-phosphate, which suggested that SPH accelerated hepatic glucose metabolism. Transcriptome analysis showed that glycolysis-related genes *HKDC1*, *PFKFB3* and *LDHB* were up-regulated by SPH. Fructose-1,6-bisphosphatase can hydrolyse fructose-1,6-bisphosphate to produce fructose-6-phosphate (3). *HKDC1* encodes a protein that catalyzes glucose to produce glucose-6-phosphate (23). The key enzyme of glycolysis, 6-phosphofructo-1-kinase, is positively regulated by fructose 2,6-bisphosphate, which is catalyzed and synthesized by PFKFB3 (24). LDHB catalyzes the interconversion of lactate and pyruvate (25). Therefore, SPH infusion accelerated hepatic glycolysis. Stepien et al. (26) found that Wistar rats fed a diet containing 50% milk protein inhibited glycolysis in the liver after 14 days of intervention. The reasons for this difference are unclear, but it is attributable to the different protein types. In addition, *GLUT2* was up-regulated by SPH. Studies on *Glut2*-deficient mice have shown that *GLUT2* is necessary for glucose uptake but not for glucose output (27). The expression of *GLUT2* indicated that the circulation of glucose uptake was promoted in the liver, which might be in response to the acceleration of glycolysis.

Triglycerides are a significant indicator when assessing lipid metabolism. Garcia et al. (28) proved that compared to a low-protein diet, HP diet reduces hepatic TG deposition in steatosis mice. Diaz-Rua et al. (2) investigated the safety of long-term intake of HP diet and found that Wistar rats fed a high-casein diet had increased liver TG deposition and exhibited health risk signs after 4 months of intervention. In the current study, SPH infusion decreased hepatic TG levels, indicating that HPP intake did not increase the risk of TG deposition. Kozaczek et al. (4) performed RNA-seq on liver samples of obese Zucker rats fed with SPI for 16 weeks and found that SPI might have inhibited the liver steatosis by enhancing lipid conversion and transport. However, the mechanisms through which HPP intake inhibited hepatic TG accumulation might be different in normal and obese animal models. In the present study, both metabolome and transcriptome analyses showed that SPH treatment affected the lipid metabolism pathways. In addition, *PPARα* was up-regulated following SPH treatment. Tovar et al. (29) showed that, in the liver of obese rats, soy protein supplementation promoted *PPARα* expression, leading to increased β-oxidation of fatty acid. In addition, SPH increased hepatic *ACSL6* expression and L-carnitine levels in this study. ACSL6 converts long-chain fatty acids to acyl-CoAs (30). Previous studies showed that HP intake increased the concentration of lysine and methionine, which increased L-carnitine production in the liver in pigs (31, 32). L-carnitine can transport acyl-CoA into the mitochondrial matrix to promote fatty acid β-oxidation, which can be biosynthesized by L-lysine and L-methionine as substrates in the body (33), suggesting that SPH infusion accelerates fatty acid β-oxidation in the liver.

Lipid reduction might also be related to the inhibition of TG synthesis. French et al. (34) showed that HP diet reduced the expression of the fatty acid synthase, thereby inhibiting hepatic fat deposition in obese Zucker rats. In their study, *DGAT1* and *DGAT2* were down-regulated by SPH, which are the key enzymes that catalyze TG formation from diacylglycerol (DG) (35). This indicates that SPH treatment inhibited TG synthesis. Furthermore, SPH infusion increased PE production and up-regulated *EPT1* expression. *EPT1* was identified and believed to be responsible for catalyzing the final step of the PE synthesis (36). Previous studies also found that dietary soy protein supplementation altered the fatty acid profile and increased PE content in the liver in mice and pigs (37, 38). The synthesis of TG and PE requires a common substrate, DG. When the synthesis of either TG or PE was blocked, DG was redistributed to synthesize the other (35). Therefore, SPH infusion can break the balance of TG and PE syntheses by regulating the hepatic gene expression, thereby reducing TG deposition. SPH infusion increased hepatic arachidonic acid levels and up-regulated *ELOVL2* expression in this study. In mammals, *ELOVL2* encodes an elongase that catalyzes the elongation of monounsaturated and polyunsaturated fatty acids (39). Unsaturated fatty acids are an important component of PE, whose synthesis provides the necessary substrate for PE synthesis.

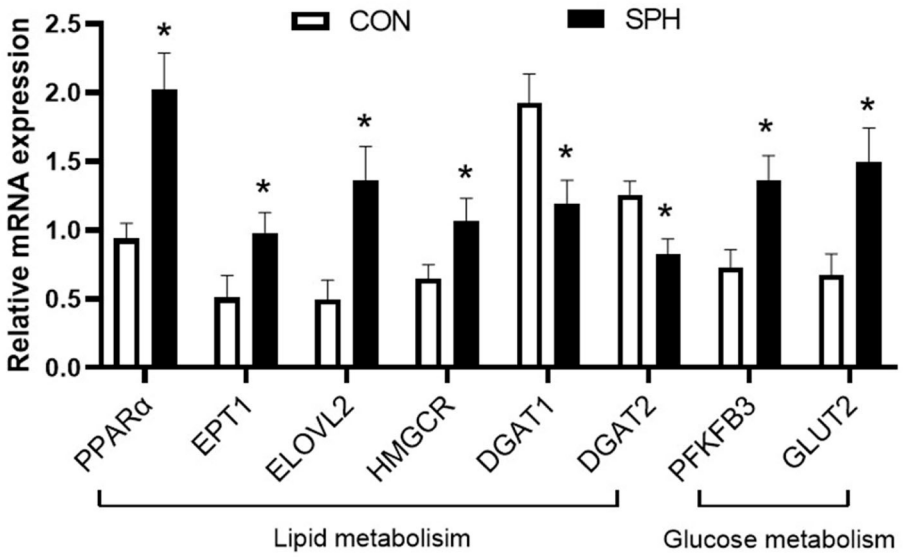

FIGURE 3 | qRT-PCR analysis of DEGs in the CON and SPH groups. Values are mean ± SEM ($n = 8$). *$P < 0.05$.

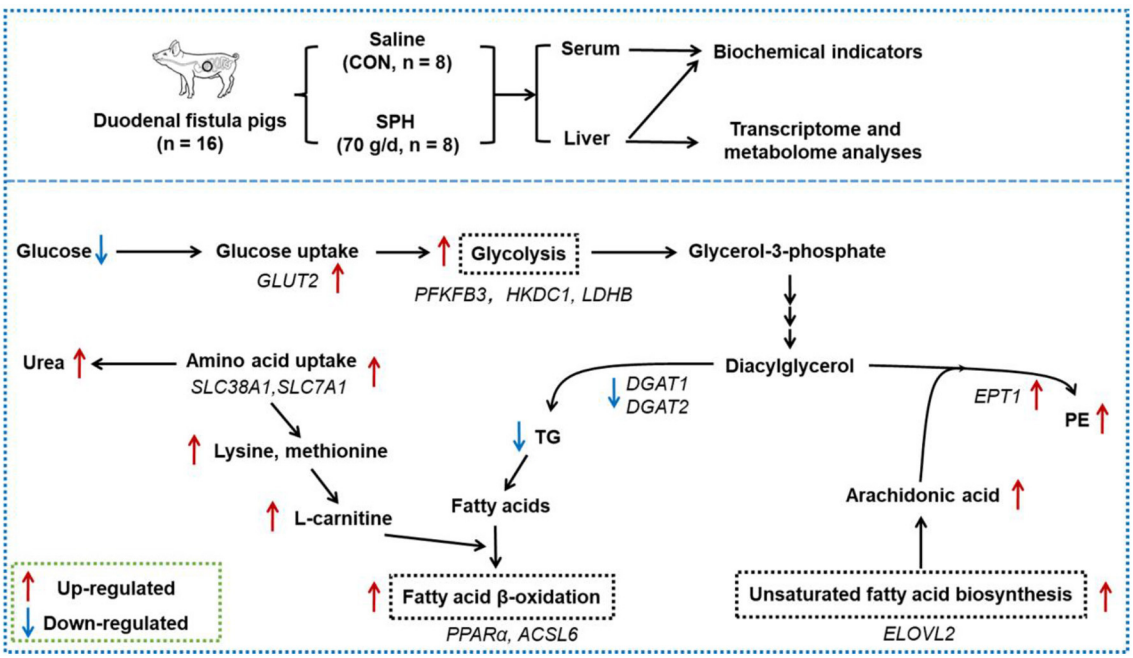

FIGURE 4 | Effects of the duodenal infusion SPH on liver metabolism. The red arrows represent the up-regulated level of genes and metabolites in the SPH group compared to the control (CON) group; the blue arrows represent the down-regulated level of genes and metabolites in the SPH group compared to the CON group.

Additionally, the effect of HPP intake on cholesterol metabolism has been evaluated. Tovar et al. (29) confirmed that compared to casein intake, soy protein supplementation significantly reduced hepatic cholesterol levels in obese mice. In their study, SPH infusion reduced the hepatic T-CHO and LDL-C levels, indicating that HPP intake did not cause hepatic cholesterol accumulation (40). A previous study demonstrated that hepatic cholesterol homeostasis was affected by lipoprotein synthesis, secretion to the blood and reuptake into the liver (9). However, the serum T-CHO and LDL-C levels were not altered in this study. Hence, the reduction in the hepatic cholesterol levels by SPH infusion might not be due to alterations in the lipoprotein flow. Yang et al. (41) revealed that soy protein intake reduced hepatic cholesterol levels of non-alcoholic steatohepatitis rats, which resulted from an increase in the bile acid synthesis and secretion. However, the

metabolome analysis showed that SPH reduced the hepatic glycocholic acid levels. Nagao et al. (42) found that SPI reduced the lipodystrophy-induced hepatic cholesterol accumulation by inhibiting the expression of cholesterol synthesis genes. Unexpectedly, *HMGCS1* and *HMGCR* were up-regulated by SPH infusion, which are the key enzymes that promote the *de novo* synthesis of cholesterol (43). This difference may be attributable to the different models used. Most previous studies have used lipid deposition models in animals to evaluate the effect of soy protein on lipid metabolism (44–46), whereas the present study used a normal pig model. Cholesterol is an indispensable macronutrient that has several significant functions in animals. Therefore, cholesterol homeostasis is vital for maintaining appropriate cellular and systemic functions. There might be a negative feedback mechanism that regulates the cholesterol synthesis. When SPH treatment reduces the cholesterol levels to the threshold, the liver initiates a negative feedback mechanism to accelerate cholesterol synthesis and inhibit cholesterol degradation.

Note that SPH infusion significantly affects the glucagon signaling pathway, which is related to glucose and lipid metabolism. Therefore, the glucagon signaling pathway might be involved in SPH-regulated hepatic metabolism; however, this needs to be further verified. These findings provide new data for evaluating the safety and hepatic metabolic effects of HPP intake in healthy animals.

CONCLUSION

High plant protein intake regulated glycaemic responses and hepatic lipid metabolism, but did not increase the risk of hepatic lipid deposition and hyperglycaemia in pigs. SPH infusion accelerated fatty acid β-oxidation and inhibited TG synthesis, thereby reducing the hepatic TG levels. The inhibition of TG synthesis by soy protein might be related to the promotion of PE synthesis. SPH infusion reduced the serum glucose levels by promoting hepatic glucose uptake and glycolysis (**Figure 4**). SPH infusion inhibited cholesterol accumulation in the liver and initiated a negative feedback mechanism, which maintains cholesterol homeostasis. These findings validated the effects of HPP on host glucose and lipid metabolism in healthy animals.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental Animal Welfare Ethics Committee of Nanjing Agricultural University.

AUTHOR CONTRIBUTIONS

SH and ZL conceived and designed the experiments. ZL and LD performed the experiments. ZL and SH analyzed the data and drafted the manuscript. SH and WZ provided the funding. All authors read and approved the final manuscript.

FUNDING

This research was funded by the National Key Basic Research Program of China (2013CB127301).

ACKNOWLEDGMENTS

We thank Jangyin Feng, Zhen Zhang, and Lvyang Wang for their assistance in sample collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.838617/full#supplementary-material

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frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 11 May 2022
doi: 10.3389/fnut.2022.888608

Testing Links of Food-Related Olfactory Perception to Peripheral Ghrelin and Leptin Concentrations

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The peptide hormones ghrelin and leptin play major roles in the regulation of appetite and food intake. However, the precise effects of these hormones on sensory processing remain a subject of debate, particularly with food related stimuli and its small body of evidence. Here, we test for relationships between ghrelin and leptin levels against olfactory performance with multiple food-related odours. Specifically, a total of 94 Caucasian males were tested for their supra-threshold sensitivity (i.e., d′), intensity, and valence perception to three odour compounds (i.e., *vanilla*, *potato*, and *dairy* odours). These sensory data were then analysed against peripheral ghrelin and leptin levels, both assessed in plasma samples. Participants' body adiposity measures were also obtained. Results lent strong support to one of our original hypotheses, with ghrelin levels being positively correlated to the supra-threshold sensitivity of the *dairy* odour, (r = 0.241, p = 0.020), and intensity ratings to most of the food odours tested [*dairy* (r = 0.216, p = *0.037*) and *vanilla* (r = 0.241, p = *0.020*)]. By contrast, peripheral leptin levels were not significantly linked to any of the olfactory measures ($p > 0.05$). These relationships remained similar after controlling for variabilities of adiposity measures. The present study brings novel insights by identifying positive links between supra-threshold olfactory perception and ghrelin. This new knowledge is highly relevant for future research linking olfactory shifts to hormonal dysregulation and obesity.

Keywords: ghrelin, leptin, obesity, olfaction, food odour

OPEN ACCESS

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

Received: *03 March 2022*
Accepted: *19 April 2022*
Published: *11 May 2022*

Citation:
Ginieis R, Abeywickrema S, Oey I and Peng M (2022) Testing Links of Food-Related Olfactory Perception to Peripheral Ghrelin and Leptin Concentrations. Front. Nutr. 9:888608. doi: 10.3389/fnut.2022.888608

INTRODUCTION

Understanding the aetiology of obesity remains an important research direction (1). Over-responsiveness to food cues is considered a key contributor to obesity in the current food environment (2, 3). Research over the last two decades has shown that maladaptive eating behaviour is often accompanied by major alterations in peptide hormones, such as ghrelin and leptin (4, 5). However, mechanisms underpinning these observed relationships remain unclear. Recent research has postulated that sensory processing plays an important role in mediating hormonal effects on eating (6–8). The current study adds to the emerging body of research by testing for links between peripheral leptin and ghrelin levels and olfactory perception of food-related stimuli.

Previous research has consistently observed links between obesity and resistance to leptin and ghrelin [e.g., (9, 10)]. Notably, peripheral ghrelin and leptin have been increasingly used as

biomarkers for obesity (9, 11). Individuals with obesity were shown to have reduced ghrelin (1/3–1/2 time lower) (11, 12), and increased leptin levels (2–8 times higher), compared to normal-weight controls (13–15). In addition to links to obesity, neurological evidence suggests that both ghrelin and leptin can modulate neural responsiveness to food rewards (4, 5, 16). Specifically, ghrelin is an orexigenic agent (i.e., promoting food intake) (17, 18) and leptin is an anorexigenic agent (i.e., inhibiting food intake) (19, 20). Based on these previous findings, it is intuitive to propose that changes of ghrelin and leptin alter food-related neurological and behavioural responses, and correspondingly influence one's body weight overtime (5, 21–23). However, the precise mechanism underpinning these effects remains unclear. Recent research suggests that sensory processing may play a key role in mediating ghrelin and leptin effects on eating [cf. (24)], although such findings remain controversial.

Across the five special senses, olfaction is the least understood, despite its vital function in flavour perception and food acceptance (25). Olfactory processing emerges from first-order neurons at the olfactory mucosa (OM) toward the olfactory bulb (OB) (25). The OB then conveys olfactory information to the olfactory cortex (26–28), which includes the piriform cortex, anterior olfactory nucleus, lateral entorhinal cortex, periamygdaloid cortex and the cortical nucleus of the amygdala (26, 29–31). Further higher order projections from the olfactory cortex to the orbitofrontal cortex, amygdala, and hippocampus encode for executive, emotional, motivational, and memory-related processes associated with human olfaction (32). Thus, higher-order processing confers its specificity to the stimulus perceived and reveal odour features, including odour intensity and valence (29). This temporal cascade of olfactory processes is shown to start with odour detection and discrimination, followed by the identification of odour quality (e.g., the smell of a rose) and ends with the hedonic perception of this stimulus (33). Altogether, these complex neuroanatomical pathways govern different aspects of an individual's olfactory perception, from detection sensitivity to hedonic valence, highlighting a fundamental difference between these sensory measures.

Ghrelin and leptin can traverse the blood-brain barrier and reach several cerebral areas that are directly involved with feeding behaviour [e.g., hypothalamus: (34–36); mesolimbic reward system: (37)], as well as other sensory-related regions that are indirectly linked to eating (38–40). Recent research further indicated that ghrelin and leptin were particularly involved in olfactory transduction (41). Specifically, ghrelin receptors are found in olfactory structures such as the glomeruli, mitral cells, and granule cells located in the OB (39). More recent research indicated that ghrelin is able to modulate olfactory information transmission from the mitral cells to the amygdala and hypothalamus (42). Similarly, leptin and its associated receptor have been found in the OM (43), olfactory epithelium (44) and OB (45). More functional evidence indicated that leptin was involved in olfactory-related mechanisms such as the mucus production (41, 46). Furthermore, ghrelin and leptin signalling, and olfactory transduction were shown to be co-modulated in shared cerebral structures that are closely related to feeding (35, 42). These observed links point to the possibility that ghrelin and leptin influence eating behaviour *via* shaping individual olfactory perception.

A few studies have tested for links between olfactory perception and peripheral ghrelin levels, with findings remaining controversial. Specifically, a subset of these studies attempted to test for links between peripheral ghrelin and detection threshold to neutral odours (e.g., n-butanol) (47–50). However, most of these studies failed to detect any significant relationship (47–49). Only a recent study found a significant positive correlation between peripheral ghrelin levels and detection threshold scores to *n-butanol* (50). Importantly, the study from Uygun et al. (50) was performed only on women with obesity while other studies considered all weight groups for analysis, which may have contributed to these differential results. Although no study tested for links between peripheral ghrelin and olfactory sensitivity to food odours, a few studies showed that systemic ghrelin infusions significantly increased individual sniffing magnitude in response to food odours (39) and generated a greater response to food odour conditioning task (48). In line with these findings, peripheral ghrelin levels were shown to be significantly associated with valence ratings (VR) of food odours (e.g., black pepper oil) (48, 49). Such inter-study inconsistencies may indicate the important role of the nature of odorants in the relationship between peripheral ghrelin and olfaction.

With regards to the link between leptin and olfactory acuity, animal studies have consistently observed a negative relationship [e.g., (51)], while human studies revealed mixed findings. Specifically, several rodent studies observed that leptin-deficient animals exhibited a heightened sensitivity to food-related odours, compared to matched controls (44, 52, 53). Such findings were later explained as leptin reduces neural activity in the olfactory epithelium and OB (45, 54–56). Accordingly, a recent human study highlighted a negative correlation between OB volume and peripheral leptin levels (57). On the other hand, studies with human subjects regarding olfactory sensitivity revealed either positive (49, 58) or negative correlations with peripheral leptin levels (58, 59). Karlsson et al. (58) further pointed out differential results being dependent on the sex of participants with peripheral leptin levels and olfactory sensitivity being negatively linked in females and positively linked in males. Notably, these studies mainly focussed on identification, detection and discrimination of a neutral olfactory compound, *n-butanol*. The study from Trellakis et al. (49) was the only experiment on human subjects incorporating food odours, and their results revealed a significant correlation between pleasantness to black pepper oil smell and peripheral levels of leptin. Given that numerous animal studies using food odours observed a consistent negative relationship between leptin and olfaction, the use of food odours to assess olfactory functions in humans may reveal a consistent link with peripheral leptin levels.

The present paper aims to test for links of peripheral ghrelin and leptin levels to olfactory functions. Due to the close relationship between olfactory supra-threshold functions and eating behaviour [e.g., (60)], the present study compares peripheral hormone levels to supra-threshold sensitivities, intensity ratings (IR), and VR to three food-related odours.

Building upon previous literature, we hypothesised that individuals with elevated levels of peripheral ghrelin show heightened olfactory supra-threshold sensitivity and IR, but reduced VR of food odours. By contrast, we hypothesised that individuals with high levels of peripheral leptin levels show weakened olfactory supra-threshold sensitivity and IR, but heightened VR for food odours.

MATERIALS AND METHODS

Participants

A total of 94 Caucasian males [25.2 ± 5.7 years of age; body mass index (BMI): 26.7 ± 4.9 $kg{\cdot}m^{-2}$] undertook this study. Given the high degree of inter-individual differences in olfactory perception due to sex, only males were recruited and included in the present study [cf. (61)]. Sample size was determined using G*Power 3.1.9.7 Software, with calculations being based on the effect sizes reported in previous studies testing for links between peripheral hormone levels and olfactory performances [e.g., (49, 50)]. The analysis produced a sample size of 84 to achieve an 80% power and an α-level of 0.05, based on bivariate normal model of correlations (effect size r = 0.300). We decided to recruit an additional 10 participants to allow for at least 10% of predicted rate of participants' withdrawal. All participants were non-smokers, and were free from sensory dysfunctions, chronic medical conditions, or food allergies. Participants were required to abstain from food or non-water beverage after 10 p.m. of the night prior to each laboratory session and the phlebotomy appointment. In addition, participants were asked not to wear any cologne or scented cosmetic on the day of the testing. All participants gave informed written consents. The study was approved by the University of Otago Ethics Committee for Human Participation – health panel (Reference: H18/111). Each participant received a monetary compensation upon completion of the study.

Study Overview

Each participant attended six 30-min experimental sessions over consecutive weekdays, from 7.00 to 9.30 a.m., and a separate phlebotomy test for blood sample collection. All experimental sessions were carried out in standard individual sensory booths, at 20°C and under red light, in the Sensory Neuroscience Laboratory at the University of Otago, New Zealand. The six experimental sessions included replicated assessments of individual supra-threshold sensitivities, intensity, and valence perception of three food-related odours. Testing orders were randomised across participants using a Latin Square design (62). Participants' weight and height were measured in laboratory for calculating anthropometric measures. On a separate morning after the completion of sensory tests, participants were asked to attend a phlebotomy appointment for collection of a fasting blood sample.

Stimuli

Information of the olfactory stimuli used in this study are described in **Table 1**. These odorants were selected due to their close relevance to common snack foods in New Zealand (63, 64). Each odorant was made into solutions of 11 concentrations following an additive logarithmic steps, with the middle concentration being the reference sample. The five lower concentration levels referred to the *decremental series,* and the five higher concentration levels to the *incremental series*. Serial dilution method was used to make these solutions, with filtered water (0.5 μm) being the solvent. According to previous reports and pilot tests, the selected concentration range of the olfactory compounds should be above the recognition thresholds associated with the odorant (60, 65, 66). During the sensory tests, all olfactory samples were presented at a volume of 5 mL in 50 mL glass bottles (73 mm height, 42 mm diameter, Arthur Holmes, New Zealand).

Olfactory Supra-Threshold Sensitivity Measures

Each odorant was tested twice over two 30-min sessions on separate days. The supra-threshold sensitivity test was constructed based on the method of constant stimuli, with a two-alternative forced choice (2-AFC) presentation. In a single 2-AFC task, the participant was presented with one reference sample and one testing sample (from either the *incremental* or *decremental series*), following a pre-determined randomised order. The participants were required to sniff the two samples for two seconds each and then indicate the "most intense sample." Each testing session contains 50 2-AFC comparisons, comprising five replicated testing of each concentration level. Across the two sessions, for each odorant, each concentration level was compared to the reference sample for 10 replicates. Participants were given non-flavoured crackers and a glass of water for inter-trial palate cleansing. Olfactory supra-threshold sensitivity tests were performed on *Compusense* Cloud software (Guelph, ON, Canada).

Olfactory Intensity and Valence Ratings

Intensity and hedonic general Labelled Magnitude Scale (gLMS) were used to measure intensity and valence perception to each testing odorant. The intensity gLMS is a 100-point scale, marked with semi-logarithmically spaced descriptors (*no sensation* = 0, *weak* = 6, *moderate* = 17, *strong* = 35, *very strong* = 53, *strongest imaginable sensation of any kind* = 100) (67). The hedonic gLMS is a double scale ranging from −100 to 100, where −100 represents the *strongest imaginable dislike,* and 100 represents the *strongest imaginable like* (68). Other interval labels are similar to the intensity gLMS. Both scales are widely accepted and were shown to be adapted to scale olfactory sensations (67–69). On the first day of the six laboratory sessions, participants were instructed on how to use these scales (69). For each odorant, intensity and valence were rated against the reference sample (see **Table 1**). These ratings were performed on *Compusense* Cloud software (Guelph, ON, Canada).

Measurements of Hormones

Blood samples were collected between 7.30 and 10 a.m. following overnight fasting. Samples were then centrifuged in a refrigerated centrifuge (4°C) for 10 min at 1,000 × g within 30 min of blood collection. The plasma was pipetted and transferred in K_2

TABLE 1 | Summary of olfactory stimuli characteristics including description, compound, suppliers, purity, and reference concentrations.

Odorant code	Odour descriptor	Chemical name	CAS number	Purity	Supplier	Reference (Concentration/log value)	Dilution log step
O1	Vanilla	4-Hydroxy-3-methoxybenzaldehyde *Vanillin*	121–33–5	> 99%	Vanesse, Camlin Fine Sciences, India	0.39 $g{\cdot}L^{-1}$ /–0.40	0.221
O2	Potato	3-(Methylthio) Propionaldehyde *Methional*	3268–49–3	> 96%	Sigma–Aldrich United States	1.86×10^{-4} $mL{\cdot}L^{-1}$ /–3.73	0.146
O3	Dairy	4h-Pyran-4-One, 3-Hydroxy-2-Methyl-	118–71–8	–	Firmenich, Switzerland	0.08 $mL{\cdot}L^{-1}$ /–1.10	0.221
		3(2h)-Furanone, 4-Hydroxy-2,5-Dimethyl- *Maltol/Furaneol* mixture	3658–77–3				

EDTA tubes and stored at −80°C until analysis. Leptin and acyl ghrelin (active ghrelin) were both measured in duplicate using a commercially available multiplex kit (Milliplex Map kit, Human Metabolic Hormone Magnetic Bead Panel HMHEMAG-34K; Millipore Corp., St. Louis, MO, United States). Milliplex map kits are specific to the Luminex Magpix Analyzer and are analytically validated for sensitivity, specificity, and reproducibility. These kits offer a great sensitivity for active ghrelin and leptin with respective detection thresholds of 14 pg/mL (intra-assay CV < 10%, inter-assay CV < 15%) and 41 pg/mL (intra-assay CV < 10%, inter-assay CV < 15%). Additionally, when a sample contained a lower concentration than the lowest detection threshold, standard curves were extrapolated to determine the sample concentration. The measurements of the 94 blood samples required three Milliplex map kits in total. The protocol was performed over two consecutive days, and the exact same procedure was followed for each kit.

Anthropometric Measurements

Participants' weight and height were measured using a standard scale and stadiometer to the nearest 0.1 unit. Participants were asked to stay in standing position wearing light clothing without shoes. Participants' BMI were classified as normal weight for a value between 18.5 and 24.9 $kg{\cdot}m^{-2}$, overweight from 25 to 29.9 $kg{\cdot}m^{-2}$, and obese over 30 $kg{\cdot}m^{-2}$. Additionally, body fat percentage was also measured using skinfold thickness measurements on four body sites (biceps, triceps, subscapular area, and suprailiac area) as a complementary measure of BMI.

Sensitivity Calculations

Individual supra-threshold sensitivities were calculated based on the results obtained from 2-AFC tasks. Specifically, Hit rates (H; correctly recognising the higher concentration positioned on the left side) and False Alarm rates (F; mistakenly recognising the lower concentration positioned on the left side as the higher concentration) were calculated for *decremental* and *incremental series* separately. A decremental d′ value – $d'_{(Decremental,Reference)}$ – as well as an incremental d′ value – $d'_{(Reference,Incremental)}$ – were calculated using the equation from Macmillan and Creelman (70), for each sensory stimulus and each individual. The individual value of d′ resulted from the addition of $d'_{(D,R)}$ and $d'_{(R,I)}$ for each participant and each sensory stimulus. Additionally, extreme values of d′ were corrected using the 1/(2N) rule (71). In line with the rule, extreme proportions of H and F reaching a value of 1 or 0 were replaced with 1–1/(2N) and 1/(2N), respectively, with N being the number of trials used in the experimental design. The calculations of $d'_{(D,R)}$, $d'_{(R,I)}$, and d′ were performed on Excel (Microsoft office, 2018, United States).

Statistical Analyses

Pearson's correlations were firstly calculated to assess the relationships between continuous values of BMI and peripheral levels of ghrelin and leptin. Pearson's correlations were also calculated between sensory measures of supra-threshold sensitivity (d′), IR and VR for each olfactory compound. Subsequently, Pearson's and partial correlations were calculated between olfactory functions (d′, IR, and VR) and peripheral ghrelin levels, with and without BMI as a covariate. Similar analyses were then performed to assess the correlations between olfactory functions (d′, IR, and VR) and peripheral leptin levels. For all correlation analyses, the strength of the correlation observed was reported as weak, moderate, and strong for an absolute value of the correlation coefficient in the ranges of 0–0.4, 0.4–0.7, and 0.7–1, respectively (72). All statistical analyses used an α level of 0.05 for detecting significant differences, and analyses were performed on SPSS Statistics (V26 – IBM Corp., Armonk, NY, United States) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, United States).

RESULTS

Summary of Testing Measures

The present study included a total of 94 Caucasian male participants with an average age of 25.81 ± 5.74 years old, body fat percentage of 19.99 ± 5.69% and BMI of 26.78 ± 4.93 $kg{\cdot}m^{-2}$ (**Figure 1**). **Table 2** summarises the mean values and standard deviations of leptin and ghrelin concentrations in periphery, as

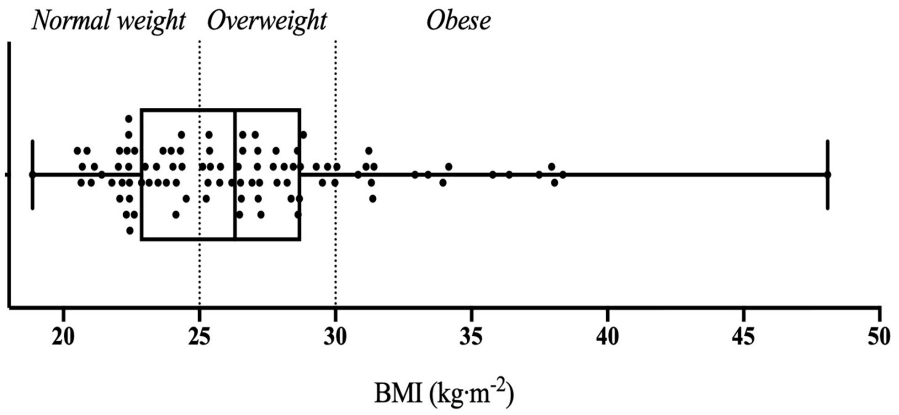

FIGURE 1 | Whisker plot illustrating individuals' BMI across three BMI groups classified as normal weight, overweight, and obese.

TABLE 2 | Summary of Mean ± SD values of hormonal measures and olfactory functions across BMI groups with results from One-way ANOVAs assessing the effect of BMI groups on each testing variables.

	Normal weight	Overweight	Obese	ANOVA	Total
N	**38**	**37**	**19**	***F*-statistics; *p*-value**	**94**
Ghrelin (pg·mL^{-1})	20.17 ± 18.33	13.07 ± 6.76	9.51 ± 2.62	$F_{(2,91)}$ = 5.51; $p = 0.005$	15.22 ± 13.08
Leptin (pg·mL^{-1})	788.76 ± 800.37	2011.63 ± 1754.01	8968.26 ± 6442.86	$F_{(2,91)}$ = 46.35; $p < 0.001$	2923.40 ± 4374.69
Olfactory supra-threshold sensitivity					
d′O1	2.51 ± 0.68	2.42 ± 0.97	2.40 ± 0.98	$F_{(2,91)}$ = 0.15; p = 0.859	2.45 ± 0.86
d′O2	1.41 ± 0.67	1.49 ± 0.90	1.44 ± 0.55	$F_{(2,91)}$ = 0.09; p = 0.912	1.45 ± 0.74
d′O3	1.42 ± 0.88	1.24 ± 0.61	1.08 ± 0.61	$F_{(2,91)}$ = 1.46; p = 0.236	1.28 ± 0.74
Olfactory intensity ratings					
IRO1	26.16 ± 13.74	24.05 ± 11.39	29.39 ± 14.03	$F_{(2,91)}$ = 1.08; p = 0.344	25.98 ± 12.93
IRO2	36.65 ± 15.67	32.08 ± 14.22	35.37 ± 18.18	$F_{(2,91)}$ = 0.83; p = 0.441	34.60 ± 15.63
IRO3	26.15 ± 14.02	19.81 ± 11.90	22.50 ± 12.62	$F_{(2,91)}$ = 2.25; p = 0.111	22.92 ± 13.12
Olfactory valence ratings					
VR O1	30.80 ± 19.57	26.57 ± 19.43	32.56 ± 16.02	$F_{(2,91)}$ = 0.78; p = 0.459	29.49 ± 18.82
VR O2	−14.86 ± 25.35	−15.18 ± 26.50	−15.75 ± 29.33	$F_{(2,91)}$ = 0.01; p = 0.993	−15.16 ± 26.35
VR O3	−4.24 ± 17.80	−1.91 ± 17.82	0.39 ± 15.36	$F_{(2,91)}$ = 0.47; p = 0.623	−2.39 ± 17.25

O1: vanilla odour, O2: potato odour, O3: dairy odour, d′: supra-threshold sensitivity (0–4), IR: intensity rating (0–100), VR: valence rating (−100 to 100).

well as olfactory performances for three food-related odorants (O1, O2, and O3) across BMI groups.

A total of eleven separate One-way ANOVAs examining the effect of BMI groups on each testing variables were performed. Results revealed significant main effects of BMI groups on peripheral ghrelin and leptin levels (**Table 2**). *Post hoc* tests, based on simple effects tests with *Bonferroni* corrections revealed that normal-weight individuals had a significantly higher level of ghrelin than overweight individuals ($p = 0.048$) and individuals with obesity ($p = 0.009$, see mean values in **Table 2**). Additionally, individuals with obesity had higher levels of leptin in periphery than normal weight ($p < 0.001$) and overweight individuals ($p < 0.001$, see mean values in **Table 2**). On the other hand, there was no significant main effect of BMI groups on olfactory measures (**Table 2**).

Associations Between Peripheral Ghrelin and Leptin Levels and Body Mass Index

Pearson's correlations were calculated to assess the relationship between continuous values of BMI and peripheral levels of ghrelin and leptin. Results revealed a significant negative correlation between peripheral ghrelin concentration and BMI ($r = -0.302$; $p = 0.003$) and a positive and strong significant correlation between leptin and BMI ($r = 0.755$; $p < 0.001$). Similar analyses were performed between body fat percentage measures and peripheral levels of ghrelin and leptin, which revealed comparable results (see details in **Supplementary Table 1**).

Associations Between Olfactory Measures and Body Mass Index

A series of Pearson's correlations were calculated to assess for associations between continuous values of BMI and olfactory sensory measures including supra-threshold sensitivity (d′), IR and VR for three food-related odorants. Results revealed that BMI and suprathreshold sensitivity for O3 (dairy smell) were significantly negatively correlated ($r = -0.217$; $p = 0.036$). In contrast, sensitivities to other odorants (O1 and O2) as well as all IR and VR were not significantly correlated to BMI ($p > 0.05$, see details in in **Supplementary Table 2**). Similar analyses were performed between body fat percentage and olfactory measures, which revealed comparable results (see details in **Supplementary Table 2**).

Links Between Olfactory Sensory Measures

Pearson's correlations were calculated between sensory measures of supra-threshold sensitivity (d′), IR and VR for each olfactory compounds tested. For all three odorants, sensitivity scores were not shown to be significantly correlated to any of the rating measures (IR and VR) (**Figure 2**). Regarding O1, a significantly positive and strong correlation was observed between both rating measures IRO1 and VRO1 ($r = 0.65$, $p < 0.001$, see **Figure 2A**). Then, when looking at the olfactory compound O2, a significantly negative correlation was noted between IRO2 and VRO2 ($r = -0.35$, $p < 0.001$, see **Figure 2B**). In contrast, IR and VR were not significantly correlated for the olfactory compound O3 (see **Figure 2C**).

Relationships Between Peripheral Ghrelin Levels and Olfactory Functions

Pearson's correlations were calculated between ghrelin concentrations and olfactory supra-threshold sensitivities, olfactory IR, and olfactory VR, for all three food-related odorants O1, O2, and O3 (**Figure 3**). These analyses revealed two significantly positive correlations, both involving the olfactory compound O3. Specifically, peripheral ghrelin levels were shown to be significantly positively correlated to d′O3 ($r = 0.289$, $p = 0.005$, **Figure 3A**) and IRO3 ($r = 0.217$, $p = 0.035$, **Figure 3B**). On the other hand, no correlation was observed between olfactory VR for any of the odorant tested and peripheral ghrelin levels (**Figure 3C**).

Subsequently, partial correlations were calculated between peripheral ghrelin levels and olfactory performances, accounting for BMI. Results from partial correlations also revealed that ghrelin concentrations were significantly and positively correlated to different measures of olfactory function, with a specific emphasis on IR. Specifically, positive correlations were observed between peripheral levels of ghrelin and IRO1 ($r = 0.241$, $p = 0.020$) and IRO3 ($r = 0.216$, $p = 0.037$), and a trend toward statistical significance was observed with IRO2 ($r = 0.188$, $p = 0.071$). Furthermore, a significant positive relationship was still observed between peripheral ghrelin levels and d′O3 after adjusting for BMI ($r = 0.241$, $p = 0.020$). Finally, while ghrelin levels were not significantly correlated to olfactory VR, a tendency toward significance was noted with VRO1 ($r = 0.198$, $p = 0.058$).

Relationships Between Peripheral Leptin Levels and Olfactory Functions

Pearson's correlations were calculated between peripheral leptin levels and olfactory supra-threshold sensitivities, olfactory IR, and olfactory VR, for all three food-related odorants O1, O2, and O3 (**Figure 4**). Regarding results on sensitivity measures, a trend toward statistical significance was noted between d′O3 and peripheral leptin concentrations ($r = -0.192$, $p = 0.064$), but not with other odorants O1 and O2 (**Figure 4A**). Additionally, results did not reveal any significant correlation between peripheral leptin and olfactory IR (**Figure 4B**), nor VR (**Figure 4C**).

Subsequently, partial correlations were calculated between peripheral leptin levels and olfactory performances, accounting for BMI. Results from partial correlations revealed similar outcomes as the ones from Pearson's correlations, with no significant correlation between peripheral leptin levels and any of the olfactory functions tested ($p > 0.05$, **Figure 3**). Notably, the tendency toward significance between d′O3 and peripheral leptin concentrations was no longer observed after adjusting the correlation for BMI ($r = -0.044$, $p = 0.673$).

DISCUSSION

The present study investigated the links between fasting individual olfactory functions and peripheral concentrations of ghrelin and leptin, two hormones highly involved in eating behaviour and previously used as biomarkers for obesity. Results from our study support the role of ghrelin in modulating individual olfactory sensitivity and intensity perception of specific food odours (i.e., *dairy* and *vanilla* odours), whereas no significant relationships were observed for leptin.

Firstly, the present study confirmed previously reported links between adiposity and hormonal balance, with individuals with obesity having lower levels of ghrelin (12, 15) and higher levels of leptin compared to normal-weight individuals (73, 74). Additionally, the present study also demonstrated that different sensory measures represent distinct phases of perception, with no evidence for correlations between olfactory sensitivity and ratings, in line with previous observations of gustation [e.g., (75)].

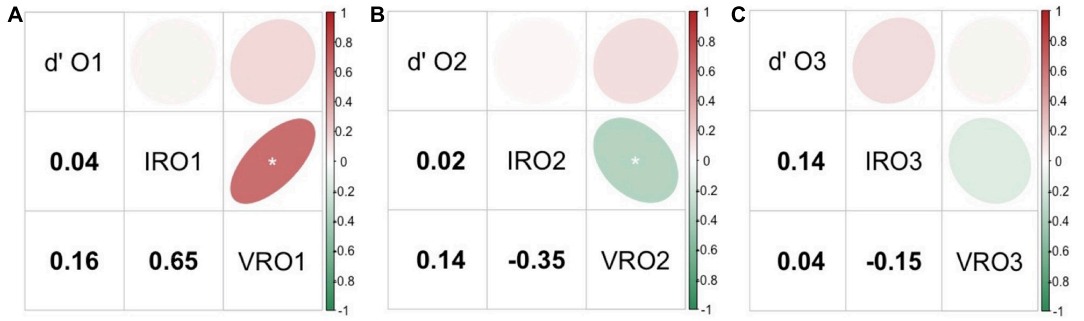

FIGURE 2 | Pearson's correlations across three types of olfactory measures: supra-threshold sensitivity (d'), intensity ratings (IR) and valence ratings (VR) for the odorants O1 **(A)**, O2 **(B)**, and O3 **(C)**. The upper part of each figure illustrates the strength of the correlation *via* an elliptical shape, and the lower part shows the correlation coefficient value for each pair of sensory measures compared. Positive correlations are represented in a red gradient and negative correlation in a green gradient. O1: vanilla odour, O2: potato odour, O3: dairy odour. Significant results are represented by *.

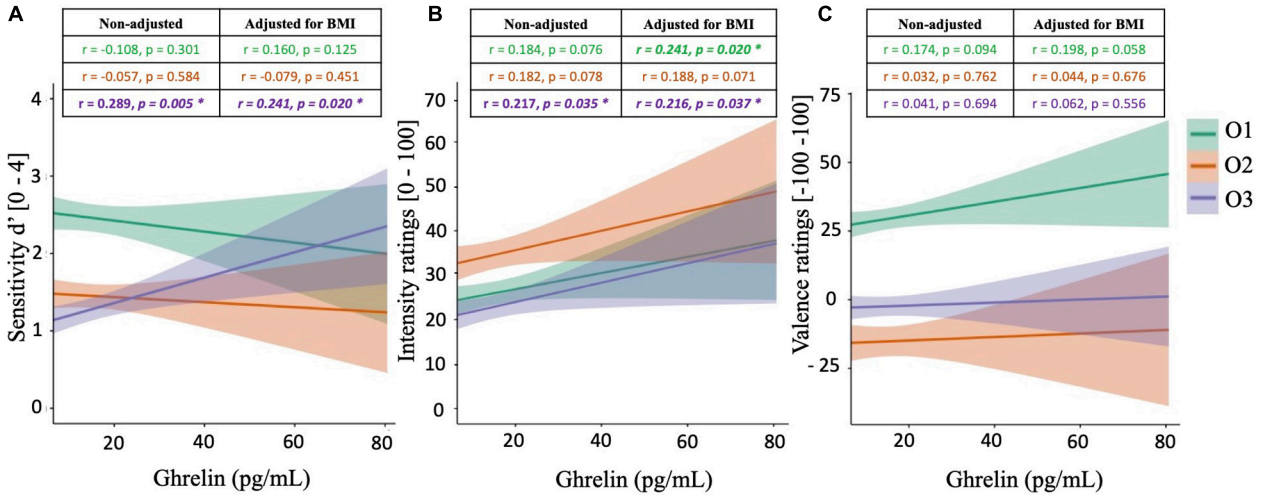

FIGURE 3 | Correlation plots between peripheral ghrelin levels and olfactory supra-threshold sensitivity **(A)**, olfactory intensity ratings **(B)**, and olfactory valence ratings **(C)** of the odorants O1 (vanilla odour), O2 (potato odour), and O3 (dairy odour). All graphical plots illustrate results obtained from Pearson's correlations (non-adjusted). Significant results are represented by *.

The present analyses showed a positive link between fasting peripheral ghrelin levels and supra-threshold sensitivities with the *dairy* odour, with additional positive relationships based on IR of the *dairy* and *vanilla* odour. The observed positive relationships to two of these odours were in line with a recent study of Uygun et al. (50), in which peripheral levels of ghrelin was correlated to odour detection scores. By contrast, no relationship was found between ghrelin and olfactory measures of the *potato* odour, regardless of controlling for BMI. The inconsistent results across olfactory compounds imply an odour-specific relationship of ghrelin effects. Similarly, Trellakis et al. (49) investigated the link between ghrelin levels and olfactory VR of six odours, and observed only one significant correlation (with black pepper oil). Moreover, studies using a neutral odour of *n-butanol* failed to observe a ghrelin-olfaction link (47–49), while other studies using food-related odours did (39, 48). These previously observed inconsistencies may be attributed to choices of neutral versus food-related odours. The current findings further highlight discrepant results across different food odours (e.g., *vanilla* versus *potato* odours).

It is important to note that the observed ghrelin-olfaction links were subject to the sensory measure. Specifically, significant results were found with supra-threshold sensitivity and intensity rating (to specific odours). However, none of the analyses based on odour valence showed significance (although correlation for the vanilla odour was close to significance after BMI being controlled). Ghrelin links to odour valance had been tested in two separate studies, with findings remaining controversial (48, 49). For instance, Trellakis et al. (49) found that ghrelin was significantly correlated with valence to an odour of black pepper oil, but did not find evidence for relationships based on odour discrimination nor identification. Recent research has found that different types of sensory functions, such as intensity and valence perception, involve distinct brain regions (29). Specifically, evidence has shown that odour intensity perception activates the amygdala, while valence emerges from activations

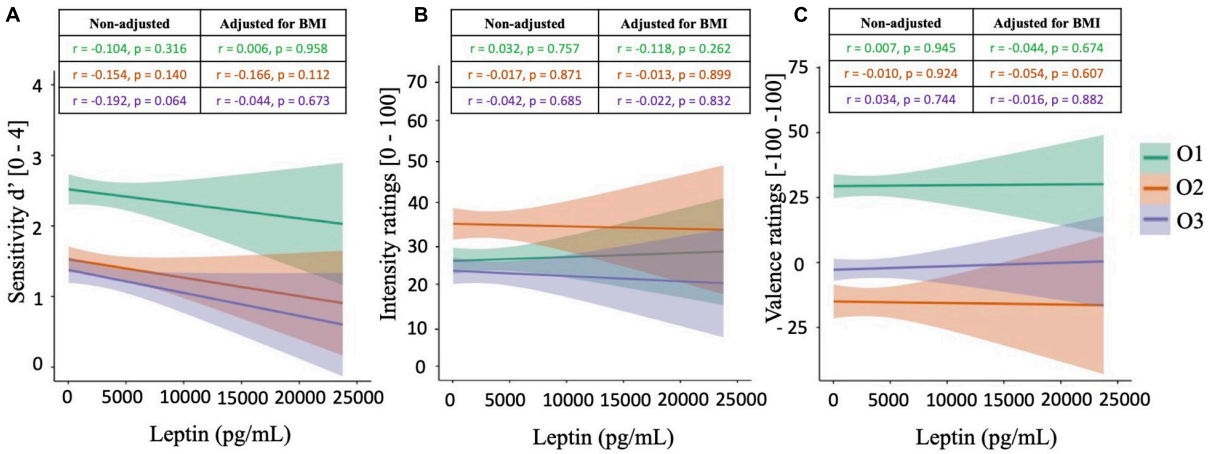

FIGURE 4 | Correlation plots between peripheral leptin levels and olfactory supra-threshold sensitivity **(A)**, olfactory intensity ratings **(B)**, and olfactory valence ratings **(C)** of the odorants O1 (vanilla *odour*), O2 (potato *odour*), and O3 (dairy *odour*). All graphical plots illustrate results obtained from Pearson's correlations (non-adjusted). Significant results are represented by *.

of the orbitofrontal cortex (76). Moreover, Russo et al. (42) demonstrated that ghrelin injections were able to modulate rat's sense of smell, by altering olfactory transduction from the mitral cells to the amygdala – a cortical area thought to be important for intensity perception. In this context, our study offers new behavioural data on close links between ghrelin and odour intensity perception in humans. Additionally, these findings point to the importance of considering specific sensory functions when evaluating links to ghrelin levels.

In general, the present study failed to detect strong associations between leptin levels and olfactory measures, which was in line with findings from Uygun et al. (50). In contrast, numerous previous investigations have reported either positive (49, 58) or negative associations between leptin levels and olfactory sensitivities (51, 54, 55, 58, 59). In addition, one study reported negative correlations between leptin and odour valence (49). Previously, obesity has been linked to increased leptin levels and declined olfactory functions [cf. (7, 77)]. Building upon these findings, we originally proposed that leptin should be negatively correlated to odour sensitivity. Notably in our results, correlation between leptin and sensitivity to the *dairy* odour was close to significance (p = 0.06) but increased drastically with correlations controlling for BMIs (p = 0.67). Such change in correlation coefficients suggests that the link between leptin and odour sensitivity was prominently mediated by individual BMIs. In this tripartite relationship, BMI plays a more substantial role in linking leptin and olfactory sensitivities. While future studies are needed to confirm this proposal, our data appear to suggests that leptin and olfactory performance are not directly linked.

The current findings point to the important role of olfactory processing in mediating ghrelin influences on food intake (78). Elevated peripheral ghrelin levels have been previously observed in individuals experiencing food reward anticipation, which is characterised by an increased cerebral activity in reward-related areas, such as the orbitofrontal cortex (79, 80). Furthermore, evidence suggests that peripheral ghrelin concentration following consumption of energy-dense food maintains at a high level in sated healthy-weight individuals, in contrary to the expected post prandial ghrelin profile (5, 22). Our study has found that individuals with fasting elevated peripheral ghrelin levels show heightened olfactory supra-threshold sensitivity and intensity perception for specific food odours, which may facilitate food-seeking behaviour. This finding may be related to neural evidence for convergence of ghrelin and sensory functions, as evidence indicates that sensory food cues and systemic ghrelin administration activate the same subset of hypothalamic neurons (81). These connexions directly imply a co-action of ghrelin signalling and olfactory perception, resulting in increased appetite and food intake. Notably, recent research indicated that plasma ghrelin only targets specific brain regions, including the hypothalamus, the mesolimbic pathway, as well as the OB [cf. (82)]. The latter brain area, which is directly involved in olfactory coding processes (25, 27), was shown to be one of the regions with the highest uptake of systemically injected ghrelin (83). Inversely, a recent study from Riera et al. (84) suggested that olfactory activity directly modulates fat mass and hormonal alteration associated with obesity. Therefore, findings from both previous research and the present study support the concept of bidirectional relationship between olfactory perception and metabolic regulation through ghrelin signalling. Overall, these findings bring new insights into understanding individual susceptibility to overeating and obesity.

A limitation of the present study was that no serine protease inhibitor was added to the K_2 EDTA blood samples before measuring acyl ghrelin concentrations. The addition of serine protease inhibitor is typically used to limit the degradation of acyl ghrelin due to deacylation (85, 86). A common serine protease inhibitor is aprotinin, which has been widely used as a standardized procedure to prevent from acyl ghrelin degradation in previous research (87). The absence of a protease inhibitor in the present experiment may explain the rather low concentrations of acyl ghrelin reported in this study. However,

Blatnik and Soderstrom (86) observed no significant difference in the degree of acyl ghrelin degradation, when plasma samples were stored in K_2 EDTA tubes only or in K_2 EDTA tubes containing aprotinin, with both storage leading to an approximate loss of 50% of acyl ghrelin. Therefore, concentrations of acyl ghrelin presented in this study should be interpreted with a certain degree of caution.

CONCLUSION

Overall, the present study investigated links between individual olfactory functions and two major peptide hormones. The findings point to the important role of ghrelin in influencing olfactory performances. Specifically, strong relationships were observed between peripheral ghrelin levels and olfactory functions, in particular intensity perception, of specific food odours. Furthermore, peripheral leptin levels were not linked to any of the tested olfactory performances. Results from the present study brings new data supporting the role of olfaction in mediating hormonal effects on eating.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Otago Human Ethics Committee (Health). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RG, SA, and MP contributed to conception and design of the study. RG and SA organized the database. RG performed the statistical analysis and wrote the first draft of the manuscript. MP and IO edited the manuscript. RG, SA, IO, and MP contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This study was supported by the New Zealand Royal Society Te Apārangi *via* Marsden Fund (UOO_1720).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.888608/full#supplementary-material

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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 Nutrition

ORIGINAL RESEARCH
published: 16 May 2022
doi: 10.3389/fnut.2022.819106

Nutrient Intake and Nutrition Status in Vegetarians and Vegans in Comparison to Omnivores - the Nutritional Evaluation (NuEva) Study

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Introduction: In recent years, vegetarian and vegan diets became increasingly important as they are associated with beneficial health outcomes. Therefore, the NuEva study compares the impact of flexitarian, vegetarian, or vegan diets with omnivorous nutritional habits on nutrient intake and risk factors for non-communicable diseases.

Methods: A dietary protocol was kept over five days and blood and 24h urine samples were collected to examine the impact of dietary habits [omnivores, $n = 65$ (Median/Interquartile range: 33/17 yrs.), flexitarians, $n = 70$ (30/17 yrs.), ovo-lacto vegetarians, $n = 65$ (28/14 yrs.), vegans, $n = 58$ (25/10 yrs.)] on nutrient intake, nutrient concentrations in plasma, serum or 24h urine, body composition, and blood lipids.

Results: The increased exclusion of animal based foods in the diet (omnivores < flexitarians < vegetarians < vegans) is associated with a decreased intake of energy, saturated fat, cholesterol, disaccharides, and total sugar as well an increased intake of dietary fibers, beta carotene, vitamin E and K. The combined index of the B12 status (4cB12 score) in vegetarians (0.02/0.75) was lower compared to omnivores (0.34/0.58; $p \leq 0.05$) and flexitarians (0.24/0.52; $p \leq 0.05$). In omnivores vitamin A, vitamin E, ferritin, and the urinary excretion of selenium, iodine, and zinc were higher than in vegans ($p \leq 0.05$). In contrast, vegans had the highest concentrations of biotin, folate, and vitamin C. Flexitarians, vegetarians, and vegans had a lower body weight, BMI, and body fat percentage in comparison to omnivores ($p \leq 0.05$). In omnivores the concentrations on total cholesterol, total cholesterol/HDL cholesterol ratio, LDL cholesterol, LDL cholesterol/HDL cholesterol ratio, apolipoprotein B, and apolipoprotein B/ apolipoprotein A1 ratio were higher than in vegetarians and vegans ($p \leq 0.05$).

Conclusion: The NuEva study confirms the position of the Academy of Nutrition and Dietetics that adequately planned vegetarian diets are healthy, nutritionally adequate, and may provide health benefits in the prevention and treatment of non-communicable

OPEN ACCESS

Edited by:
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Maastricht University, Netherlands

Reviewed by:
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University of Florida, United States

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

Received: *20 November 2021*
Accepted: *31 January 2022*
Published: *16 May 2022*

Citation:
Dawczynski C, Weidauer T, Richert C, Schlattmann P, Dawczynski K and Kiehntopf M (2022) Nutrient Intake and Nutrition Status in Vegetarians and Vegans in Comparison to Omnivores - the Nutritional Evaluation (NuEva) Study. Front. Nutr. 9:819106. doi: 10.3389/fnut.2022.819106

diseases. Nevertheless, critical nutrients were identified for all groups studied. This highlights the need to develop individual nutritional concepts to ensure an adequate nutrient intake.

Keywords: vegans, vegetarians, omnivores, nutrient intake, blood lipids, body weight

INTRODUCTION

Cardiovascular diseases (CVD) are one of the leading health problems worldwide and the main cause of death in German and the European region (1). Up to 40% of cardiovascular disease can be avoided by changing to a healthier diet (2). In recent years, vegetarian and vegan diets became increasingly important as they are associated with beneficial effects on blood lipid profile and a reduced risk of cardiovascular diseases (3, 4). On the other side, vegetarian and vegan diets typically avoid such foods as meat, sausage, fish (vegetarians) and eggs, dairy products, and honey (vegans) which bear the risk of undersupply of essential nutrients such as long-chain n-3 fatty acids (n-3 LC-PUFA), vitamin B2, vitamin B12, vitamin D, calcium, potassium, selenium, and zinc (5). In addition, the availability of ultra-processed meat and dairy alternatives on the supermarkets is growing (6, 7). Vegetarians and vegans consumed more ultra-processed foods such as industrial plant-based meat and dairy substitutes than omnivores. A higher consumption of ultra-processed foods is associated with higher risks of cardiovascular, coronary heart, and cerebrovascular diseases (8).

The predominant dietary pattern in Germany is characterized by high intake of foods of animal origin whereas the consumption of foods of plant origin is comparable low (9). This dietary pattern results in a low intake of dietary fibers, polyunsaturated fatty acids (PUFA), and secondary plant compounds which is associated with an increase in cardiovascular risk factors such as blood lipids (10–12).

The hype surrounding the vegetarian and vegan diet and the high prevalence of the omnivorous dietary pattern in combination with the likelihood for over- and undersupply of nutrients following the adoption of these eating habits highlights the need of extensive data collection to develop evidence-based recommendations.

In this context, a central objective of the NuEva study is to assess nutrient intake in the studied diets and compare of nutrient concentrations in plasma, serum and 24 h urine as well as the impact on cardiovascular risk factors.

MATERIALS AND METHODS

Study Design (Screening)

In summer/autumn 2018, healthy women and men between 18 to <70 years were recruited by press release and flier. The flier was distributed on the universities in Jena, Halle and Leipzig and in refectories, cafeterias, fitness studios, youth clubs, restaurants and coffee shops. Interested individuals initially complete a telephone pre-screening. As precondition, one of the four diets studied had been implemented since at least 1 year before enrollment. The adherence to one of the four diets (omnivores, flexitarians, vegetarians, vegans) was assessed by a self-created questionnaire and a dietary protocol over 5d. The study protocol listed the following exclusion criteria (13):

- Patients with diseases of the parathyroid, diseases necessitating regular phlebotomies, or patients with acute or chronic disease, which could affect the results of the present study. In addition, the following treatments precluding participation (at least 3 months prior to study start) resulted in an exclusion from the NuEva study
- Weight loss or weight gain (> 3 kg).
- Fundamental changes in dietary habits.
- Hormone replacement therapy.
- Elite athletes (>15 h of strenuous physical activity per week).
- Pregnancy or lactation.

Following the informed consent and confirmation of the in- and exclusion criteria, participants are scheduled for the baseline assessment. In total, 65 omnivores (daily consumption of meat and sausage, inclusive chicken/poultry, beef, pork etc.; consumption of fish), 70 flexitarians (occasional consumption of meat and sausage, inclusive chicken/poultry, beef, pork etc. (pre-dominantly high-quality products, ≤ two times/week); consumption of fish), 65 ovo-lacto vegetarians (no consumption of meat, sausage, fish), and 58 vegans (no consumption of foods of animal origin) participate on the NuEva screening (**Table 1**; **Figure 1**). The diet groups ovo-lacto vegetarians and vegans are partially summarized under the term vegetarian/vegan diets in the further course of the manuscript.

To record and document the variety in dietary practices within and between the groups, the run-in phase of the NuEva study included full self-reporting of individual dietary intake over 5 days. The dietary record based on the template "Freiburger Ernährungsprotokoll" which was provided by PRODI® version 6.4 (Nutri-Science, Stuttgart, Germany) and includes common foods and usual portion sizes. The template was adapted on the NuEva study by adding foods which are favored in vegetarian and vegan diets such as tofu, vegan yogurt alternatives and plant drinks, soy products, seitan, tempeh, maple and agave syrup (further foods could be added individually). Foods which were basically not contained in PRODI® were created and the nutritional information was taken from the packaging (inclusive fortification with e.g., vitamin B12, calcium). The daily energy and nutrient intake was calculated by the software package PRODI®. The nutrient intake from supplements was not considered in the calculation of nutrient intake by the dietary protocols (reason: irregular intake and great variety of the supplements, no information on nutrient bioavailability from the used supplements).

Available questionnaires from the German National Consumption Survey II (NVS II) and the German health

TABLE 1 | Characteristics of the study collective - NuEva-screening [Median/Interquartile range (IQR); (Min - Max)].

	Group 1 40 w, 25 m		Group 2 56 w, 14 m		Group 3 47 w, 18 m		Group 4 41 w, 17 m	
Age (years)	33.0 / 17.0 (18–61)	a	29.5 / 16.8 (19–69)	a	28.0 / 14.0 (18–65)	a,b	25.0 / 9.8 (19–56)	b
Implementation of the diet (years)	32.0 / 20.0 (1–61)	a	8.0 / 17.8 (1–68)	b	6.0 / 10.0 (1–34)	b	3.0 / 3.0 (1–34)	c

*Groups: 1 = omnivores, 2 = flexitarians, 3 = vegetarians, 4 = vegans. *Diet groups with different indices differ significantly ($p < 0.05$).*

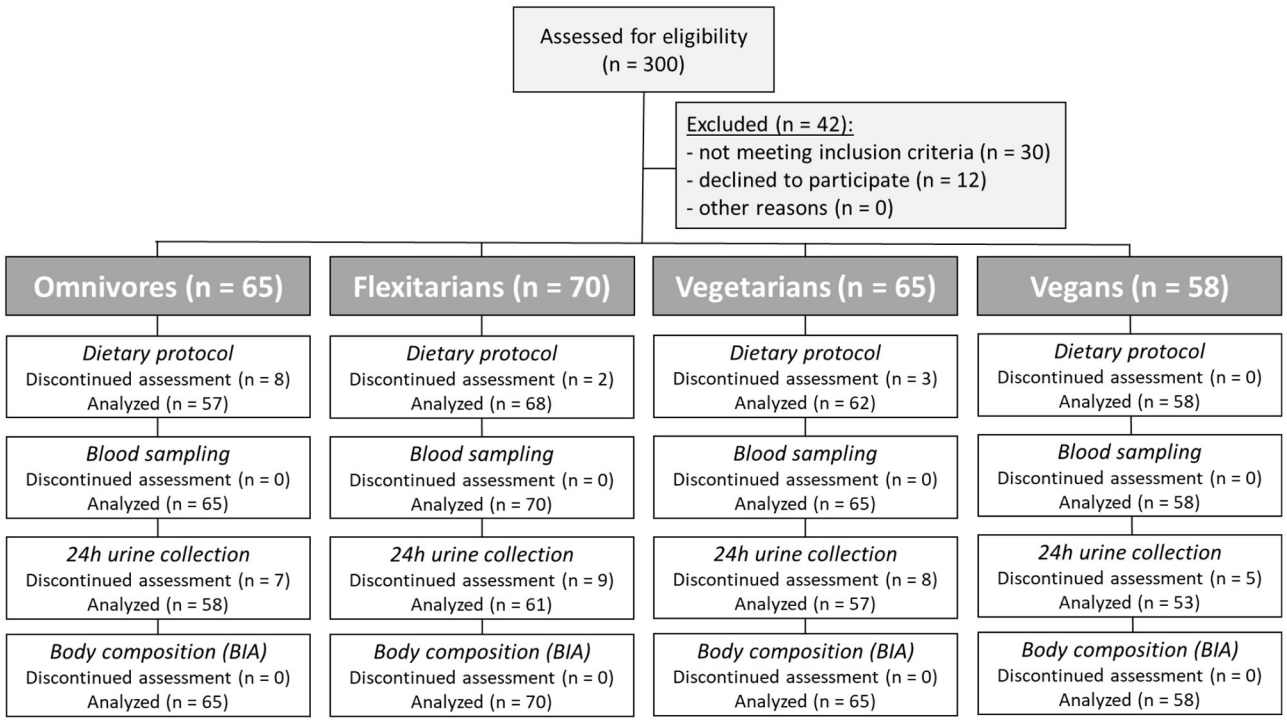

FIGURE 1 | Flow diagram of the participants. Three hundred subjects were enrolled in this study. Forty-two subjects were excluded since they did not meet the inclusion criteria or declined to participate. Based on their eating habits in advance, the participants were divided into four groups (omnivores, flexitarians, vegetarians, and vegans).

interview and examination survey for adults (DEGS1) are used to consider the socio-economic status as a confounding factor (14). In detail, the questionnaires include a set of questions about marital status, household size, educational achievement, income, and occupation as well as employment status. In addition to this, participants filled out questionnaires to assess physical activity (15), and health and disease status (inclusive medication use).

Blood was taken by venipuncture between 7:30 AM and 10:30 AM after at least 12 h overnight fast. The urine was collected over 24h directly before phlebotomy. The collection began after the morning urine on the day before. Afterward the urine was completely collected in a special container for the next 24 h. After the morning urine on day 2 (day of phlebotomy) the collection was finished. The total volume was documented, and eight Sarstedt® tubes á 9 ml were filled by the participants according to a standardized operating procedure. The aliquots were stored on a cool place and had to be hand at the study center until 10:00 am. Here the aliquots were frozen immediately (−20°C). Body weight, height, and waist circumferences were measured, respectively, by the same trained study nurse to the nearest half-kilogram or half-centimeter, with patients wearing light clothing with bare feet (one measurement). Waist circumference was measured midway between the lower rib margin and the iliac crest (a thumb's breadth above the navel). For measurement calibrated instruments were used (scale with integrated stadiometer: seca813, Hamburg, Germany; ergonomic tape measure: seca212, Hamburg, Germany).

Body composition was assessed by Body Impedance Analyzer [Data Input, Germany; exactness of measurement: 0.5 % of measurement value (Reactance)/± 2.0 % of measurement value (Resistance)]. The study protocol was reviewed and approved by the Ethical Committee of the Friedrich-Schiller-University

of Jena (number: 5504-03/18). The NuEva study was registered before launching (Clinical-Trials.gov Identifier: NCT03582020).

Sample Collection and Biochemical Analyses

Fasting peripheral venous blood samples were collected and centrifuged (10 min, 2,762 g, 4°C) for separation of plasma and serum. The 24h urine was aliquoted. Study parameters were analyzed immediately after blood sampling or urine collection or by using aliquots from serum, plasma, and 24h urine collections which were stored at −20°C (24h urine) or −80°C (serum, plasma) until analysis. The samples were prepared according to standard operation procedures. The analyses of biotin, methylmalonic acid, vitamin B2, vitamin C, and iodine were performed by Dianovis GmbH (**Supplementary Table S1**). Further chemical parameters in serum, plasma and urine were measured by using an Abbott Architect CI 16200 analyzer or HPLC according to the manufacturer's recommendations (**Supplementary Table S1**). Blood count was analyzed by XN 1000 (Sysmex®). Selenium and zinc were quantified by atomic absorption spectroscopy (**Supplementary Table S1**).

Statistical Methods

Statistical analyses were performed using the statistical software 'R' (version R i386 3.5.2). The same procedures were used for all studied diet groups. If the data of the four groups follow a normal distribution (tested with Shapiro-Wilk), one-way ANOVA was applied and the differences between specific groups were investigated using pairwise comparison with a two-sample *t*-test (using Benjamini-Hochberg correction). Otherwise, Kruskal-Wallis test with pairwise comparisons using Wilcoxon signed-rank tests (using Benjamini-Hochberg correction) were used.

The same analysis was performed for men and women subgroups and adjusted data sets as well. A standard ANCOVA was applied to the data to detect if a covariate (age, BMI, sex) influenced a specific variable. This influence meant that the correlation of the variable and the covariate was similar in all four groups, large enough (correlation coefficient larger than 0.3 or lower than −0.3) and significant. If the conditions were met, the values of that variable were adjusted for age (i.e., all values were adjusted as if the participants were all 30 years old) or BMI (i.e., all participants had the same BMI of 22). If sex had a significant influence, the statistical analysis was performed for men and women separately. All tests in this section were evaluated with $\alpha = 0.05$.

The power calculation was conducted for LDL cholesterol/HDL cholesterol ratio. The calculation based on data by Li et al. (16). A sample size of 44 participants per group had 80% power. We assume a drop-out rate of 25%. Thus, we enrolled at least 55 participants per group. The other parameters are examined exploratively. The power calculation for the NuEva study was conducted with G*Power 3.1.9.2 as described in (13). The details on study design, power-calculation, recruitment procedures, study assessments, and intervention protocol have been published previously (13).

RESULTS

The NuEva participants' age ranged between 18 and 69 years and the collective consisted of 70% women and 30% men (**Table 1**). In men, the age did not differ significantly between the four studied diet groups. The omnivorous women were slightly older than the women in the other three groups ($p \leq 0.05$). In the NuEva study population, the higher age in omnivores and flexitarians differed significantly from the lower age in the vegan group ($p \leq 0.05$; **Table 1**). The data were adjusted for age and for BMI, if a significant influence was observed (marked in **Tables 2–6**).

Generally, the omnivorous diet is practiced since birth or childhood whereas the flexitarian, vegetarian, and vegan diets were practiced on average for 13, 10, and 4 years (**Table 1**).

Socio-Economic Data

Marital and educational status differed significantly between the groups (**Supplementary Table S2a**). The participants of group 1 to 3 were married or living together with a partner while most vegans were single ($p \leq 0.05$). Most participants in all groups had a university entrance qualification, whereas the proportion with a secondary school leaving certificate was higher in omnivores. In omnivores and flexitarians, the number of subjects with a completed vocational training was higher than in the vegetarian/vegan groups ($p \leq 0.05$; **Supplementary Table S2a**). The NuEva study population's household size varied in size ranging from 1 to 4 without differences between the groups under consideration. In omnivores and flexitarians, the proportion of participants with a household net income > 3000 Euro per month was higher than in the vegetarian/vegan groups. On the other hand, the part with a household net income between 501–800 Euro per month was higher in vegetarians and vegans ($p \leq 0.001$). The intake of nutritional supplements was comparably high in vegans and low in omnivores ($p \leq 0.001$; **Supplementary Table S2b**). The regular intake of vitamin B12 and iron supplements was higher in the vegans than in the other groups studied ($p \leq 0.05$).

Nutrient Intake

The energy intake varied between the four studied groups with the highest intakes in both the omnivores and flexitarians and the lowest intake in vegans ($p \leq 0.01$). The intake of carbohydrates, dietary fibers, protein, and fat also varied with substantial differences between the omnivores and the vegetarian/vegan diets ($p \leq 0.05$). The intake of carbohydrates and dietary fibers increased in the following order: omnivores < flexitarians < vegetarians < vegans, the intake of protein and fat decreased in parallel (**Table 2**). The consumed dietary fibers consisted of approx. 30% water-soluble fibers and approx. 70% non-water-soluble fibers (**Table 2**). In vegans, the intake of water-soluble fibers and oligosaccharides (non-absorbable; data not shown) was higher than in omnivores and flexitarians ($p \leq 0.05$). The intake of monosaccharides was similar in all the four diets. However, the intake of disaccharides was markedly lower in vegans than in flexitarians and vegetarians ($p \leq 0.05$; data not shown).

TABLE 2 | Daily intake of energy and macronutrients (self-reports, 5 days)–NuEva-screening [Median/IQR; (Min–Max)].

		Group 1		Group 2		Group 3		Group 4	
	Sex	**Median / IQR**	**p**	**Median / IQR**	**p**	**Median / IQR**	**p**	**Median / IQR**	**p**
Energy (kcal)	All	2,325 / 902	a	2,114 / 789	a	2,088 / 875	a,b	1,829 / 573	b
Men (2,100–3,100)/ Women (1,700–2,500)§		(898–5,526)		(1,123–4,499)		(1,029–4,155)		(977–4,169)	
Carbohydrates (%)	All	42.2 / 11.7	a	47.2 / 8.1	b	49.2 / 9.3	b	55.1 / 9.2	c
>50% of total energy§		(23.5–54.2)		(2.4–63.4)		(16.8–65.9)		(30.8–70.1)	
Total dietary fiber (g)	All	24.4 / 13.2	a	27.0 / 10.9	a	30.3 / 15.2	b	36.8 / 10.7	c
30 g/day§		(9.5–54.6)		(12.5–63.4)		(6.4–85.0)		(14.8–115.5)	
Dietary fiber	All	7.9 / 4.0	a	8.2 / 3.8	a	9.0 / 4.6	a	10.1 / 4.6	b
(water-soluble) (g)		(3.3–17.7)		(3.3–24.7)		(1.8–24.6)		(1.8–30.8)	
Dietary fiber	All	16.0 / 8.3	a	16.4 / 6.6	a,b	17.8 / 9.5	a,b	22.0 / 8.8	c
(not water-soluble) (g)		(6.0–38.0)		(7.3–40.0)		(3.7–63.2)		(9.2–79.2)	
Protein (%)	All	16.5 / 4.4	a	14.0 / 4.2	b	13.3 / 2.9	c	12.8 / 2.8	c
Approx. 15% of total energy§		(9.5–27.8)		(9.8–24.6)		(9.4–23.5)		(8.8–23.0)	
Fat (%)	All	35.5 / 10.5	a	32.3 / 6.9	b	33.0 / 7.9	b	26.6 / 7.3	c
Approx. 30% of total energy§		(19.9–52.9)		(18.2–47.4)		(18.1–55.8)		(14.3–49.3)	
Σ saturated fatty acids (%)	All	15.3 / 4.4	a	12.9 / 4.2	b	11.8 / 3.6	b	6.2 / 3.3	c
<10% of total energy§		(6.6–23.8)		(4.9–20.6)		(4.3–20.8)		(2.9–14.7)	
Σ monounsaturated fatty acids (%)	All	11.2 / 4.2	a	9.2 / 2.6	b	9.6 / 4.6	b	8.5 / 3.9	c
≥10% of total energy§		(5.9–21.0)		(3.9–20.5)		(3.1–25.1)		(3.2–16.6)	
Σ polyunsaturated fatty acids (%)	All	4.3 / 1.8	a	4.2 / 2.0	a	5.0 / 2.9	b	6.3 / 2.7	c
≥10% of total energy§		(2.0–8.3)		(1.5–11.6)		(1.5–14.7)		(1.3–17.3)	
Oleic acid (g)	All	27.6 / 17.4	a	18.8 / 11.7	b	18.7 / 12.1	b	15.8 / 9.0	c
		(7.2–81.6)		(7.8–54.3)		(5.0–94.2)		(3.9–40.8)	
Palmitic acid (g)	All	18.9 / 10.3	a	13.2 / 8.0	b	12.1 / 6.9	b	5.3 / 3.3	c
		(7.3–46.1)		(4.7–32.9)		(2.5–24.9)		(1.6–18.4)	
Stearic acid (g)	All	8.1 / 6.0	a	5.3 / 3.5	b	4.4 / 2.8	c	1.6 / 1.1	d
		(2.3–23.6)		(1.1–15.9)		(0.8–17.7)		(0.4–9.1)	
Alpha linolenic acid, ALA (g)	All	1.5 / 1.2	a	1.2 / 0.8	a	1.3 / 0.9	a	1.4 / 1.1	a
		(0.3–7.1)		(0.5–10.5)		(0.4–7.7)		(0.3–7.7)	
Linoleic acid, LA (g)	All	8.4 / 5.3	a	8.0 / 5.4	a	9.2 / 8.0	a	9.9 / 5.7	a
		(1.6–33.3)		(2.0–18.2)		(2.3–36.9)		(2.1–20.7)	
Arachidonic acid, ARA (g)	All	0.21 / 0.25	a	0.11 / 0.15	b	0.04 / 0.04	c	0.02 / 0.02	d
		(0.04–1.11)		(0.01–0.47)		(0.01–0.17)		(0.01–0.78)	
Eicosapentaenoic acid, EPA (g)	All	0.08 / 0.18	a	0.04 / 0.07	b	0.01 / 0.34	c	0.00 / 0.01	d
		(0.01–1.50)		(0.01–0.61)		(0–1.17)		(0–0.01)	
Docosapentaenoic acid, DPA (g)	All	0.05 / 0.09	a	0.04 / 0.05	a	0.03 / 0.05	b	0.00 / 0.01	c
		(0.01–0.42)		(0.00–0.27)		(0–0.44)		(0–0.42)	
Docosahexaenoic acid, DHA (g)	All	0.16 / 0.29	a	0.09 / 0.13	b	0.04 / 0.05	c	0.01 / 0.01	d
		(0.01–1.60)		(0.01–0.99)		(0.01–1.90)		(0–0.43)	
Cholesterol (mg)	All	395.4 / 193.4	a	223.4 / 194.6	b	131.3 / 136.8	c	28.8 / 42.6	d
<300 mg§		(79–1,010)		(0–605)		(16–471)		(0–331)	

Groups: 1 = omnivores, 2 = flexitarians, 3 = vegetarians, 4 = vegans.
Adjusted for age: Σ monounsaturated fatty acids (%).
§*Reference intake: (17).*
**Diet groups with different indices differ significantly ($p < 0.05$).*

In addition to the observed differences in the amount of dietary fat, its composition differs also markedly between the four study groups. The intake of saturated fatty acids (SFA) was high in omnivores and notably lower in both flexitarians and vegetarians ($p \leq 0.001$). The lowest amounts of SFA were consumed in the vegan group. The omnivores consumed the highest amounts of monounsaturated fatty acids (MUFA) and vegan participants had the lowest intake ($p \leq 0.001$). In contrast

TABLE 3 | Daily intake of vitamins (self-reports, 5 days)–NuEva-screening [Median/IQR; (Min–Max)].

		Group 1		Group 2		Group 3		Group 4	
	Sex	**Median / IQR**	**p**	**Median / IQR**	**p**	**Median / IQR**	**p**	**Median / IQR**	**p**
Biotin (µg)	All	58.3 / 30.7	a	53.0 / 27.1	a	51.6 / 25.7	a	47.8 / 22.1	a
30–60 µg/day§		(7.4–122.7)		(22.0–137.1)		(20.9–189.2)		(16.0–134.0)	
Folic acid (µg)	All	299 / 180	a	305 / 158	a	277 / 147	a	312 / 133	a
300 µg/day§		(52–833) (101–671)		(103–1,209) (144–1,606)					
Niacin equivalent (mg)	All	35.1 / 18.7	a	25.3 / 13.0	b	23.6 / 12.5	b,c	22.1 / 8.5	c
12–15 mg/day§		(5.7–85.2)		(11.3–56.1)		(7.0–97.6)		(6.7–51.6)	
Pantothenic acid (mg)	All	5.7 / 2.1	a	4.8 / 2.3	a,b	4.3 / 1.8	b	4.3 / 1.8	b
6 mg/day§		(0.82–13.50)		(1.77–24.0)		(1.55–15.97)		(1.95–12.94)	
Vitamin A (mg)	All	0.52 / 0.94	a	0.39 / 0.36	b	0.32 / 0.24	c	0.07 / 0.06	d
0.8–1.0 mg/day§		(0.11–3.48)		(0.02–4.07)		(0.08–0.78)		(0.01–0.57)	
Beta carotene (µg)	All	5,210 / 8,833	a	6,872 / 7,602	a,b	6,912 / 6,380	a	9,797 / 8,705	b
		(145–43,991)		(236–67,029)		(485–22,581)		(1,357–37,951)	
Retinol equivalent (µg)	All	1,614 / 2,379	a	1,592 / 1,418	a	1,513 / 1,079	a	1,742 / 1,522	a
800–1,000 µg/day§		(231–14,661)		(300–11,563)		(289–4,129)		(297–6,356)	
Vitamin B_1 (mg)	m	1.94 / 0.89	a	1.60 / 0.70	a	1.48 / 0.91	a	1.54 / 0.94	a
1.0–1.2 mg/day§		(0.50–3.85)		(1.05–2.78)		(0.50–3.36)		(0.87–3.18)	
	w	1.34 / 0.46	a	1.27 / 0.46	a	0.93 / 0.67	a	1.26 / 0.68	a
		(0.50–3.12)		(0.17–2.37)		(0.22–4.74)		(0.46–2.84)	
	All	1.47 / 0.81	a	1.33 / 0.64	a,b	1.22 / 0.90	b	1.34 / 0.74	a,b
		(0.50–3.85)		(0.17–2.78)		(0.22–4.74)		(0.46–3.18)	
Vitamin B_2 (mg)	m	1.84 / 0.70	a	1.79 / 0.76	a	1.44 / 0.53	a,b	1.01 / 0.35	b
1.1–1.4 mg/day§		(0.33–5.16)		(1.27–3.57)		(0.69–2.38)		(0.55–2.53)	
	w	1.66 / 0.56	a	1.36 / 0.63	b	1.20 / 0.50	b	0.79 / 0.32	c
		(0.56–5.28)		(0.58–3.18)		(0.44–3.09)		(0.33–2.22)	
	All	1.69 / 0.77	a	1.42 / 0.68	b	1.25 / 0.56	c	0.85 / 0.32	d
		(0.33–5.28)		(0.58–3.57)		(0.44–3.09)		(0.33–2.53)	
Vitamin B_6 (mg)	All	2.04 / 0.96	a	1.79 / 0.96	b	1.47 / 0.64	b	1.77 / 0.87	b
1.1–1.4 mg/day§		(0.38–3.95)		(0.64–4.09)		(0.26–4.29)		(0.67–4.41)	
Vitamin B_{12} (µg)	m	6.43 / 4.38	a	3.39 / 2.29	b	2.05 / 2.06	c	0.39 / 1.09	d
4 µg/day§		(0.00–35.27)		(1.82–8.60)		(0.86–8.97)		(0–2.49)	
	w	5.36 / 3.74	a	2.81 / 1.92	b	1.77 / 1.33	c	0.37 / 0.57	d
		(1.61–42.63)		(0.36–10.83)		(0.26–9.81)		(0–3.75)	
	All	6.25 / 4.48	a	2.92 / 2.32	b	1.83 / 1.22	c	0.37 / 0.82	d
		(0.20–42.63)		(0.36–10.83)		(0.26–9.81)		(0–3.75)	
Vitamin C (mg)	All	131 / 79.8	a	147 / 103	a	124 / 94.6	a	161 / 83.9	a
95–110 mg/day§		(0–384)		(1–312)		(32–370)		(62–701)	
Vitamin D (µg)	All	2.28 / 1.83	a	1.71 / 1.74	b	1.67 / 1.57	b	0.94 / 1.27	c
20 µg/day§		(0.43–19.72)		(0.13–8.53)		(0.39–8.53)		(0.01–4.78)	
Vitamin E (mg)	All	9.16 / 4.80	a	11.1 / 5.79	a,b	13.3 / 8.51	b,c	14.5 / 8.00	c
12–14 mg/day§		(2.4–35.1)		(1.0–26.7)		(3.6–50.1)		(3.4–52.4)	
Vitamin K (µg)	All	154 / 167	a	198 / 180	a	195 / 181	a	243 / 184	b
60–70 µg/day§		(3–1,222)		(3–676)		(35–1,278)		(67–1,394)	

Groups: 1 = omnivores, 2 = flexitarians, 3 = vegetarians, 4 = vegans. §*Reference intake: (17). Significant influence of sex: vitamin B1, B2, B12.* **Diet groups with different indices differ significantly ($p < 0.05$).*

to SFA and MUFA, the intake of PUFA was low in omnivores, higher in vegetarians and highest in the vegan group ($p \leq 0.001$; **Table 2**).

The intake of alpha linolenic acid (ALA) and linoleic acid (LA) were similar in all four groups and the consumption of palmitic acid, oleic acid, and docosapentaenoic acid (n-3) were higher in omnivores than in the vegetarian/vegan diets ($p \leq 0.05$). The intake of stearic acid, arachidonic acid (n-6), eicosapentaenoic acid (n-3, EPA), and docosahexaenoic acid (n-3, DHA) differed also between the four groups, with decreasing of the intake in the

TABLE 4 | Daily intake of minerals and trace elements (self-reports, 5 days)–NuEva-screening [Median/IQR; (Min–Max)].

		Group 1		Group 2		Group 3		Group 4	
	Sex	**Median / IQR**	**p**	**Median / IQR**	**p**	**Median / IQR**	**p**	**Median / IQR**	**p**
Calcium (mg)	All	870 / 520	a	884 / 367	a	862 / 357	a	576 / 266	b
1,000 mg/day§		(93–3,061)		(345–2,412)		(219–1,581)		(157–1,723)	
Magnesium (mg)	All	353 / 135	a	349 / 145	a	337 / 195	a	394 / 188	a
300–350 mg/day§		(121–812)		(174–689)		(68–1,085)		(137–894)	
Sodium (mg)	All	2,584 / 1,136	a	1,982 / 754	b	1,900 / 1,027	b	1,452 / 909	c
1,500 mg/day§		(896–6,322)		(693–5,593)		(409–4,286)		(198–3,181)	
Potassium (mg)	All	3,467 / 1,454	a	3,392 / 1,285	a	2,835 / 1,090	a	3,284 / 1,242	a
4,000 mg/day§		(683–6,923)		(1,171–7,198)		(660–7,313)		(1,316–6,972)	
Chloride (mg)	m	4,502 / 2,676	a	3,118 / 1,615	a	3,286 / 2,019	a	2,244 / 1,666	b
2,300 mg/day§		(1,470–8,358)		(2,257–6,021)		(1,561–6,878)		(906–3,868)	
	w	3,823 / 1,299	a	2,713 / 1,148	b	2,380 / 1,064	c	1,801 / 1,301	d
		(1,821–8,503)		(1,040–5,322)		(824–5,575)		(500–3,319)	
	All	3,926 / 1,764	a	2,731 / 1,157	b	2,418 / 1,304	b	1,898 / 1,349	c
		(1,470–8,503)		(1,040–6,021)		(824–6,878)		(500–3,868)	
Phosphor (mg)	All	1,476 / 461	a	1,271 / 586	b	1,156 / 520	b	940 / 372	c
700 mg/day§		(460–3,218)		(602–2,740)		(358–2,674)		(333–2,192)	
Iron (mg)	m	13.0 / 5.8	a	12.1 / 3.6	a	13.3 / 6.7	a	14.9 / 6.4	a
10–15 mg/day§		(4.5–28.8)		(7.7–17.0)		(6.8–27.7)		(7.5–27.8)	
	w	11.4 / 3.7	a	10.2 / 4.9	a	9.9 / 5.0	a	11.1 / 6.4	a
		(4.7–34.4)		(4.8–18.2)		(3.6–22.0)		(4.5–24.7)	
	All	12.1 / 4.2	a,b	10.9 / 4.6	a	10.6 / 6.2	a,b	12.6 / 6.5	b
		(4.5–34.4)		(4.8–18.2)		(3.6–27.7)		(4.5–27.8)	
Copper (μg)	m	1,956 / 909	a	1,919 / 796	a	2,361 / 1,502	a	2,161 / 865	a
1,000–1,500 μg/day§		(542–3,551)		(1,094–2,915)		(1,117–4,217)		(1,406–5,570)	
	w	1,654 / 630	a	1,649 / 653	a	1,615 / 912	a	1,804 / 633	a
		(741–3,725)		(773–2,927)		(376–5,145)		(627–3,422)	
	All	1,771 / 826	a	1,650 / 679	a	1740 / 1135	a	1,896 / 696	a
		(542–3,725)		(773–2,927)		(376–5145)		(627–5,570)	
Manganese (μg)	All	3,889 / 1,806	a	4,120 / 2,629	a	4553 / 3,642	a,b	5,511 / 3,421	b
2,000–5,000 μg/day§		(1,887–10,538)		(1,581–16,342)		(1,375–18,376)		(1,923–13,948)	
Zinc (mg)	m	12.6 / 9.6	a	11.1 / 2.7	b	9.9 / 5.9	b	9.3 / 3.3.	b
11–16 mg/day§		(3.7–25.0)		(7.0–13.9)		(4.6–22.2)		(4.0–15.4)	
	w	11.2 / 4.7	a	9.2 / 4.0	b	7.9 / 3.5	b	6.7 / 2.5	c
		(4.0–24.8)		(4.2–18.0)		(2.8–20.2)		(2.6–17.7)	
	All	12.0 / 5.9	a	9.7 / 4.2	b	8.1 / 3.6	b	7.1 / 3.3	c
		(3.7–25.0)		(4.2–18.3)		(2.8–22.2)		(2.6–17.7)	

*Groups: 1 = omnivores, 2 = flexitarians, 3 = vegetarians, 4 = vegans. Adjusted for BMI: Iodine (μg). §Reference intake: (17). Significant influence of sex: chloride, iron, copper, zinc. The selenium intake was not calculated because the nutritional software (PRODI®) does not provide any information on the selenium levels in foods. The iodine intake was not calculated because the additional intake by fortified table salt was unknown. *Diet groups with different indices differ significantly ($p < 0.05$).*

following order: omnivores > flexitarians > vegetarians > vegans ($p \leq 0.01$; **Table 2**).

The vegan group was characterized by the lowest daily intake of cholesterol which was ten times higher in omnivores ($p \leq 0.001$). The average cholesterol intake in flexitarians, vegetarians and vegans matches the recommendations of the German Society of Nutrition (**Table 2**). The intakes of biotin, folic acid, vitamin B1, retinol equivalent, and vitamin C were similar between all studied diets and the average intakes complied with the recommendations of the German Society of Nutrition (**Table 3**), (17). The intake of niacin equivalent was the highest in omnivores ($p \leq 0.001$). The average intake of pantothenic acid and vitamin A matches the recommendations only in omnivores. The lowest intake of vitamin A in the vegan group was partly compensated by a higher intake of beta carotene ($p \leq 0.001$). Except for vitamin B2 and B12, the average intake of the B-vitamins complied with the recommendations for daily intake. The intake of vitamin B2 and B12 decrease as follows omnivores > flexitarians > vegetarians > vegans ($p \leq 0.05$). In the vegan group, the average intake of vitamin B2 was below recommended levels. The

TABLE 5 | Anthropometric data, body composition and blood lipids – NuEva-screening [Median/IQR; (Min–Max)].

		Group 1		Group 2		Group 3		Group 4	
Parameter	**Sex**	**Median / IQR**	**p**	**Median / IQR**	**p**	**Median / IQR**	**p**	**Median / IQR**	**p**
Anthropometric data									
Body weight	m	80.7 / 14.6	a	68.4 / 7.5	b	79.2 / 16.1	a,b	73.2 / 8.6	a,b
(kg)		(68.0–124.6)		(62.6–90.8)		(56.5–94.6)		(50.3–92.9)	
	w	71.8 / 17.8	a	62.6 / 8.6	b	62.6 / 13.6	b,c	57.9 / 10.0	c
		(46.8–100.9)		(51.1–91.4)		(47.5–92.5)		(47.6–80.0)	
	All	73.7 / 15.7	a	64.2 / 9.3	b	65.4 / 14.4	b	59.4 / 17.0	b
		(46.8–124.6)		(51.1–91.4)		(47.5–94.6)		(47.6–92.9)	
BMI	m	24.6 / 4.0	a	22.5 / 2.1	b	23.6 / 3.9	a,b	22.6 / 2.4	b
(kg/m^2)		(19.8–40.5)		(18.8–24.9)		(18.8–28.6)		(17.0–26.9)	
	w	25.4 / 5.7	a	22.1 / 3.5	b	21.6 / 3.3	b,c	20.8 / 3.1	c,d
		(18.5–33.7)		(18.3–32.2)		(17.3–29.9)		(17.1–27.3)	
	All	24.6 / 4.9	a	22.1 / 3.4	b	22.4 / 3.5	b	21.6 / 3.4	b
		(18.5–40.5)		(18.3–32.2)		(17.3–29.9)		(17.0–27.3)	
Waist	All	80.0 / 14.0	a	73.0 / 11.8	b	74.0 / 11.0	b	72.5 / 11.5	b
circumferences (cm)		(53–165)		(40–123)		(51–116)		(52–99)	
Body composition									
Body cell mass	m	36.6 / 5.4	a	33.1 / 6.0	a	33.3 / 4.2	a	34.6 / 6.1	a
(BCM, kg)		(30.1–52.1)		(28.7–40.6)		(29.0–39.5)		(24.2–43.3)	
	w	25.9 / 4.9	a	23.9 / 3.9	b	24.5 / 2.7	b	23.5 / 4.6	b
		(20.0–33.8)		(18.8–32.3)		(19.4–29.9)		(18.0–28.7)	
	All	28.9 / 10.4	a	25.0 / 6.8	b	25.5 / 6.3	b	24.5 / 6.3	b
		(20.0–52.1)		(18.8–40.6)		(19.4–39.5)		(18.0–43.3)	
Extracellular mass	m	25.8 / 6.3	a	22.8 / 4.3	a	28.4 / 6.8	a	25.0 / 6.7	a
(ECM, kg)		(18.6–35.1)		(18.6–36.3)		(18.4–34.4)		(19.6–33.0)	
	w	21.9 / 4.3	a	21.1 / 3.0	a	20.3 / 3.7	a	20.2 / 3.2	a
		(13.2–30.8)		(14.9–27.0)		(14.6–30.1)		(14.0–27.4)	
	All	22.9 / 5.6	a	21.6 / 3.4	a	21.6 / 6.1	a	20.9 / 5.1	a
		(13.2–35.1)		(14.9–36.3)		(14.6–34.4)		(14.0–33.0)	
ECM/BCM	m	0.72 / 0.15	a	0.71 / 0.11	a,b	0.85 / 0.20	c	0.78 / 0.19	a,c
		(0.5–1.1)		(0.5–1.1)		(0.6–1.1)		(0.5–1.0)	
	w	0.86 / 0.23	a	0.88 / 0.16	a	0.86 / 0.23	a	0.93 / 0.18	a
		(0.5–1.1)		(0.5–1.2)		(0.6–1.3)		(0.5–1.2)	
	All	0.80 / 0.30	a	0.85 / 0.24	a	0.86 / 0.23	a	0.86 / 0.25	a
		(0.5–1.1)		(0.5–1.2)		(0.6–1.3)		(0.5–1.2)	
Metabolic rate	m	1,770 / 170	a	1,665 / 190	a	1,670 / 128	a	1,710 / 190	a
(kcal)		(1,570–2,260)		(1,520–1,900)		(1,530–1,860)		(1,380–1,980)	
	w	1,440 / 150	a	1,370 / 123	b	1,390 / 88	b	1,355 / 143	b
		(1,250–1,680)		(1,210–1,640)		(1,230–1,560)		(1,190–1,520)	
	All	1,530 / 333	a	1,405 / 215	b	1,425 / 205	b	1,390 / 195	b
		(1,250–2,260)		(1,210–1,900)		(1,230–1,860)		(1,190–1,980)	
Body fat (kg)	m	18.1 / 8.0	a	12.4 / 4.3	b	14.1 / 6.1	a,b	13.0 / 5.7	b
		(8.7–39.9)		(8.6–19.7)		(7.0–31.3)		(4.7–18.9)	
	w	23.1 / 13.4	a	17.5 / 8.2	b	16.5 / 8.5	b	14.5 / 6.5	b
		(8.8–44.0)		(8.4–38.7)		(7.7–38.3)		(8.3–31.6)	
	All	20.4 / 12.6	a	16.6 / 7.6	b	16.1 / 9.0	b	14.4 / 6.6	b
		(8.7–44.0)		(8.4–38.7)		(7.0–38.3)		(4.7–31.6)	
Body water (l)	m	46.5 / 7.8	a	41.1 / 6.2	a	44.0 / 6.5	a	43.7 / 6.9	a
		(37.4–62.0)		(35.5–53.1)		(36.2–53.6)		(33.5–55.4)	
	w	35.2 / 4.5	a	32.4 / 4.0	b	32.8 / 4.1	b	31.4 / 3.6	b

(Continued)

TABLE 5 | Continued

Parameter	Sex	Group 1 Median / IQR	p	Group 2 Median / IQR	p	Group 3 Median / IQR	p	Group 4 Median / IQR	p
		(27.3–44.3)		(27.6–41.7)		(26.8–39.7)		(26.6–39.0)	
	All	37.5 / 9.5	a	33.8 / 7.4	b	34.2 / 9.9	b	32.7 / 7.0	b
		(27.3–62.0)		(27.6–53.1)		(26.8–53.6)		(26.6–55.4)	
Lean body mass	m	63.6 / 10.6	a	56.0 / 8.5	a	60.2 / 8.7	a	59.7 / 9.5	a
(LBM, kg)		(51.0–84.7)		(48.5–72.5)		(49.5–73.3)		(45.8–75.6)	
	w	48.0 / 6.1	a	44.2 / 5.4	b	44.7 / 5.7	b	42.9 / 4.9	b
		(37.3–60.5)		(37.6–56.9)		(36.6–54.2)		(36.4–53.2)	
	All	51.1 / 12.9	a	46.1 / 10.1	b	46.7 / 13.6	b	44.7 / 9.5	b
		(37.3–84.7)		(37.6–72.5)		(36.6–73.3)		(36.4–75.6)	
Phase angle	m	7.50 / 1.50	a	7.60 / 0.95	a,b	6.45 / 1.25	c	7.00 / 1.73	a,c
(°)		(5.3–10.5)		(5.4–9.9)		(5.2–9.0)		(5.7–9.9)	
	w	6.40 / 1.50	a	6.25 / 1.03	a	6.45 / 1.55	a	6.00 / 1.03	a
		(5.1–9.8)		(4.9–10.0)		(4.6–8.8)		(4.7–9.5)	
	All	6.85 / 2.15	a	6.40 / 1.70	a	6.45 / 1.50	a	6.40 / 1.63	a
		(5.1–10.5)		(4.9–10.0)		(4.6–9.0)		(4.7–9.9)	
Cell amount	m	58.3 / 5.4	a	58.7 / 3.9	a,b	54.2 / 5.6	c	56.2 / 6.6	a,c
(amount BCM in LBM		(48.3–68.1)		(48.9–66.4)		(47.9–63.8)		(50.6–66.5)	
%)	w	54.0 / 6.6	a	53.3 / 4.7	a	54.1 / 6.8	a	52.0 / 4.7	a
		(47.5–66.1)		(45.8–66.8)		(44.5–63.1)		(44.7–65.4)	
	All	55.7 / 9.1	a	54.0 / 7.2	a	54.1 / 6.8	a	53.8 / 7.1	a
		(47.5–68.1)		(45.8–66.8)		(44.5–63.8)		(44.7–66.5)	
Blood lipids	All	4.90 / 1.18	a	4.63 / 1.14	a,b	4.54 / 1.02	b	3.71 / 0.77	c
Total cholesterol									
(mmol/l)		(3.5–7.6)		(3.0–8.6)		(2.9–7.1)		(2.6–5.5)	
HDL cholesterol (mmol/l)	m	1.27 / 0.36	a	1.38 / 0.21	a	1.28 / 0.32	a	1.32 / 0.36	a
		(0.8–2.0)		(0.9–2.0)		(1.0–2.0)		(0.9–1.9)	
	w	1.59 / 0.52	a	1.62 / 0.52	a	1.65 / 0.45	a	1.53 / 0.47	a
		(1.0–2.6)		(1.0–2.3)		(1.0–2.8)		(0.8–2.3)	
	All	1.47 / 0.53	a	1.59 / 0.49	a	1.57 / 0.56	a	1.47 / 0.47	a
		(0.8–2.6)		(0.9–2.3)		(1.0–2.8)		(0.8–2.3)	
Total cholesterol /	All	3.16 / 1.39	a	2.91 / 1.00	a,b	2.80 / 1.22	b	2.58 / 0.64	c
HDL cholesterol		(1.9–8.5)		(1.9–6.4)		(1.9–5.1)		(1.6–4.4)	
LDL cholesterol	All	2.83 / 0.93	a	2.62 / 1.05	a,b	2.66 / 1.17	b	2.06 / 0.72	c
(mmol/l)		(1.8–5.3)		(1.2–6.4)		(1.4–4.4)		(0.7–3.4)	
LDL cholesterol /	All	1.86 / 1.22	a	1.74 / 0.92	a,b	1.53 / 1.13	b,c	1.40 / 0.69	c
HDL cholesterol		(0.7–5.0)		(0.8–4.7)		(0.8–3.5)		(0.4–2.9)	
Triacylglycerols	All	0.82 / 0.72	a	0.87 / 0.51	a	0.82 / 0.36	a	0.71 / 0.35	a
(mmol/l)		(0.4–3.3)		(0.4–3.0)		(0.4–2.8)		(0.3–2.4)	
Malondialdehyde- modified LDL	All	48.7 / 25.5	a	43.2 / 34.9	a,b	48.9 / 28.6	a	37.6 / 19.0	b
(U/l)		(13.5–132)		(4.28–128)		(15.9–103)		(13.9–96.1)	
Apolipoprotein A1	All	1.48 / 0.37	a	1.51 / 0.36	a	1.50 / 0.30	a	1.43 / 0.36	b
(g/l)		(1.0–2.5)		(0.9–2.3)		(1.0–2.7)		(1.0–2.0)	
Apolipoprotein B	All	0.85 / 0.32	a	0.83 / 0.34	a,b	0.80 / 0.35	b	0.64 / 0.19	c
(g/l)		(0.5–1.8)		(0.5–2.1)		(0.5–1.4)		(0.4–1.1)	
Apolipoprotein B /	m	0.61 / 0.29	a	0.63 / 0.43	a	0.64 / 0.32	a	0.51 / 0.17	a
Apolipoprotein A1		(0.4–1.3)		(0.4–1.1)		(0.3–1.0)		(0.3–0.8)	
	w	0.53 / 0.24	a	0.52 / 0.22	a	0.46 / 0.18	a,b	0.43 / 0.17	b
		(0.3–1.1)		(0.3–1.3)		(0.3–1.0)		(0.2–0.7)	
	All	0.59 / 0.26	a	0.54 / 0.25	a,b	0.49 / 0.26	b,c	0.45 / 0.17	c
		(0.3–1.3)		(0.3–1.3)		(0.3–1.0)		(0.2–0.8)	

*Adjusted for age: BMI, total cholesterol, LDL cholesterol, apolipoprotein A1, apolipoprotein B. Adjusted for BMI: waist circumferences. Significant influence of sex: weight, BMI, body cell mass, extracellular mass, BCM/ECM, metabolic rate, body fat, body water, lean body mass, phase angle, cell amount, HDL cholesterol, apolipoprotein A1/ apolipoprotein B. *Diet groups with different indices differ significantly ($p < 0.05$).*

TABLE 6 | Vitamins, minerals and trace elements in plasma/serum and 24h urine – NuEva-screening (Median / IQR; (Min - Max)).

		Group 1		Group 2		Group 3		Group 4	
Parameter	**Sex**	**Median / IQR**	***p***	**Median / IQR**	***p***	**Median / IQR**	***p***	**Median / IQR**	***p***
Plasma / serum									
Biotin	All	249 / 108	a	305 / 161	b	284 / 136	a,b	291 / 166	b
(ng/l)		(94–1,000)		(143–1,000)		(62–1,000)		(101–1,000)	
Folate	All	7.20 / 6.00	a,b	8.65 / 4.18	a,b	8.10 / 3.90	a	10.40 / 5.03	b
(µg/l)		(2.2–16.9)		(3.2–16.5)		(2.9–16.9)		(3.7–18.3)	
Vitamin B_{12}	All	242 / 94	a	246 / 119	a	208 / 110	b	213 / 161	a,b
(pmol/l)		(109–567)		(116–508)		(110–966)		(128–712)	
Holo-Transcobalamine	All	80.8 / 44.1	a	73.9 / 35.1	a	54.9 / 29.8	b	54.9 / 47.6	c
(pmol/l)		(39–227)		(26–180)		(11–356)		(14–327)	
Homocysteine	All	9.5 / 4.4	a	10.5 / 4.1	a	10.2 / 4.4	a	10.0 / 3.7	a
(µmol/l)		(4.4–21.2)		(5.3–19.2)		(5.2–33.5)		(3.7–37.8)	
Methyl malonic acid	All	17.0 / 8.5	a	20.0 / 10.0	a	21.0 / 13.0	a	18.5 / 12.3	a
(µg/l)		(9–65)		(8–57)		(9–82)		(7–64)	
4cB12 score[§]	All	0.34 / 0.58	a	0.24 / 0.52	a,c	0.02 / 0.75	c	0.08 / 0.89	b,c
		(−0.51 to 1.33)		(−0.66 to 1.45)		(−2.05 to 2.07)		(−1.44 to 1.52)	
Vitamin B_1	All	137.2 / 34.2	a,b	140.0 / 37.6	a	130.3 / 37.6	b	133.0 / 33.3	a,b
(nmol/l)		(79 – 235)		(72–215)		(63–275)		(91–208)	
Vitamin B_2	All	230 / 54.3	a	247 / 37.0	b	225 / 56.0	a,c	220 / 44.5	a,c
(µg/l)		c (150–334)		(175–343)		(155–335)		(147–318)	
Vitamin B_6	All	51.7 / 40.8	a	54.6 / 28.6	a	48.7 / 29.1	a	54.8 / 30.8	a
(nmol/l)		(20–264)		(18–187)		(14–257)		(15–194)	
Vitamin C	All	6.9 / 3.7	a	7.8 / 5.8	a,b	8.8 / 4.7	b	10.4 / 4.1	c
(mg/l)		(0.4–13.1)		(1.6–19.5)		(0.6–16.6)		(3.0–20.4)	
Vitamin A	All	1.61 / 0.62	a	1.75 / 0.58	a	1.67 / 0.59	a	1.35 / 0.42	b
(µmol/l)		(0.9–3.1)		(1.0–3.0)		(1.0–2.9)		(0.9–2.9)	
Vitamin D	All	70.7 / 21.6	a	65.4 / 26.6	a	68.3 / 34.3	a	65.0 / 22.3	a
(nmol/l)		(17–134)		(34–118)		(18–145)		(16–181)	
Vitamin E	All	26.7 / 8.9	a	27.1 / 7.8	a	25.0 / 7.3	a,b	24.0 / 6.8	b
(µmol/l)		(17–72)		(17–60)		(14–44)		(13–47)	
Ferritin	All	80.1 / 89.6	a	31.3 / 44.2	b	31.2 / 19.6	b	29.9 / 39.8	b
(µg/l)		(3.1–455)		(2.5–223)		(4.5–267)		(1.5–169)	
Transferrin	All	2.5 / 0.5	a	2.8 / 0.78	b	2.8 / 0.5	b	2.8 / 0.5	b
(g/l)		(2.0–3.9)		(1.9–4.7)		(2.0–3.9)		(1.8–4.1)	
Transferrin saturation	All	28.5 / 13.2	a	26.2 / 18.6	a	27.0 / 13.3	a	30.9 / 20.1	a
(%)		(6.4–88.0)		(2.9–57.7)		(6.6–60.0)		(7.8–73.0)	
24h urine Magnesium	All	4.30 / 2.10	a	4.40 / 1.93	a	4.80 / 1.60	a	4.90 / 2.20	a
(mmol/24h)		(1.0–10.6)		(1.4–9.5)		(1.0–8.7)		(1.3–9.9)	
Sodium	All	143 / 79	a	113 / 71	a	146 / 80	a	128 / 88	a
(mmol/24h)		(61–291)		(40–299)		(48–282)		(42–346)	
Selenium	All	0.25 / 0.19	a	0.19 / 0.13	b	0.20 / 0.09	b	0.16 / 0.12	b
(µmol/ 24h)		(0.07–0.77)		(0.06–0.76)		(0.07–0.66)		(0.06–0.91)	
Zinc	m	10.75 / 3.33	a	8.30 / 8.00	a	8.25 / 4.53	a	6.05 / 3.55	a
(µmol/24h)		(3.6–32.8)		(3.4–19.7)		(2.8–13.6)		(4.3–13.4)	
	w	5.85 / 4.23	a	5.20 / 3.08	a	5.60 / 4.20	a	4.20 / 2.70	b
		(3.2–27.2)		(1.8–14.6)		(1.7–18)		(0.8–9.5)	

(Continued)

TABLE 6 | Continued

Parameter	Sex	Group 1 Median / IQR	p	Group 2 Median / IQR	p	Group 3 Median / IQR	p	Group 4 Median / IQR	p
	All	7.85 / 5.58 (3.2–32.8)	a	5.50 / 4.60 (1.8–19.7)	b,c	6.10 / 3.90 (1.7–18)	b	5.00 / 3.30 (0.8–13.4)	c
Iodine (μg/l)	All	53.0 / 47.5 (17–268)	a	52.0 / 35.5 (13–192)	a,b	42.0 / 27.0 (6–335)	a,b	21.5 / 16.8 (8–509)	b

Significant influence of sex: zinc. Adjusted for age: vitamin E. [*] *Diet groups with different indices differ significantly ($p < 0.05$).* [§] *4cB12 score–combined index of B12 deficiency (normal range:−0.5 - 1.0).*

recommended daily intake of Vitamin B12 (4 μg) was also not reached in vegetarians, flexitarians, and vegans (**Table 3**).

The dietary intake of vitamin D was far below the recommendations in all four groups, with the lowest daily intake in vegans. The average intake of vitamin E failed to reach the recommended values in both omnivores and flexitarians. The vitamin E intake in the vegetarian/vegan groups was higher than in omnivores ($p \leq 0.05$). In all groups, vitamin K intake was 3 to 4 times higher than recommended, with the highest intake in vegans ($p \leq 0.01$; **Table 3**).

The average intake of magnesium, potassium, and cooper were comparable between the four diets under consideration. In all study groups, the daily intake complied with recommended levels, except for potassium whose intake was subpar. The lower iron intake in flexitarians differ from the higher intake in vegans ($p \leq 0.05$; **Table 4**). For most women, the recommended iron intake was not reached.

In group 1 to 3, the average calcium intake was almost at recommended level but was lower in vegans (576/266 mg; $p \leq 0.05$). In women, the intake of chloride varied in the following order: omnivores > flexitarians/vegetarians > vegans ($p \leq 0.05$). The comparably high intake of phosphor in omnivores, but also in flexitarians and in vegetarians differed from the lower intake in the vegan group ($p \leq 0.01$).

In groups 2 to 4 the average intake of zinc was lower than recommended, whereby the higher zinc intake in omnivores differed significantly from the lower intakes in the groups 2 to 4 ($p \leq 0.001$). The lowest zinc intake in the vegan subjects varied from the higher intake in omnivores, flexitarians, and vegetarians, respectively ($p \leq 0.01$). In total, the average intakes of the vitamins B1, B2, B12, chloride, iron, copper, and zinc were higher in men than in women ($p \leq 0.01$; **Tables 3**, **4**).

Anthropometric Data

The highest body weight in omnivores differed significantly from the lower ones in groups 2 to 4 ($p \leq 0.05$; **Table 5**). BMI depended on age. After adjustment for age, the higher BMI values in omnivores differed from the lower ones in groups 2 to 4 ($p \leq 0.05$). Comparable differences were detected for waist circumference ($p \leq 0.05$; **Table 5**).

The body composition differed significantly between men and women (**Table 5**). Extracellular mass (ECM) includes all the metabolically inactive body components, whereas the body cell mass (BCM) describes the metabolically active tissues of the body. Thus, the ECM/BCM ratio is a highly sensitive index of malnutrition (18). In men, the BCM did not differ significantly between the four studied groups. In women and the entire collective, the higher values in omnivores varied from the lower ones in group 2 to 4 ($p \leq 0.05$). The ECM and the ECM/BCM ratio did not differ between the four diet groups, except the slightly lower ratios in omnivorous and flexitarian men in comparison to the higher ratios in vegetarian men ($p \leq 0.05$; **Table 5**). In omnivores, the basal metabolic rate was on average 100 kcal higher than in flexitarians, vegetarians, and vegans ($p \leq 0.05$). The body fat mass was higher in omnivorous men in comparison to flexitarian and vegan men. In women and the entire study population, the higher values in omnivores differed from the values measured in the groups 2 to 4 ($p \leq 0.05$).

In men of the studied diets, the body water fraction was similar. The highest amounts of approx. 35 L in omnivorous women differed from the values measured in the other three groups which varied between 28–42 L ($p \leq 0.05$; **Table 5**). Comparable differences between the diets studied were also identified for the lean body mass (LBM) which highly correlates with muscle mass. LBM is defined as the difference between total body weight and body fat. The highest LBM was measured in omnivores with approx. 53 kg which differed from the lower LBM in groups 2 to 4 ($p \leq 0.05$; **Table 5**).

The phase angle (PhA; °) normally ranges between 5 to 7°. Values below the reference limit were found in groups 2 to 4 (**Table 5**). The lowest PhA in vegetarian men differed significantly from the higher PhA measured in omnivorous and flexitarian men ($p \leq 0.05$). Similar differences were found for the cell amount which describes the amount of BCM in the LBM (**Table 5**). The highest cell amount in omnivorous and flexitarian men differed from the lower levels in vegetarian men ($p \leq 0.05$; **Table 5**). In women and the entire study population cell amount and PhA were comparable between the four groups investigated (**Table 5**).

Blood Lipids and Vitamin B12 Status

The highest concentrations on total cholesterol, total cholesterol/HDL cholesterol ratio, LDL cholesterol, LDL cholesterol/HDL cholesterol ratio, apolipoprotein B, and apolipoprotein B/apolipoprotein A1 ratio in omnivores differ from the lower values in vegetarians and vegans ($p \leq 0.05$). Apolipoprotein A1 concentration in groups 1 to 3 were higher than in vegans ($p \leq 0.05$). HDL cholesterol and triacylglycerols did not differ between the four groups (**Table 6**). The higher concentrations of malondialdehyde-modified LDL in omnivores

and vegetarians differ from the lower ones in vegans ($p \leq 0.05$; **Table 6**).

Vitamin B12 concentrations in plasma were higher in omnivores and flexitarians compared to vegetarians (**Table 6**). Vitamin B12 concentrations below the reference range were found in all groups studied. Holotranscobalamin (holoTC) varied between 11 to 356 pmol/l with the lowest concentrations in vegetarians and vegans and the highest means in both omnivores and flexitarians ($p \leq 0.05$; **Table 6**). Concentrations below the reference range of 37.5 pmol/l were found in individuals from all studied groups, whereby the lowest concentrations observed in groups 1 and 2 ranged between 26 to 39 pmol/l and in the vegetarian/vegan groups lowest concentration between 11 to 14 pmol/l were detected.

Plasma methylmalonic acid and homocysteine concentrations were comparable between the studied groups (**Table 6**). The 4cB12 score was calculated from the above-mentioned parameters as a combined indicator of vitamin B12 status (19). Altogether, the lowest 4cB12 score in the vegetarian group (0.02/0.75) differed from the higher index calculated in omnivores (0.34/0.58) and flexitarians (0.24/0.52; $p \leq 0.05$; **Table 6**). The lower score in vegans (0.08/0.89) varied also from the score in omnivores ($p \leq 0.05$; **Table 6**).

Concentrations of Further Vitamins, Minerals, and Trace Elements in Plasma or Serum

In omnivores concentrations of vitamin A and vitamin E were higher than in vegans ($p \leq 0.05$ **Table 6**). The lowest vitamin A concentration observed in the vegan group differed from the higher means in the groups 1 to 3 ($p \leq 0.05$; **Table 6**). Vitamin E concentrations were adjusted for age and were on average 29 μmol/l in both omnivores and flexitarians, respectively and thus higher compared to the vegan group ($p \leq 0.01$; **Table 6**).

The lowest vitamin B1 concentrations were detected in the vegetarian group which differ from the higher concentrations in flexitarians ($p \leq 0.05$; **Table 6**). Vitamin B2 concentrations ranged from 147 to 343 μg/l whereby the higher concentrations detected in flexitarians differ from the lower concentrations in omnivores and vegetarian/vegan diets ($p \leq 0.001$; **Table 6**).

The folate concentrations varied between 2.2 to 18.3 μg/l with the highest values recorded in the vegan group (on average 10 μg/l; **Table 6**). The folate concentrations in vegetarians were lower than in vegans ($p \leq 0.05$). Biotin values in omnivores were lower compared to flexitarians and vegans, respectively (**Table 6**). In comparison to omnivores the detected vitamin C concentrations were higher in vegetarian/vegan diets ($p \leq 0.05$; **Table 6**).

Vitamin B6 and vitamin D concentrations ranged between 14 to 264 nmol/l and 16 to 181 nmol/l, respectively, without significant differences between the studied groups.

In the NuEva study population, concentrations of calcium, potassium, iron, and the iron saturation were comparable between the four diets studied.

Highest ferritin values were observed in the omnivores compared to flexitarians, vegetarians, and vegans ($p \leq 0.01$; **Table 6**). The lowest ferritin concentration of 1.5 μg/l was detected in a vegan woman, whereas low levels between 2.5 to 4.5 μg/l were also detected in women of group 1, 2 and 3. Ferritin levels above the upper limit of normal were observed in both men and women of the omnivorous group only. The lowest transferrin concentrations were measured in omnivores compared to groups 2, 3 and 4 ($p \leq 0.01$; **Table 6**).

Concentrations of Albumin, Creatinine, Minerals, and Trace Elements in 24h Urine Collection

Albumin and creatinine concentrations in 24h urine ranged from 5–82 mg/l and 1.4–21.9 mmol/l, respectively. The lowest values observed in vegetarians and vegans, diffed from omnivorous group's concentrations, respectively ($p \leq 0.05$; data not shown).

Magnesium and sodium in 24h urine was comparable between the groups studied. With regard to trace elements, highest zinc, and selenium concentrations were identified in omnivores differing from the lower ones in flexitarians, vegetarians, and vegans ($p \leq 0.05$; **Table 6**). The highest urinary iodine concentrations in the omnivores differ from the lower values in vegans ($p \leq 0.05$; **Table 6**).

DISCUSSION

Nutrient Intake and Cardiovascular Risk Factors

The data on nutrient intake showed substantial differences between meat-eaters and the vegetarian/vegan diets, with the strongest differences between omnivores and vegans. In line with data from Clarys et al. (20) the comparable high energy intake in omnivores differs from the lower energy content of the diet in vegans. In contrast, Weikert et al. (21) found no differences in energy intake between German vegans and omnivores. The higher energy intake effects body weight as in comparison to flexitarians, vegetarians, and vegans, omnivores showed a higher body weight (approx. 10 kg), associated with a 3 to 4 points increase of the BMI index and a 10 to 13 cm wider waist circumference. Matsumoto et al. (22) described also lower values of BMI and waist circumference in the vegetarian subgroup of the non-Hispanic whites in the Adventist Health Study-2. Overall, in the NuEva screening differences for body weight, BMI, LBM, BCM, body fat, and basal metabolic rate were detected in the following order: omnivores > flexitarians > vegetarians > vegans (omnivores vs. vegans: ≤ 0.05). We assume that the differences of anthropometric parameters and body composition relates to the characteristic intake of energy, fat, and protein which was calculated based on the dietary protocols. We would neglect the influence of physical activity as strenuous physical activity (> 15 h per week) was an exclusion criterion (13). The data available from the activity protocol indicate for a low to moderate physical activity of the NuEva participants (data not shown).

Our findings on weight and body composition are in accordance with previous data and highlighted the potential of plant-oriented dietary diets for weight management and therefore prevention of CVD (23, 24).

In line with previous findings, the intake of energy, major nutrients, dietary fibers and SFA differs strongly between the four groups with the most significant dissimilarity between omnivores and vegans (20, 21, 25). These characteristic differences may have an impact on the development of risk factors for non-communicable diseases, particularly CVD (26–29). In this context, the reduction of energy and SFA intake plays a key role in prevention of CVD. Thus, consuming less than 10% or in case of hypercholesterolemia, even less than 7% of total calorie intake (en%) from SFA is recommended by both European and American experts (17, 30). In the NuEva study population, only the vegans met these recommendations.

The NuEva study was able to show the sharp contrast between the intake of energy, dietary fibers, fat and SFA between omnivores and vegans which is accompanied by significantly higher concentrations of blood lipids in omnivores. Malondialdehyde-modified LDL is a marker for oxidative stress which is associated with atherosclerotic cardiovascular diseases (31). The lowest concentrations detected in vegans may related to a higher intake of antioxidative compounds, such as carotenoids, vitamin C, vitamin K, and vitamin E in this group.

In summary, our data indicate for the highest cardioprotective potential of the vegan diet.

Critical Nutrients in Omnivores, Flexitarians, Vegetarians, and Vegans

In omnivores, the average intake of energy, total fat, SFA, cholesterol, disaccharides, total sugar, and purines was higher than recommended by the German Society of Nutrition (17) and the intake of carbohydrates, particularly dietary fiber, PUFA mainly n-3 PUFA, potassium, vitamin D, vitamin E was lower than recommended.

For flexitarians, the following critical nutrients were identified with intakes higher than recommended: total fat, SFA, disaccharides, total sugar. In this group, the average intake of carbohydrates, particularly dietary fiber, PUFA particularly n-3 PUFA, pantothenic acid, vitamin B12, vitamin D, vitamin E, iron (woman), potassium, and zinc fell below the DGE recommendations (17).

In vegetarians, the intake of total fat, SFA, disaccharides, and total sugar was also above the recommendations while the intake of total protein, PUFA particularly n-3 PUFA, pantothenic acid, vitamin B12, vitamin D, calcium, iron (women), potassium, and zinc were below the recommendations for adequate nutrient intake (17).

In vegans, the mean intake of total sugar was also higher than recommended. In this diet form, the sugar intake mainly arose from fruits while consumption of sugar such as chocolate or gummy bears was comparably lower, as these foods often contain animal-based ingredients (20, 32). Still, the average intake of total protein, PUFA, particularly n-3 PUFA, pantothenic acid, vitamin B2, vitamin B12, vitamin A, vitamin D, calcium, potassium, iron (women), and zinc was markedly lower than recommended by the DGE (17). The intake of calcium was especially low in the vegan diet, as no dairy products are consumed. Clarys et al. (20) reported a mean vegans' calcium intake of 738 ± 456 mg/day and Weikert et al. (21) described a mean intake of 899 mg/day. The lower intakes in the present study are in accordance with data available from the EPIC-Oxford vegans (men: 603 ± 232 mg/day; women: 586 ± 226 mg/day), (33). In the EPIC oxford cohort, the percentage of subjects consuming less than 700 mg/day calcium was 15.0 for meat eaters, 15.9 for fish eaters, 18.6 for vegetarians and 76.1 for vegans which is similar to the NuEva screening. Appleby et al. (33) described a 30% higher fracture rate in vegans which disappeared when the analysis was conducted with all participants who consume less than 525 mg calcium/day.

The average intake of iron was below the recommendations in flexitarians and vegetarians but almost reached the optimal levels in both omnivores and vegans. Considering the lower bioavailability of non-heme iron (iron from plant origin), the iron intake in vegetarian/vegan diets should be 1.8 times higher than in omnivores diets (34). In accordance with the data from Kristensen et al. (25), vegan men nearly reached these recommended amounts.

In the diets under consideration, the average concentrations of all vitamins, minerals and trace elements analyzed, except for vitamin D and iodine, were within the reference range. Previous data indicate undersupply for vitamin D as a general problem independently from the diets studied (35, 36).

Evident from the dietary records, the intake of vitamin B2 and B12 decreased significantly in the following order omnivores > Flexitarians > vegetarians > vegans, because dairy products, fish and meat are the main food sources of these vitamins. Schüpbach et al. (37) found also higher average vitamin B2 levels in omnivores (92.0 ± 44.8 nmol/l; $n = 100$), whereby the difference to the lower values in vegetarians (82.4 ± 42.4 nmol/l; $n = 53$) and vegans (79.8 ± 41.7 nmol/l; $n = 53$) was not significant.

Undersupply with vitamin of B12 is a well-known problem in vegetarian/vegan diets as only animal-based foods deliver relevant amounts of active vitamin B12 (38). A 4cB12 score between −1.5 and −0.5 indicates for a low vitamin B12 supply and was calculated in one omnivorous participant, two flexitarians, ten vegetarians and ten vegans. A 4cB12 index between −1.5 and −2.5 indicates a potential B12 deficiency and was found in one vegetarian woman. In contrast to the findings from Weikert et al. (21) which described comparable 4cB12 indices between vegans (0.54) and omnivores (0.42; $p = 0.62$), the low 4cB12 indices in vegetarians and vegans differed significantly from the index calculated in omnivores. Thus, our data indicate for the risk of undersupply with vitamin B12 in the vegetarian/vegan groups which can manifests in macrocytic anemia or neurological impairments and can lead to irreversible neurological damage if undetected (39, 40).

Depletion of iron stores, defined by ferritin concentrations below 20/30 μg/l for women and men, were detected in 20 and 0% of the omnivorous group, 43/14% flexitarians, 28/17% vegetarians, and 42/6% vegans, respectively. Since, ferritin is also an acute-phase reactant, its synthesis is upregulated by

infection or inflammation (41). In the NuEva collective, c-reactive protein (CRP) concentrations between 3,5 mg/l and 22 mg/l were observed in six participants of the omnivorous group, four flexitarians, seven vegetarians and two vegans (data not shown). In this subgroup, hemoglobin, MCV and transferrin saturation were within the normal range ($n = 15$), except for two participants showing hemoglobin values below 7.6 mmol/l, one omnivorous participant and one vegan with an additional transferrin saturation below 16%. Moreover, in one vegetarian and one vegan only transferrin saturation was below 16%, indicating latent iron deficiency.

In accordance with the literature, men were marked by two to threefold higher ferritin concentrations than women (42). In addition, the NuEva screening shows significantly higher ferritin concentrations and lower transferrin concentrations in omnivores in comparison to the other studied diets. The obvious difference in ferritin levels between omnivores and vegetarian/vegan diets were also described by Schüpbach et al. (37) and Weikert et al. (21).

An iron deficiency anemia defined by decreased hemoglobin concentrations were found in 13% of women and 28% of men in the omnivorous group, 20/14% flexitarians, 21/17% vegetarians, and 29/18% vegans. Of the participants with anemia, reduced MCV values (<80 fl) were observed in 9% flexitarian women and one vegan (2%) woman. In men of this subgroup, MCV below the reference range was only detected in one vegetarian (6%).

Although the data on nutrient intake from self-reports showed a comparable iron intake on average 11–14 mg/d in the four studied diets, the iron status was worse in flexitarians, vegetarians, and vegans. This can be attributed to the low bioavailability of non-heme (approx. 3.7%) vs. heme iron (approx. 25%) (43). The absorption of non-heme iron varies strongly in dependence of dietary factors. Whereas phytic acid, calcium, polyphenols from coffee and tea reduce absorption of non-heme iron, simultaneously intake of ascorbic acid or other organic acids increase bioavailability of non-heme iron (43–45). While the dietary intake of vitamin C seems to be comparable between the four diets, the lower vitamin C concentrations measured in omnivores differ from the higher amounts in the vegetarian/vegan diets ($p \leq 0.05$). This indicates for a higher intake of vitamin C and thereof an improvement of the bioavailability of non-heme iron (43).

Besides iron, iodine, selenium, and zinc are further critical trace elements because of their lower content in vegetarian/vegan diets and particularly their lower bioavailability from plants, due to the presence of e.g., phytic acid (44). Since it has been shown that urinary selenium is a reliable biomarker for assessing selenium status (46), our findings are in line with actual data on plasma concentrations of selenium and selenoproteins P in vegans vs. omnivores (21). The lower urinary zinc concentrations in flexitarians, vegetarians, and particularly vegans are in accordance with the markedly lower calculated dietary intake and thus indicate a poorer supply in comparison to omnivorous group. As zinc and vitamin A interact, the lowest level of urinary zinc in combination with the lowest average plasma concentrations of vitamin A in the vegan group point to an additional impairment of the physiological functions in the vegan diet (25). The average vitamin A concentrations were in line within the reference range, but the lowest concentrations in vegans differed significantly from the other groups. In twenty-one participants of the omnivorous group, fourteen flexitarians, nineteen vegetarians, and thirty-two vegans the vitamin A concentration fell below the reference range of 1.46 μmol/l. Evident from the dietary records, the fact that vegan diets are very low in vitamin A is partially compensated by the high intake of beta carotin in this group. A high intake of carotenoids is associated with a reduction of CVD risk (47). The lower urinary iodine levels in vegetarians, and vegans in comparison to omnivores are comparable to the data described by Weikert et al. (21). In the four diets under consideration, the mean values of iodine excretion are below the WHO cut-off values (<100 μg/l), indicating for an iodine deficiency. Thus, an adequate iodine intake by the diet must be promoted to avoid development of goiter (48).

CONCLUSIONS

Most of the Europeans practice one of the before mentioned dietary patterns, each of which varies in their amount of animal-based products. The Academy of Nutrition and Dietetics postulates that adequately planned vegetarian diets are healthy, nutritionally adequate, and may provide health benefits in the prevention and treatment of non-communicable diseases (32). Overall, the data from the NuEva screening confirm this statement. The reduced consumption of animal products in the following order omnivores > flexitarians > vegetarians > vegans is associated with a decreased intake of energy, fat, and particularly SFA, cholesterol, disaccharides, and total sugar as well an increased intake of soluble and non-soluble dietary fibers, vitamin E, K, beta carotene, and manganese. In detail, the data suggests that flexitarian, vegetarian and vegan diets are nutrient-dense and could be recommended for weight management. The prevention of body weight gain and the observed blood lipid lowering effect of vegetarian and in particular vegan diets contribute to the prevention of CVD (49, 50).

However, the NuEva screening reveals an insufficient dietary intake of selenium, zinc, potassium, iron (women), calcium, vitamin B12, n-3 LC-PUFA, and vitamin D particularly in vegetarian and vegan diets.

Recommendations

A regular consumption of the following foods can counteract the weak points detected in the NuEva screening (**Table 7**):

i) nuts, seeds, wheat bran and barley flakes as sources for zinc and selenium,
ii) paprika, pistachio, pumpkin seeds, cocoa etc. for optimal potassium intake,
iii) sesame seeds or tahini, cocoa, amaranth, cashews, pine nuts, and oats in combination with vitamin C or other organic acids to ensure an adequate iron intake,
iv) dairy products or plant-based foods enriched with vitamin B12, B12 supplements,

TABLE 7 | Critical nutrients in vegetarian/vegan diets and suitable food sources.

Critical nutrients and reference range for daily intake	Suitable food sources	Content per 100 g	Reference
Zinc	Poppy seeds	7.9 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/171330/nutrients
7–16 mg/d	Sesame seeds, dried	7.75 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170150/nutrients
	Wheat bran	7.26 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169722/nutrients
	Cashew, raw	5.78 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170162/nutrients
	Pecan nuts	4.53 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170182/nutrients
	Linseeds	4.34 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169414/nutrients
	Brazil nuts	4.06 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170569/nutrients
	Barley flakes	2.77 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170283/nutrients
	Pistachio	2.2 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170184/nutrients
Selenium	Brazil nuts	1920 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170569/nutrients
60–70 μg/d	Wheat bran	77.6 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169722/nutrients
	Barley flakes	37.7 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170283/nutrients
	Sesame seeds, dried	34.4 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170150/nutrients
	Cheese, parmesan, grated	34.4 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/171247/nutrients
	Linseeds	25.4 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169414/nutrients
	Cashew, raw	19.9 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170162/nutrients
	Poppy seeds	13.5 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/171330/nutrients
	Pecan nuts	3.8 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170182/nutrients
Potassium	Paprika	2280 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/171329/nutrients
4000 μg/d	Cocoa	1520 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169593/nutrients
	Pistachio	1020 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170184/nutrients
	Pumpkin seeds	919 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170188/nutrients
	Almonds	733 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170567/nutrients
	Potatoes	417 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170027/nutrients
	Lentils, mature seeds, cooked, boiled	369 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/172421/nutrients
	Carrots	320 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170393/nutrients
	Kohlrabi	320 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/168424/nutrients
	Mushrooms	318 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169251/nutrients
	Lettuce	247 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169247/nutrients
	Cabbage	170 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169975/nutrients
Iron*	Sesame seeds, dried	14.6 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170150/nutrients
10–15 mg/d	Cocoa	13.9 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/169593/nutrients
	Sesame tahini	9 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/446287/nutrients
	Amaranth, grain, uncooked	7.61 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170682/nutrients
	Cashew, raw	6.68 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170162/nutrients
	Pine nuts	5.53 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170591/nutrients
	Quinoa, uncooked	4.57 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/168874/nutrients
	Oats	4.5 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/368739/nutrients
	Chickpeas	2.89 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/173757/nutrients
	Spinach, raw	2.71 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/168462/nutrients
Calcium	Sesame seeds, dried	975 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170150/nutrients
1,000 mg/d	Cheese, parmesan, grated	853 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/171247/nutrients
	Almonds	269 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170567/nutrients
	Spinach, raw	99 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/168462/nutrients
	Broccoli	47 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170379/nutrients
	Fortified foods, such as oat milk		
Vitamin B12	Cheese, swiss	3.02 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/746767/nutrients
4 μg/d	Cheese, mozzarella, low moisture, part-skim	1.65 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/329370/nutrients
	Cheese, parmesan, grated	1.35 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/325036/nutrients

(Continued)

TABLE 7 | Continued

Critical nutrients and reference range for daily intake	Suitable food sources	Content per 100 g	Reference
	Cheese, cheddar	1.06 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/328637/nutrients
	Cheese, ricotta, whole milk	0.78 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/746766/nutrients
	Yogurt, plain, low fat	0.56 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/170886/nutrients
	Milk, whole	0.54 μg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/1097512/nutrients
	Plant-based foods enriched with vitamin B12		
	B12 supplements (chewable tablets, drops, toothpaste etc.)		
Long-chain n-3 PUFA 250–500 mg/d	Nori (seaweeds)	80 mg	https://fdc.nal.usda.gov/fdc-app.html#/food-details/168458/nutrients
	Supplements, capsules, or microalgae oils, e.g., from schizochytrium sp., ulkenia sp.		
Vitamin D 20 μg/d	Adequate sun exposure or supplementation		

** Combination of plant-based foods with sources of vitamin C or other organic acids and avoiding the simultaneous consumption of foods and beverages containing polyphenols (tea, coffee), phytic acid to increase iron bioavailability.*

v) seaweeds (Nori) or microalgae oils, e.g., from schizochytrium sp. or ulkenia sp. for an optimal n-3 LC-PUFA status,
vi) adequate sun exposure is recommended to improve vitamin D status,
vii) supplementation of calcium and vitamin D and ensuring an adequate intake of high-quality protein to avoid an elevated fracture risk or the development of osteoporosis.

Future R&D activities should focus on the improvement of nutrient profiles of traditional plant food, e.g., by optimal variety selection of seeds, improvement of the soil quality, and reduction in processing steps of plant-based foods to avoid loss of their valuable nutrients.

In summary, the NuEva study highlights the need for development and distribution of practical nutritional concepts adapted to individual dietary preferences to ensure an adequate nutrient intake and to avoid deficiency symptoms and risk of associated disorders for all diets under consideration.

Strengths and Limitations

The NuEva study is designed to identify critical nutrients relating to the implementation of one of the studied diets (omnivores, flexitarians, vegetarians, vegans,) by evaluation of a self-reported dietary protocol and measured parameters reflecting nutrient status in serum, plasma, and 24h urine. Due to a comprehensive assessment of biomarkers and data on body composition, the NuEva study allows evaluating physiological benefits or possible physiological consequences resulting from the implementation of the studied nutritional habits with focus on cardiovascular risk.

A one-time self-reported dietary protocol over a defined period is one of the limitations of the NuEva screening. Self-reports on dietary intake bear the risk of over-reporting of healthy foods and under-reporting of high-energy, low nutrient foods. The dietary protocols were calculated with the software PRODI® version 6.4 (Nutri-Science, Stuttgart, Germany) for professional dietary counseling and therapy. The calculations on nutrients composition based on the "Bundeslebensmittelschlüssel" and further nutrition tables. In this context, differences between nutrient profiles calculated with the software and the nutrient composition of the consumed foods, adding to the limitations encountered in this study. Variations of nutrient profiles can depend on types, preparation conditions and feeding conditions.

A further limitation is the generalizability of the data which represent a regional sample as the participants were recruited from central East Germany.

The calculated nutrient intake form self-reports was related to the recommendations for nutrient intake from the German Society of Nutrition (DGE e.V.). Currently, these guidelines do not consider the implementation of diets differing in their proportion of animal-based foods.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of an ongoing evaluation of the datasets by the study team. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the Friedrich Schiller University of Jena. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CD was responsible for acquisition of funding, conceptualization and trial design, conduction of the NuEva study, data acquisition,

and she wrote the original draft. As study physician KD was responsible for medical care of the NuEva participants during the study. TW performed the statistical analysis of the data sets based on a constituted statistical plan. PS and CD supervised the statistical analysis. CR supported the data acquisition and sample management. MK was responsible for data acquisition and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the German Federal Ministry of Research and Education (Grant Number: 01EA1708). The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

ACKNOWLEDGMENTS

We are grateful to thank the Federal Ministry of Education and Research for financing the NuEva study. We would like to thank Muriel Steiner for blood sampling and Ute Helms for her excellent technical assistances. In addition, we thank Mrs. Sybille Bremer-Streck for analysis of trace elements and Jaqueline Muthumbi-Yomade for proof-reading the manuscript. Special thanks go to NuEva subjects for participating in the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.819106/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Correction
PUBLISHED 27 July 2022
DOI 10.3389/fnut.2022.975159

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

RECEIVED 21 June 2022
ACCEPTED 15 July 2022
PUBLISHED 27 July 2022

CITATION
Dawczynski C, Weidauer T, Richert C, Schlattmann P, Dawczynski K and Kiehntopf M (2022) Corrigendum: Nutrient intake and nutrition status in vegetarians and vegans in comparison to omnivores—the nutritional evaluation (NuEva) study. *Front. Nutr.* 9:975159. doi: 10.3389/fnut.2022.975159

Corrigendum: Nutrient intake and nutrition status in vegetarians and vegans in comparison to omnivores—the nutritional evaluation (NuEva) study

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KEYWORDS

vegans, vegetarians, omnivores, nutrient intake, blood lipids, body weight

A corrigendum on
Nutrient intake and nutrition status in vegetarians and vegans in comparison to omnivores—the nutritional evaluation (NuEva) study

by Dawczynski, C., Weidauer, T., Richert, C., Schlattmann, P., Dawczynski, K., and Kiehntopf. M. (2022). Front. Nutr. 9:819106. doi: 10.3389/fnut.2022.819106

In the published article, there was an error in the legend for "**Table 1**. Characteristics of the study collective - NuEva-screening (Median/Interquartile range (IQR); (Min - Max))." as published. The Information "*Diet groups with different indices differ significantly ($p < 0.05$)" was lost. The corrected legend appears below.

"Groups: 1 = omnivores, 2 = flexitarians, 3 = vegetarians, 4 = vegans.

*Diet groups with different indices differ significantly ($p < 0.05$)."

In the published article, there was an error in the legend for "**Table 2**. Daily intake of energy and macronutrients (self-reports, 5 days) - NuEva-screening (Median/IQR; (Min - Max))." as published. The Information "*Diet groups with different indices differ significantly ($p < 0.05$)" was lost. The corrected legend appears below.

"Groups: 1 = omnivores, 2 = flexitarians, 3 = vegetarians, 4 = vegans.

Adjusted for age: Σ monounsaturated fatty acids (%).

§Reference intake: DGE, 2019.

*Diet groups with different indices differ significantly ($p < 0.05$)."

In the published article, there was an error in the legend for "**Table 3**. Daily intake of vitamins (self-reports, 5 days) - NuEva-screening (Median / IQR; (Min - Max))." as published. The Information "*Diet groups with different indices differ significantly ($p < 0.05$)" was lost. The corrected legend appears below.

"Groups: 1 = omnivores, 2 = flexitarians, 3 = vegetarians, 4 = vegans.

§Reference intake: DGE, 2019.

Significant influence of sex: vitamin B1, B2, B12.

*Diet groups with different indices differ significantly ($p < 0.05$)."

In the published article, there was an error in the legend for "**Table 4**. Daily intake of minerals and trace elements (self-reports, 5 days) - NuEva-screening (Median / IQR; (Min - Max))." as published. The Information "*Diet groups with different indices differ significantly ($p < 0.05$)" was lost. To complete the data, we would like to insert the information that the calculation of iodine and selenium intake was not possible. The corrected legend appears below.

"Groups: 1 = omnivores, 2 = flexitarians, 3 = vegetarians, 4 = vegans.

Adjusted for BMI: Iodine (µg).

§Reference intake: DGE, 2019.

Significant influence of sex: chloride, iron, copper, zinc.

The selenium intake was not calculated because the nutritional software (PRODI®) does not provide any information on the selenium levels in foods.

The iodine intake was not calculated because the additional intake by fortified table salt was unknown.

*Diet groups with different indices differ significantly ($p < 0.05$)."

In the published article, there was an error in the legend for "**Table 5**. Anthropometric data, body composition and blood lipids – NuEva-screening (Median / IQR; (Min - Max))." as published. The Information "*Diet groups with different indices differ significantly ($p < 0.05$)" was lost. The corrected legend appears below.

"Adjusted for age: BMI, total cholesterol, LDL cholesterol, apolipoprotein A1, apolipoprotein B.

Adjusted for BMI: waist circumferences.

Significant influence of sex: weight, BMI, body cell mass, extracellular mass, BCM/ECM, metabolic rate, body fat, body water, lean body mass, phase angle, cell amount, HDL cholesterol, apolipoprotein A1/ apolipoprotein B.

*Diet groups with different indices differ significantly ($p < 0.05$)."

In the published article, there was an error in the legend for "Table 6. Vitamins, minerals and trace elements in plasma/serum and 24h urine – NuEva-screening (Median / IQR; (Min - Max))." as published. The Information "*Diet groups with different indices differ significantly ($p < 0.05$)" was lost. In addition, the information on 4cB12score [§4cB12 score - combined index of B12 deficiency (normal range:−0.5 - 1.0)] was also lost. The corrected legend appears below.

"Significant influence of sex: zinc.

Adjusted for age: vitamin E.

*Diet groups with different indices differ significantly ($p < 0.05$).

§4cB12 score - combined index of B12 deficiency (normal range:−0.5 - 1.0)."

In the published article, there was an error in "Table 6. Vitamins, minerals and trace elements in plasma/serum and 24h urine – NuEva-screening (Median / IQR; (Min - Max))." as published. The units for ferritin (µg/l), transferrin (g/l) and transferrin saturation (%) were lost in Table 6. The corrected "Table 6. Vitamins, minerals and trace elements in plasma/serum and 24h urine – NuEva-screening (Median / IQR; (Min - Max))." and its legend appear below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

Publisher's note

TABLE 6 Vitamins, minerals and trace elements in plasma/serum and 24h urine – NuEva-screening (Median / IQR; (Min - Max)).

		Group 1		Group 2		Group 3		Group 4	
Parameter	**Sex**	**Median / IQR**	***p***	**Median / IQR**	***p***	**Median / IQR**	***p***	**Median / IQR**	***p***
Plasma / serum									
Biotin	All	249 / 108	a	305 / 161	b	284 / 136	a,b	291 / 166	b
(ng/l)		(94–1,000)		(143–1,000)		(62–1,000)		(101–1,000)	
Folate	All	7.20 / 6.00	a,b	8.65 / 4.18	a,b	8.10 / 3.90	a	10.40 / 5.03	b
(µg/l)		(2.2–16.9)		(3.2–16.5)		(2.9–16.9)		(3.7–18.3)	
Vitamin B_{12}	All	242 / 94	a	246 / 119	a	208 / 110	b	213 / 161	a,b
(pmol/l)		(109–567)		(116–508)		(110–966)		(128–712)	
Holo-Transcobalamine	All	80.8 / 44.1	a	73.9 / 35.1	a	54.9 / 29.8	b	54.9 / 47.6	c
(pmol/l)		(39–227)		(26–180)		(11–356)		(14–327)	
Homocysteine	All	9.5 / 4.4	a	10.5 / 4.1	a	10.2 / 4.4	a	10.0 / 3.7	a
(µmol/l)		(4.4–21.2)		(5.3–19.2)		(5.2–33.5)		(3.7–37.8)	
Methyl malonic acid	All	17.0 / 8.5	a	20.0 / 10.0	a	21.0 / 13.0	a	18.5 / 12.3	a
(µg/l)		(9–65)		(8–57)		(9–82)		(7–64)	
4cB12 score[§]	All	0.34 / 0.58	a	0.24 / 0.52	a,c	0.02 / 0.75	c	0.08 / 0.89	b,c
		(−0.51 to 1.33)		(−0.66 to 1.45)		(−2.05 to 2.07)		(−1.44 to 1.52)	
Vitamin B_1	All	137.2 / 34.2	a,b	140.0 / 37.6	a	130.3 / 37.6	b	133.0 / 33.3	a,b
(nmol/l)		(79 – 235)		(72–215)		(63–275)		(91–208)	
Vitamin B_2	All	230 / 54.3	a	247 / 37.0	b	225 / 56.0	a,c	220 / 44.5	a,c
(µg/l)		c (150–334)		(175–343)		(155–335)		(147–318)	
Vitamin B_6	All	51.7 / 40.8	a	54.6 / 28.6	a	48.7 / 29.1	a	54.8 / 30.8	a
(nmol/l)		(20–264)		(18–187)		(14–257)		(15–194)	
Vitamin C	All	6.9 / 3.7	a	7.8 / 5.8	a,b	8.8 / 4.7	b	10.4 / 4.1	c
(mg/l)		(0.4–13.1)		(1.6–19.5)		(0.6–16.6)		(3.0–20.4)	
Vitamin A	All	1.61 / 0.62	a	1.75 / 0.58	a	1.67 / 0.59	a	1.35 / 0.42	b
(µmol/l)		(0.9–3.1)		(1.0–3.0)		(1.0–2.9)		(0.9–2.9)	
Vitamin D	All	70.7 / 21.6	a	65.4 / 26.6	a	68.3 / 34.3	a	65.0 / 22.3	a
(nmol/l)		(17–134)		(34–118)		(18–145)		(16–181)	
Vitamin E	All	26.7 / 8.9	a	27.1 / 7.8	a	25.0 / 7.3	a,b	24.0 / 6.8	b
(µmol/l)		(17–72)		(17–60)		(14–44)		(13–47)	
Ferritin	All	80.1 / 89.6	a	31.3 / 44.2	b	31.2 / 19.6	b	29.9 / 39.8	b
(µg/l)		(3.1–455)		(2.5–223)		(4.5–267)		(1.5–169)	
Transferrin	All	2.5 / 0.5	a	2.8 / 0.78	b	2.8 / 0.5	b	2.8 / 0.5	b
(g/l)		(2.0–3.9)		(1.9–4.7)		(2.0–3.9)		(1.8–4.1)	
Transferrin saturation	All	28.5 / 13.2	a	26.2 / 18.6	a	27.0 / 13.3	a	30.9 / 20.1	a
(%)		(6.4–88.0)		(2.9–57.7)		(6.6–60.0)		(7.8–73.0)	
24h urine	All	4.30 / 2.10	a	4.40 / 1.93	a	4.80 / 1.60	a	4.90 / 2.20	a
Magnesium									
(mmol/24h)		(1.0–10.6)		(1.4–9.5)		(1.0–8.7)		(1.3–9.9)	
Sodium	All	143 / 79	a	113 / 71	a	146 / 80	a	128 / 88	a
(mmol/24h)		(61–291)		(40–299)		(48–282)		(42–346)	
Selenium	All	0.25 / 0.19	a	0.19 / 0.13	b	0.20 / 0.09	b	0.16 / 0.12	b
(µmol/ 24h)		(0.07–0.77)		(0.06–0.76)		(0.07–0.66)		(0.06–0.91)	
Zinc	m	10.75 / 3.33	a	8.30 / 8.00	a	8.25 / 4.53	a	6.05 / 3.55	a
(µmol/24h)		(3.6–32.8)		(3.4–19.7)		(2.8–13.6)		(4.3–13.4)	

(Continued)

TABLE 6 Continued

Parameter	Sex	Group 1 Median / IQR	*p*	Group 2 Median / IQR	*p*	Group 3 Median / IQR	*p*	Group 4 Median / IQR	*p*
	w	5.85 / 4.23 (3.2–27.2)	a	5.20 / 3.08 (1.8–14.6)	a	5.60 / 4.20 (1.7–18)	a	4.20 / 2.70 (0.8–9.5)	b
	All	7.85 / 5.58 (3.2–32.8)	a	5.50 / 4.60 (1.8–19.7)	b,c	6.10 / 3.90 (1.7–18)	b	5.00 / 3.30 (0.8–13.4)	c
Iodine (µg/l)	All	53.0 / 47.5 (17–268)	a	52.0 / 35.5 (13–192)	a,b	42.0 / 27.0 (6–335)	a,b	21.5 / 16.8 (8–509)	b

Significant influence of sex: zinc.
Adjusted for age: vitamin E.
* Diet groups with different indices differ significantly ($p < 0.05$).
§ 4cB12 score–combined index of B12 deficiency (normal range:−0.5 - 1.0).

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ORIGINAL RESEARCH
published: 30 May 2022
doi: 10.3389/fnut.2022.838613

Novel Strategies for Assessing Associations Between Selenium Biomarkers and Cardiometabolic Risk Factors: Concentration, Visit-to-Visit Variability, or Individual Mean? Evidence From a Repeated-Measures Study of Older Adults With High Selenium

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OPEN ACCESS

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

Received: *18 December 2021*
Accepted: *11 May 2022*
Published: *30 May 2022*

Citation:
Li A, Zhou Q, Mei Y, Zhao J, Zhao M, Xu J, Ge X and Xu Q (2022) Novel Strategies for Assessing Associations Between Selenium Biomarkers and Cardiometabolic Risk Factors: Concentration, Visit-to-Visit Variability, or Individual Mean? Evidence From a Repeated-Measures Study of Older Adults With High Selenium. Front. Nutr. 9:838613. doi: 10.3389/fnut.2022.838613

Background and Aims: Previous studies have focused only on the cardiometabolic effects of selenium concentrations. We explored whether selenium levels and their visit-to-visit variability (VVV) and individual mean (IM) are independently associated with cardiometabolic risk factors.

Methods: A three-wave repeated-measures study of older adults with high selenium (n = 201) was conducted in Beijing from 2016 to 2018. Whole blood selenium and urinary selenium concentrations were measured. VVV and IM were used to profile the homeostasis of the selenium biomarkers. Four indicators, namely standard deviation, coefficient of variation, average real variability, and variability independent of the mean, were employed to characterize VVV. We considered 13 cardiometabolic factors: four lipid profile indicators, three blood pressure indices, glucose, uric acid, waistline, hipline, waist-hip ratio, and sex-specific metabolic syndrome score. Linear mixed-effects regression models with random intercepts for the participants were employed to explore the associations of the selenium concentrations, VVV, and IM with the cardiometabolic factors.

Results: The geometric mean whole blood and urinary selenium levels were 134.30 and 18.00 μg/L, respectively. Selenium concentrations were significantly associated with numerous cardiometabolic factors. Specifically, whole blood selenium was positively associated with total cholesterol [0.22, 95% confidence interval (CI): 0.12, 0.33],

low-density lipoprotein cholesterol (LDL-C; 0.28, 95% CI: 0.13, 0.42), glucose (0.22, 95% CI: 0.10, 0.34), and uric acid (0.16, 95% CI: 0.04, 0.28). After adjustment for VVV, the IM of whole blood selenium was positively correlated with total cholesterol (0.002, 95% CI: 0.001, 0.004), triglycerides (0.007, 95% CI: 0.004, 0.011), and LDL-C (0.002, 95% CI: 0.000, 0.004). However, we did not observe any robust associations between the VVV of the selenium biomarkers and cardiometabolic risk factors after adjustment for IM.

Conclusion: Our findings suggest that selenium concentrations and their IMs are significantly associated with cardiometabolic risk factors among older adults with high selenium. Longer repeated-measures studies among the general population are required to validate our findings and elucidate the relevant underlying mechanisms.

Keywords: concentration, visit-to-visit variability (VVV), individual mean (IM), cardiometabolic risk factors, selenium

INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of death in East Asia and the world. In 2019, an estimated 18.6 million [95% confidence interval (CI): 17.1–19.7 million] CVD-related deaths occurred globally (1). Cardiometabolic risk factors, including dyslipidaemia, hypertension, diabetes, and obesity, are the primary causes of CVD (2, 3). Because of the antioxidant effects of selenium, selenium supplements and high-selenium foods are widely consumed to prevent the development of cardiometabolic risk factors (4). However, evidence on the influence of selenium on cardiometabolic risk factors remains inconclusive, and selenium may even increase cardiometabolic risk (5–7). Thus, whether selenium should be promoted for its cardiometabolic protective effects remains uncertain.

A cross-sectional study of healthy adults in two Chinese counties with different selenium intake habits indicated that serum selenium was positively correlated with serum glucose in those with selenium deficiency (median: 58 μg/L). However, no significant relationship between serum selenium and glucose was observed in those without such deficiency (median: 103 μg/L) (8). Additionally, a cross-sectional analysis using data from the National Health and Nutrition Examination Survey (NHANES) reported a U-shaped association between plasma selenium (mean: 137.1 μg/L) and the likelihood of diabetes, with the lowest risk noted for a concentration of ∼122 μg/L (9). An experimental study discovered that the dose-dependent effects of selenium range from antioxidation and anti-inflammation to the promotion of oxidative stress and insulin resistance (10). Therefore, two crucial factors must be emphasized regarding the effect of selenium on cardiometabolic risk: the *U*-shaped associations between selenium levels and cardiometabolic risk factors and the broad individual variation in selenium concentrations. According to the study of Reference Man by the International Commission on Radiological Protection, a mean whole blood selenium exceeding 130 μg/L is defined as high selenium (11). However, most relevant epidemiological studies have been conducted on populations with moderate or deficient selenium levels, with means of blood selenium concentrations ranging from 47 to 127.5 μg/L (12–14). Few epidemiological studies have been conducted in populations with high selenium (>130 μg/L), limiting the understanding of the role of high selenium in the development of cardiometabolic risk factors (15, 16). Selenium-rich soil with concentrations exceeding 0.4 mg/kg has been discovered in areas across Beijing (17), providing an excellent opportunity to investigate the association between high selenium levels and cardiometabolic risk in a real-world setting. Compared with young adults, fewer older adults (aged 50 years or older) smoke or consume alcohol (18, 19). In addition, the metabolic and physiological functions, including excretion function, of older adults are gradually impaired during the aging process (20, 21). Therefore, compared with other age groups, older adults have higher selenium levels and more cases of high selenium (22–24). Moreover, aging adults experience multisystem functional impairment and increasing susceptibility to multiple chronic diseases (25). Therefore, examining older adults in a study of the cardiometabolic health effects of selenium is warranted.

Selenium assessment is mainly based on selenium concentrations in blood and urine. Increasing evidence suggests inconsistent associations among urinary selenium, circulating selenium (whole blood selenium, plasma selenium, or serum selenium), and cardiometabolic risk factors. A case–control study conducted in Wuhan, China, indicated no association between urinary selenium (mean: 20.47 μg/g) and blood pressure (BP) (26). However, another case–control study conducted in Wuhan demonstrated a U-shaped association between plasma selenium (median: 92.66 μg/L) and hypertension (12). Therefore, the associations of multiple selenium measures (e.g., whole blood and urinary selenium) with cardiometabolic risk factors should be examined simultaneously. Three cross-sectional analyses using data from the NHANES reported diverse associations of serum selenium with blood pressure and glucose and lipid profiles. *U*-shaped associations of serum selenium (mean: 137.1 μg/L) with systolic BP (SBP) and pulse pressure were observed (27). In addition, high serum selenium (mean: 137.1 μg/L) was associated with high glucose levels (9). Another study discovered that total cholesterol and triglyceride levels increased with serum selenium (median:

192.99 μg/L); furthermore, low-density lipoprotein cholesterol (LDL-C) was non-linearly associated with serum selenium, but high-density lipoprotein cholesterol (HDL-C) did not vary with serum selenium (5). This evidence suggests that multiple cardiometabolic risk factors should be measured to comprehensively characterize the associations of selenium levels with cardiometabolic health and evaluate the consistency of results to draw robust conclusions.

Changes in diet, lifestyle, or daily activities dynamically affect biomarkers and physiological parameters and these variations influence health endpoints (28). Visit-to-visit variability (VVV) in BP and glucose and lipid profiles has been associated with CVD and mortality (29–31). Selenium homeostasis is essential for a wide range of cellular functions, such as modulation of the cell cycle and apoptosis, redox balancing, and protein and DNA synthesis (32). Therefore, investigating the VVV or individual mean (IM) of selenium levels may be beneficial, while exploring the associations between selenium and cardiometabolic risk factors. Such considerations may elucidate a crucial means of reducing misclassifications in exposure assessment and further understanding the associations between selenium and cardiometabolic health. Because VVV and IM data cannot be calculated in a traditional cross-sectional study or a cohort study measuring only baseline exposure, a repeated-measures study is required.

Using a three-wave repeated-measures study of 201 older adults with high selenium residing in Beijing, China, from 2016 through 2018, we explored the associations between selenium biomarkers (i.e., whole blood and urinary selenium concentration) and cardiometabolic risk factors. On the basis of the selenium concentrations measured during three clinic visits, selenium homeostasis factors, namely VVV and IM, were considered. Furthermore, we investigated the associations of the VVV and IM of selenium levels with cardiometabolic risk factors.

METHODS

Study Setting and Population

The study was conducted in Beijing, which is in the northern North China Plain. As previously mentioned, selenium-rich soil has been discovered across the Beijing area from north to south. We selected five communities (Qian Nantai, Liu Hegou, Dongcheng, Chaoyang, and Fangshan) from four regions of Beijing from north to south to serve as the study settings. Among five communities, Qian Nantai, Liu Hegou are located in rural area. Dongcheng, Chaoyang and Fangshan are located in urban area. Further details regarding the study setting and design are presented in **Figure 1**.

The inclusion and exclusion criteria were the same as those described in a previous study (33). We attempted to sample a population of older adults with stable selenium statuses and the ability to complete three clinic visits by recruiting individuals aged 50 years or older who had lived in the community for more than 5 years and were unlikely to leave during the study period. Those who were unable to complete a questionnaire survey, had received a malignant tumor diagnosis, or had CVD or liver disease were excluded. In addition, a questionnaire inquired into the participants' dietary supplement intake, and the participants did not consume any dietary selenium supplements. All three measurements were conducted in the winter to control for confounding seasonal factors. After the first visit in November or December 2016, 201 participants were enrolled. Two follow-up surveys were conducted in November 2017 and January 2018. Ultimately, 83% ($n = 167$) of the study participants completed all three visits, and 17% ($n = 34$) completed only two visits. In total, 569 observations were analyzed.

The study protocol was reviewed and approved by the Institutional Ethics Committee of the Institute of Basic Medicine at the Chinese Academy of Medical Sciences. Each participant provided written consent before participating.

Measurement of Whole Blood Selenium and Urinary Selenium

Species Sampling

The participants fasted overnight (≥8 h) before each examination. A qualified nurse obtained peripheral blood samples from all participants at the same time of day (8:00–9:00 am) to control for variations in circadian rhythm. Whole blood and serum were collected for whole blood selenium concentration measurements and serum biochemical analyses. First morning urine samples (10 mL) were collected from the participants in trace element—free containers. All urine and blood samples were stored at −80°C for subsequent analysis.

Measurements of Blood and Urine Samples

Before analysis, the whole blood samples were diluted (1:10 ratio) in a digestive solution composed of 0.5% nitric acid and 0.01% Triton X-100. The urine samples were diluted (1:10) in a digestive solution composed of 0.5% nitric acid and 0.02% Triton X-100. Both the whole blood selenium and urinary selenium concentrations were measured through inductively coupled plasma mass spectrometry (Nexion 300D, PerkinElmer, USA). In addition, we applied quality control protocols to ensure the accuracy of our analyses. First, all the samples were measured three times. Second, standard reference materials were measured every 20 samples to ensure that the whole blood and urinary selenium measurements were consistent with certified concentrations before subsequent samples were assayed. The limits of detection (LODs) for whole blood and urinary selenium were determined to be 2.15 and 0.14 μg/L, respectively. The concentrations in all samples were higher than the LODs.

Adjustment of Urinary Selenium Concentration

In epidemiological studies of environmental contaminants measured in urine, adjustment for creatinine to control for the effect of urine dilution is common, but the optimal approach is debated (34). Traditionally, urinary biomarker concentrations have been standardized through division by the urinary creatinine concentration, in accordance with the assumption that creatinine excretion is approximately constant across individuals and time (35, 36). However, increasing evidence suggests that creatinine levels can vary with time and individual characteristics, including sex, race, age, and body mass index (BMI); thus, conventional creatinine adjustment may yield

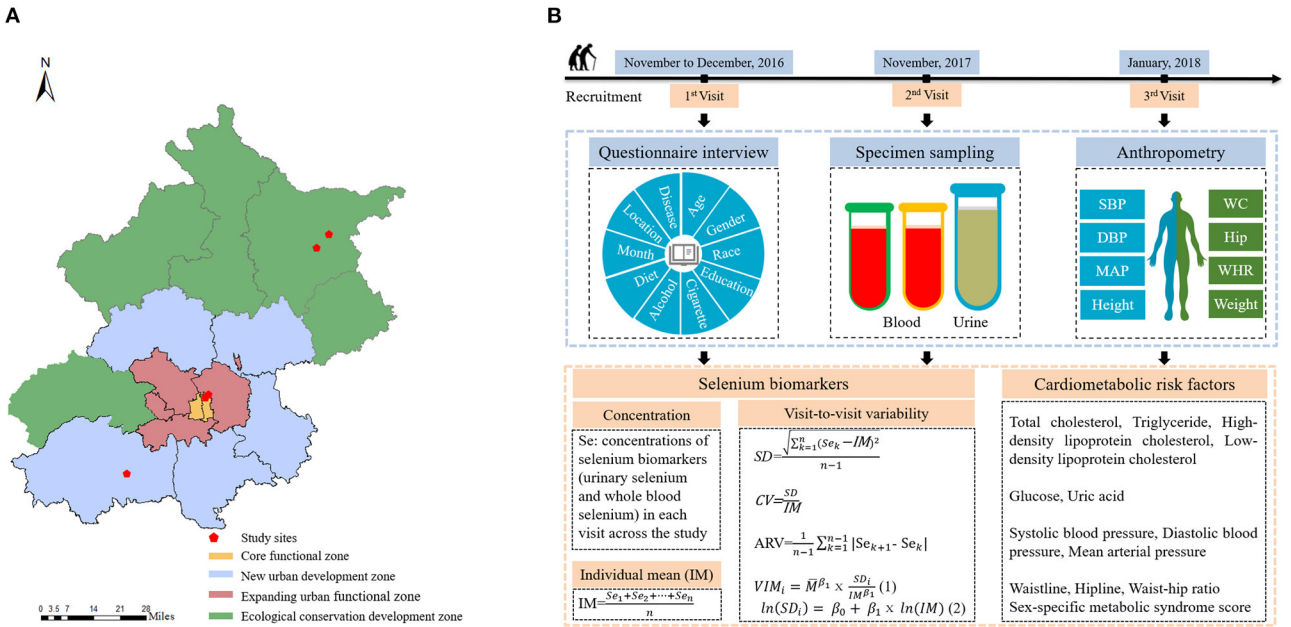

FIGURE 1 | (A) Five study locations within Beijing. **(B)** Overview of study design and data collection.

misleading conclusions. Therefore, we employed a three-step approach, enabling systematic individual differences in long-term creatinine levels to be used to estimate covariate-adjusted, standardized urinary selenium concentrations (37). Urinary creatinine concentrations were measured using the sarcosine oxidase method. First, natural log–transformed creatinine was regressed on factors that can affect urine dilution. We included age, sex, race, weight, and height as covariates in the regression model (38). The measured creatinine concentration was divided by the fitted creatinine value obtained from the regression model to obtain a ratio representing the covariate-independent residual effect of hydration on creatinine. We finally standardized the urinary selenium concentration by dividing it by this ratio. This method specifically controls the covariate-independent, short-term multiplicative effect of hydration on urine dilution, avoids potential collider bias resulting from the use of urinary creatinine or specific gravity in adjusting for urine dilution, and increases the accuracy of exposure assessment (38, 39).

Calculation of VVV and IM of Selenium Biomarkers

The VVV and IM of the selenium biomarkers were calculated to estimate the fluctuation and homeostasis of the biomarkers across three visits. The IM of the selenium biomarkers for each participant was the mean selenium concentrations from the three visits. No gold standard regarding the appropriate indicators of the VVV of selenium biomarkers exists (40). According to the concentrations of the selenium biomarkers across the three visits, we calculated four indices of VVV, namely the standard deviation (SD), coefficient of variation (CV), average real variability (ARV), and variability independent of the mean (VIM), to comprehensively assess variability. SD and CV are simple and informative indices of VVV (41). SD was used to indicate the general variation between the selenium biomarker levels and the mean values of the participants. However, the order of measurement is not considered in SD calculation (42). ARV was used to overcome the limitations of SD by accounting for the order of measurements when quantifying VVV (29). However, ARV was correlated with the selenium concentrations. The CVs of the selenium biomarkers across the three visits were computed from the mean and SD. Therefore, the CVs might be correlated with the mean selenium concentrations (43). Hence, the VIM was calculated through logarithmic curve fitting to eliminate the correlation with the biomarkers, ensuring that we could evaluate the impact of the VVV separately from the effect of the selenium biomarker levels themselves (29). The formulae for the four VVV indices are as follows:

$$SD = \frac{\sqrt{\sum_{k=1}^{n} (Se_k - IM)^2}}{n-1} \quad (1)$$

$$CV = \frac{SD}{IM} \quad (2)$$

$$ARV = \frac{1}{n-1} \sum_{k=1}^{n-1} \left| Se_{k+1} - Se_k \right| \quad (3)$$

$$\ln(SD_i) = \beta_0 + \beta_1 \ln(IM) \quad (4\text{-}1)$$

$$VIM_i = \bar{M}^{\beta_1} \frac{SD_i}{IM^{\beta_1}} \quad (4\text{-}2)$$

where Se denotes the selenium concentration; IM denotes the individual mean of the concentration; n is the total number of measurements ($n = 2$ or 3); k indicates the visit number ($k = 1$, 2, or 3); Se_K is the selenium concentration recorded during visit k; $\overline{M}$ is the overall mean of the selenium concentration for the study population; SD_i is the SD of the selenium measurements for individual i; and VIM_i is the VIM of the selenium measurements for individual i. In total, we calculated eight selenium VVV indicators: the SD, CV, ARV, and VIM of whole blood and urinary selenium (denoted as SD-WBSe, CV-WBSe, ARV-WBSe, VIM-WBSe, SD-USe, CV-USe, ARV-USe, and VIM-USe, respectively).

Outcome Assessment

Physical Measurement

The participants' upper arm BP was measured using calibrated mercury sphygmomanometer at least three times after the participants had rested for 15 min in a room. SBP and diastolic BP (DBP) were obtained at the appearance and disappearance of Korotkoff sounds, respectively. The interval between measurements was required to exceed 2 min. Additional measurements were taken if the differences among measurements exceeded 5 mmHg. The mean of the final two measurements was calculated for subsequent analysis. Mean arterial pressure (MAP) was calculated because MAP is associated with the risks of adverse cardiovascular events (44). The following formula was used to estimate MAP:

$$MAP = (2 \times DBP + SBP)/3 \quad (5)$$

Height was measured using a verified stadiometer, with each participant in a standing position with shoes removed, shoulders relaxed, head facing forwards, and back facing the wall. Weight was measured with the participants wearing little clothing, by using a certified body composition analyser. The participants' weights in kilograms were divided by their heights in meters squared to obtain their BMIs. For waistline and hipline measurements, each participant was asked to wear minimal clothing and stand with their feet close together, arms at their side, and body weight evenly distributed. Waistline was measured in centimeters at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest using stretch-resistant tape. Hipline measured in centimeters around the widest portion of the buttocks, with the tape parallel to the floor (45). The waistline measurements were divided by the hipline measurements to obtain waist-hip ratios. All the measurements were performed twice and averaged for reliability and accuracy.

Clinical Laboratory Examination

The serum samples were sent to the Clinical Laboratory Center of Peking Union Medical College Hospital for biochemical testing. Lipid (total cholesterol, triglycerides, HDL-C, and LDL-C) and glucose, uric acid, and high-sensitivity C-reactive protein (hs-CRP) levels were measured using a Beckman Coulter analyser (AU2700, Beckman Coulter, Brea, CA, USA).

Metabolic Syndrome Score

The metabolic syndrome score (MSS), calculated from various factors related to metabolic syndrome, is an accurate indicator of cardiometabolic risk (46). The MSS is correlated with long-term risk for diabetes and CVD and is widely employed in clinical practice to identify patients with high such risks (47, 48). To account for potential sex-based differences in the MSSs, we computed the sex-specific MSS (MSS-sex) for each participant to comprehensively evaluate their cardiometabolic health (49). MSS-sex was calculated by summary of sex-specific standardized Z-scores for each of five components including MAP, HDL-C, triglyceride, waistline, and glucose, generated from the population being studied (50).

Ascertainment of Covariates

Questionnaires were used to obtain the demographic characteristics, including age (years), sex (male or female), race (Han or others), and educational attainment (illiteracy or primary school, or junior school, or senior high school and above); lifestyle characteristics, such as cigarette smoking (current, former and never) and alcohol consumption (current, former and never) habits; and health status. The participants' communities were coded as categorical variables. Additionally, because this was a longitudinal study, controlling for temporal confounders was necessary. Therefore, sampling months were recorded and adjusted for as potential confounders.

In accordance with China's medical guidelines for the prevention and treatment of dyslipidaemia in adults (51), we considered hypercholesterolaemia, hypertriglyceridemia, hyperalphalipoproteinaemia, hyperbetalipoproteinaemia, or the use of any antidyslipidaemic agents (e.g., statins) , or both to indicate dyslipidaemia. Hypercholesterolaemia was defined as total cholesterol $\geq$6.2 mmol/L, hypertriglyceridemia was defined as triglycerides $\geq$2.3 mmol/L, hyperalphalipoproteinaemia was defined as HDL-C $\leq$ 1.0 mmol/L, and hyperbetalipoproteinaemia was defined as LDL-C $\geq$ 4.1 mmol/L. Hypertension was indicated by any antihypertensive medication prescription or BP $\geq$140/90 mmHg measured under standardized clinical conditions (52). Diabetes was defined as fasting glucose $\geq$7.0 mmol/L, 2-h glucose $\geq$11.1 mmol/L, or current antidiabetic medication use (both insulin or oral antidiabetic drugs) (53).

Dietary intake information was collected using a food frequency questionnaire (FFQ), which inquired into the frequency and amount of food consumed over the previous 30 days. The FFQ comprised 81 food-related items, and its reproducibility and validity were verified in a prior study (54). The daily total energy intake (kcal/day) was determined from the FFQ data and Chinese Tables of Food Composition (55).

Statistical Analysis

Statistical Description

The participants' general characteristics, demographic features, health status, VVV and IM of selenium biomarkers, and cardiometabolic risk factors were documented. The categorical variables are expressed as numbers (percentages), and the continuous variables are expressed as means $\pm$ SDs. To comprehensively profile the distribution of the selenium

biomarkers, geometric means and percentiles for the selenium biomarkers were calculated. In addition, distribution characteristics of selenium biomarkers by urban and rural areas were described. Unadjusted linear mixed-effects regression models of the selenium biomarkers by area (urban area, rural area) were developed to assess difference of selenium biomarkers concentrations between two areas.

The variability in the selenium biomarkers and cardiometabolic risk factors during the study period was also calculated. We calculated intraclass correlation coefficients (ICCs) of the selenium biomarkers, defined as the ratio of the interindividual variance to the total variance, and corresponding 95% CIs. The ICCs were used to evaluate the reproducibility of the selenium biomarkers across the three visits as follows: $0.75 \leq \text{ICC} \leq 1.00$ suggested excellent reproducibility, $0.40 \leq \text{ICC} < 0.75$ suggested fair to good reproducibility, and $0.00 \leq \text{ICC} < 0.40$ suggested poor reproducibility (56, 57). The distributions of the selenium biomarker values across the three visits were recorded and represented using violin plots. Spearman correlation coefficients between each pair of biomarkers measured at each visit were calculated. Unadjusted linear mixed-effects regression models of the selenium biomarkers and cardiometabolic risk factors by visit (modeled as a nominal categorical variable) were developed to assess global significance for each visit. The variability of the selenium biomarkers and cardiometabolic risk factors was helpful for both constructing models and explaining results in the statistical analyses.

Associations of Selenium Biomarkers With Cardiometabolic Risk Factors

Covariate Selection

We applied a directed acyclic graph to determine covariates adjusted in the models a prior, which was the minimum adjustment sets selected by graphical criteria such as the so-called "back-door" (58, 59). The covariates included were age, sex, race, educational attainment, cigarette smoking habits, alcohol consumption habits, BMI, sampling month, community, and daily total energy intake.

Exposure–Response Curves

The generalized additive mixed models (GAMMs) with embedded restricted cubic spline smoothers with three degrees of freedom were conducted to plotted exposure–response curves for the selenium biomarkers and cardiometabolic risk factors. The results of GAMMs were summarized to provide more numeric information about associations between selenium biomarkers and cardiometabolic risk factors. The linear and non-linear trends illustrated by the curves also helped guide the development and interpretation of the main analysis.

Main Analysis

To account for potentially skewed distributions, the concentrations of whole blood and urinary selenium were natural log–transformed before statistical analysis. Regarding outcomes, the levels of the four lipid indicators and of glucose and uric acid were natural log–transformed to reduce skewness in further analysis. Associations between the selenium biomarkers and cardiometabolic risk factors were assessed using linear mixed-effects regression models with participant-specific random intercepts to account for within-participant correlation in this repeated-measures study. For each model, one selenium biomarker was regressed on one cardiometabolic risk factor at the same study visit. According to the distributions of the selenium biomarkers and the non-linear and linear associations between them and the cardiometabolic risk factors, the selenium concentrations were included in the models as natural log–transformed continuous variables or categorical variables indicating tertiles, respectively. Linear trend p-values were derived by modeling the median of each selenium biomarker tertile as a continuous variable in the adjusted models. To address the multiple testing problem, in which the more inferences are made, the more likely erroneous inferences become, and reduce the probability of type I error, we adjusted the raw p-values on the basis of the false discovery rate (60).

Stratified Analysis

To account for the residual confounding and effect modification of the main analysis, we conducted analyses stratified by area (urban area or rural area), comorbidities (dyslipidaemia, diabetes, and hypertension), demographic characteristics [age (<65 or ≥ 65 years) and sex (male or female)], and BMI (<24 or ≥ 24 kg/m^2), while simultaneously controlling for the same covariates as in the main analysis. The differences between strata were examined by estimating values and 95% CIs as follows (61).

$$(\beta_1 - \beta_2) \pm 1.96 \times \sqrt{(SE_1)^2 + (SE_2)^2} \tag{6}$$

where β_1 and β_2 are the effect estimates attributed to each subgroup or stratum (e.g., the effects for participants with and without dyslipidaemia, respectively) and SE_1 and SE_2 are the corresponding standard errors. In the stratified analysis, the selenium concentrations were included in the models as natural log–transformed continuous variables.

Sensitivity Analysis

We conducted several sensitivity analyses to assess the robustness of the findings in the main analysis. First, to address any residual confounding attributed to chronic disease status, we additionally adjusted for history of hypertension, diabetes, or dyslipidaemia in the same model of main analysis. Second, blood selenium concentrations reportedly differ significantly with BMI (62), and a higher BMI increases the risk of cardiometabolic disease (63). Therefore, we repeated our analysis while excluding BMI from the covariates to elucidate the impact of BMI on the associations. Third, hs-CRP, an indicator of systemic inflammation, may also influence both selenium levels and cardiometabolic risk (64, 65). Therefore, we additionally adjusted for hs-CRP in the sensitivity analysis to explore its influence on the associations. In the sensitivity analysis, the selenium concentrations were incorporated into the models as natural log–transformed continuous variables.

Associations of VVV and IM of Selenium Biomarkers With Cardiometabolic Risk Factors

The linear mixed-effects regression models employed in the main analysis were fitted to assess the associations of the VVV and IM of the selenium biomarkers with the cardiometabolic risk factors. A single-factor models (Model 1) respectively including individual indicator of VVV or IM in selenium biomarkers were used to assess the associations of the VVV or IM of the selenium biomarkers with the cardiovascular risk factors. Furthermore, mutually adjusted models (Model 2) considering both the VVV and IM of the selenium biomarkers were used to evaluate whether the effects of VVV in selenium biomarkers on cardiovascular risk factors are independent of IM. Both models were adjusted for the same covariates as described for the main analysis.

All statistical tests were two sided, and significance was set at $p < 0.05$. All analyses were conducted using the lmerTest, gamm4, splines, corrplot, dagitty, and ggplot2 packages in R (version 4.1.0).

Quality Assurance and Control

Standard operating procedures were followed throughout the research project. The interviewers, nurses, and physicians were all trained prior to the fieldwork. To ensure that the questionnaires collected personal information accurately, the principal investigators surveyed the interviewers with the same questionnaires to measure consistency. When each visit commenced, all the related data were recorded by qualified and trained staff (i.e., physicians, registered nurses, or trained interviewers). Data cleaning, quality assessment, and processing were performed independently by two qualified statistical analysts after completion of the surveys.

RESULTS

Participant Characteristics

A summary of the participants' demographic data is presented in **Table 1**. Among the 201 participants included in the study, 125 (62.2 %) were female. The mean (SD) age and BMI of the participants were 64.60 (9.10) years and 25.10 (3.50) kg/m^2, respectively. The distributions of whole blood and urinary selenium concentration across the study period are detailed in **Table 2**. The geometric means (geometric SDs) of urinary and whole blood selenium were 18.00 (1.87) and 134.30 (1.19) μg/L, respectively. The ICCs (95% CIs) for whole blood and urinary selenium were 0.54 (0.44, 0.61) and 0.12 (0.02, 0.22), respectively, indicating good reproducibility of the whole blood measurements and poor reproducibility of the urinary measurements (**Table 2**). The characteristics of the cardiometabolic risk factors and the VVV and IM of the selenium biomarkers of the participants are presented in **Table 3**. The means (SDs) of the SD-WBSe, ARV-WBSe, and VIM-WBSe in population were 14.00 (8.70), 17.10 (12.90), 14.00 (8.10) μg/L, respectively. By comparison, the means (SD) of the SD-USe, ARV-USe, and VIM-USe in population were lower at 11.90 (12.90), 14.30 (15.80), and 10.80 (5.00) μg/L, respectively. By contrast, the mean (SD) of the CV-USe was higher than CV-WBSe in population [0.48 (0.26) vs. 0.10 (0.06)]. Furthermore, the mean (SD) of IM-WBSe and IM-USe in population were 136.00 (20.20) and 22.30 (11.60) μg/L, respectively.

TABLE 1 | Demographic characteristics and health status of participants throughout the study period.

Variables	Mean ± SD or *n* (%)
Participants	201
Age, years	64.60 ± 9.10
BMI, kg/m^2	25.10 ± 3.50
Gender	
Male	76 (37.8)
Female	125 (62.2)
Race	
Han	195 (97.0)
Others	6 (3.0)
Educational attainment	
Illiteracy or primary school	67 (33.4)
Junior school	63 (31.3)
Senior high school and above	71 (35.3)
Cigarette smoking	
Current	25 (12.4)
Former	22 (10.9)
Never	154 (76.6)
Alcohol consumption	
Current	53 (26.4)
Former	13 (6.5)
Never	135 (67.2)
Total energy intake, kcal/day	1,810 ± 529
hs-CRP, mg/L	1.95 ± 2.81
Dyslipidemia	
Yes	101 (50.2)
No	100 (49.8)
Hypertension	
Yes	114 (56.7)
No	87 (43.3)
Diabetes	
Yes	35 (17.4)
No	166 (82.6)

BMI, body mass index; hs-CRP, high-sensitivity C-reactive protein.

The variations in the selenium biomarkers and cardiometabolic risk factors across the study period were also assessed, revealing three key findings: First, the global significance test revealed that whole blood selenium and most cardiometabolic risk factors (except SBP) significantly differed across the three visits (**Supplementary Tables 1, 2**). The difference of selenium biomarkers concentrations between urban and rural area was significant (**Supplementary Table 3**). Second, the overall correlation between the urinary selenium concentrations across three visits was negligible to moderate (r_s: −0.09 to 0.56). With respect to whole blood selenium, the moderate positive correlations were found between whole

TABLE 2 | Limits of detection, distribution characteristics, and reproducibility of the selenium biomarkers.

Analyte (μg/L)					Percentiles of seleniu				ICCs (95% CI)
	LOD	GM	GSD	5th	25th	50th	75th	95th	
Whole blood selenium	2.15	134.30	1.19	103.90	120.40	132.10	150.40	180.00	0.54 (0.44, 0.61)
Urinary selenium	0.14	18.00	1.87	7.06	11.49	16.96	27.92	52.33	0.12 (0.02, 0.22)

LOD, Limit of detection; GM, geometric mean; GSD, geometric standard deviation; ICCs, intraclass correlation coefficients; 95% CI, 95% confidence interval.

TABLE 3 | Summary of cardiometabolic risk factors and selenium homeostasis of participants.

Variables	Mean ± SD
Cardiometabolic risk factors	
Total cholesterol (mmol/L)	4.96 ± 1.04
Triglyceride (mmol/L)	1.54 ± 1.08
HDL-C (mmol/L)	1.32 ± 0.33
LDL-C (mmol/L)	3.03 ± 0.86
Glucose (mmol/L)	6.07 ± 1.84
Uric acid (μmol/L)	299.00 ± 77.30
SBP (mmHg)	138.00 ± 17.70
DBP (mmHg)	83.90 ± 10.30
MAP (mmHg)	102.00 ± 11.40
Waistline (cm)	90.50 ± 9.60
Hipline (cm)	103.00 ± 7.00
Waist-hip ratio	0.88 ± 0.06
MSS-sex	0.01 ± 2.90
Visit to visit variability indicator of selenium biomarkers	
SD-WBSe (μg/L)	14.00 ± 8.70
CV-WBSe	0.10 ± 0.06
ARV-WBSe (μg/L)	17.10 ± 12.90
VIM-WBSe (μg/L)	14.00 ± 8.10
SD-USe (μg/L)	11.90 ± 12.90
CV-Use	0.48 ± 0.26
ARV-USe (μg/L)	14.30 ± 15.80
VIM-USe (μg/L)	10.80 ± 5.00
Individual mean indicator of selenium biomarkers	
IM-WBSe (μg/L)	136.00 ± 20.20
IM -USe (μg/L)	22.30 ± 11.60

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; MSS-sex, metabolic syndrome score (sex specific); IM -WBSe, individual mean of whole blood selenium; SD-WBSe, standard deviation of whole blood selenium; CV-WBSe, the coefficient of variation of whole blood selenium; ARV-WBSe, average real variability of whole blood selenium; VIM-WBSe, variability independent of the mean of whole blood selenium; IM -USe, individual mean of urinary selenium; SD-USe, standard deviation of urinary selenium; CV-USe, the coefficient of variation of urinary selenium; ARV-USe, average real variability of urinary selenium; VIM-USe, variability independent of the mean of urinary selenium.

blood selenium levels across three visits (r_s: 0.61 to 0.65) which was consistent with better reproducibility of whole blood selenium compared to urinary selenium. Furthermore, there were negligible to low positive correlations between whole blood selenium concentrations and urinary selenium concentrations across three visits (r_s: 0.07 to 0.43; **Supplementary Figure 2**). Final, greater variance in the distribution of whole blood selenium than that of urinary selenium was also observed, as indicated in **Supplementary Figure 3**.

Associations of Selenium Biomarker Concentrations With Cardiometabolic Risk Factors

Exposure–Response Curves

The exposure–response curves of the selenium biomarkers and cardiometabolic risk factors obtained from the GAMMs are presented in **Supplementary Figures 4, 5**. Whole blood selenium was linearly associated with total cholesterol, triglycerides, HDL-C, uric acid, SBP, waistline, hipline and MSS-sex but non-linearly associated with LDL-C, glucose, DBP, MAP, and waist-hip ratio (**Supplementary Figure 4**). Consist with the exposure–response curves, estimated degrees of freedom (EDF) of LDL-C, glucose, DBP, MAP, and waist-hip ratio were 2.16, 2.14, 1.55, 1.46, and 2.22 which were significantly higher than 1 (**Supplementary Table 4**). Urinary selenium was linearly associated with cardiometabolic risk factors, except for waistline (**Supplementary Figure 5**). Similarly, EDF of waistline were 1.62 (**Supplementary Table 4**). Because of the diverse linear and non-linear relations of the selenium biomarkers with the various cardiometabolic risk factors, the selenium biomarkers were respectively considered continuous variables and categorized into tertiles in the linear mixed-effects regression models.

Main Analysis

In this three-wave repeated-measures study of 201 older adults with high selenium levels, we discovered that selenium levels were significantly associated with cardiometabolic risk factors after correction for multiple comparisons and adjustment for age, sex, race, educational attainment, cigarette smoking habits, alcohol consumption habits, BMI, sampling month, community, and total daily energy intake (**Table 4**). Primarily positive associations were observed with whole blood selenium: whole blood selenium was positively associated with total cholesterol (0.22, 95% CI: 0.12, 0.33), LDL-C (0.28, 95% CI: 0.13, 0.42), glucose (0.22, 95% CI: 0.10, 0.34), and uric acid (0.16, 95% CI: 0.04, 0.28) levels. These positive associations remained significant even with whole blood selenium concentration treated as a categorical variable (p trend < 0.01 for total cholesterol, LDL-C, and glucose; p trend $= 0.02$ for uric acid). Similarly, the results of GAMMs showed that associations between whole blood selenium and total cholesterol, LDL-C, glucose and uric acid were significant.

For urinary selenium, only negative associations were observed and only with BP-related factors. Urinary selenium was negatively associated with SBP (−3.37, 95% CI: −5.68, −1.01), DBP (−1.69, 95% CI: −3.00, −0.39), and MAP (−2.32, 95% CI: −3.76, −0.88). However, these negative associations were non-significant when urinary selenium concentration was treated as a categorical variable (p trend = 0.08, 0.14, and 0.06 for SBP, DBP, and MAP, respectively). The results of GAMMs showed that associations between urinary selenium and BP indicators were significant.

Stratified Analyses

We conducted analyses stratified by area, comorbidities (dyslipidaemia, diabetes, and hypertension status) as well as by age, sex, and BMI (**Supplementary Figures 6, 7**). The associations of whole blood selenium with cardiometabolic risk factors did not differ significantly with area, BMI or dyslipidaemia status but differed significantly with sex, age, diabetes status, and hypertension status (**Supplementary Figure 6**). The associations of urinary selenium with cardiometabolic risk factors did not differ significantly with area, age, sex, or BMI but differed significantly with dyslipidaemia, diabetes, and hypertension status (**Supplementary Figure 7**).

Sensitivity Analyses

The sensitivity analyses controlling for a history of hypertension, diabetes, or dyslipidaemia (**Supplementary Figure 8**) and for hs-CRP yielded results similar to those of our main analysis (**Supplementary Figure 10**). A significant positive association between whole blood selenium and triglyceride levels was also discovered after BMI was excluded from the covariates (**Supplementary Figure 9**).

Associations Between VVV and IM of Selenium Biomarkers and Cardiometabolic Risk Factors

We observed that SD-WBSe was positively correlated with triglycerides (0.009, 95% CI: 0.000, 0.017) and MSS-sex (0.047, 95% CI: 0.005, 0.088). In addition, ARV-WBSe was positively associated with glucose (0.003, 95% CI: 0.000, 0.005) and MSS-sex (0.034, 95% CI: 0.006, 0.061) in Model 1. However, these associations were non-significant after additional adjustments for the impact of IM in Model 2. Only a marginal association of ARV-WBSe with total cholesterol (−0.003, 95% CI: −0.005, −0.000) was observed in Model 2 (**Table 5**).

Consistent with results of the main analysis, the IM of whole blood selenium was positively correlated with total cholesterol (0.002, 95% CI: 0.001, 0.004), triglycerides (0.007, 95% CI: 0.004, 0.011), and LDL-C (0.002, 95% CI: 0.000, 0.004) in Model 1, and these associations remained significant after adjustment for the impact of VVV in Model 2. The positive association between the IM of whole blood selenium and glucose (0.002, 95% CI: 0.000, 0.004) was also observed in Model 1, and this association remained significant after adjustment for SD-WBSe, CV-WBSe, and VIM-WBSe in Model 2. Furthermore, we discovered a positive association between the IM of whole blood selenium and MSS-sex (0.022, 95% CI: 0.002, 0.041) in Model 1, and this association was significant after adjustment for CV-WBSe and VIM-WBSe in Model 2 (**Table 5**).

We did not observe any robust associations of the VVV of urinary selenium with cardiometabolic risk factors, discovering only a marginal association of SD-USe with total cholesterol (−0.004, 95% CI: −0.007, −0.000) after adjusting for the IM of urinary selenium in Model 2. The IM of urinary selenium was positively related to triglycerides (0.014, 95% CI: 0.002, 0.025) and glucose (0.006, 95% CI: 0.000, 0.011) levels after adjustment for SD-USe in Model 2. Although urinary selenium concentration was negatively associated with SBP, DBP, and MAP in the main analysis, the IM of urinary selenium was not associated with SBP, DBP, or MAP. After additional adjustment for VIM-USe in Model 2, the estimated strengths of the associations of the IM of urinary selenium with SBP, DBP, and MAP were −0.029 (95% CI: −0.267, 0.208), −0.050 (95% CI: −0.175, 0.076), and −0.040 (95% CI: −0.188, 0.109), respectively. A negative association between the IM of urinary selenium and waistline (−0.105, 95% CI: −0.195, −0.010) was also discovered, and the association remained significant after adjustment for CV-USe, ARV-Use, and VIM-USe in Model 2. Moreover, a higher IM of urinary selenium was associated with a smaller hipline (−0.085, 95% CI: −0.161, −0.005) after additional adjustment for VIM-USe in Model 2 (**Table 6**).

DISCUSSION

Interpretation of Results

Associations of Selenium Biomarkers Concentrations With Cardiometabolic Risk Factors

In this three-wave repeated-measures study of older adults with high selenium levels, we discovered that whole blood selenium was positively associated with total cholesterol, LDL-C, glucose, and uric acid. Urinary selenium was not associated with any cardiometabolic risk factors except for BP among older adults with high selenium levels.

According to epidemiological studies conducted in China and other countries, the participants in our study had high selenium, with a geometric mean (geometric SD) whole blood selenium level of 134.30 (1.19) μg/L. Although this figure is lower than that recorded in the Enshi area, which is well-known for selenium poisoning in China (66), it exceeds the median level obtained in Wuhan (92.66 μg/L) (12) and mean level observed in Shandong (120 μg/L) (24). In addition, our study population had a lower selenium level than the mean of NHANES respondents in the United States (2015–2016: 191 μg/L) (16) but a higher level than that obtained in the United Kingdom (mean: 86.856 μg/L) (67). The high selenium of our participants may have two explanations: (1) As mentioned, the study settings have selenium-rich soil. (2) Our participants were older adults and they were reported to preferred cereals rich in selenium (68). The low rates of alcohol and cigarette consumption and metabolic and physiological function (e.g., excretion) impairment that occurs during the aging process may also have contributed to the participants' high selenium levels (69, 70).

TABLE 4 | Associations of selenium biomarkers with cardiometabolic risk factors, analyzed using linear mixed-effects regression models.

Cardiometabolic risk factors	Regression coefficients (95% CI) by tertiles of selenium biomarkers concentrations			Per one-unit increase of selenium biomarkers[a]	*p*-trend	*p*-value[b]
	T1	T2	T3			
Total cholesterol						
WBSe	Ref	**0.04 (0.01, 0.07)**	**0.08 (0.04, 0.12)**	**0.22 (0.12, 0.33)**	**<0.01**	**<0.01**
USe	Ref	0.02 (−0.01, 0.05)	0.02 (−0.01, 0.05)	0.01 (−0.02, 0.03)	0.25	0.64
Triglyceride						
WBSe	Ref	0.04 (−0.05, 0.14)	0.05 (−0.06, 0.18)	0.29 (0.00, 0.63)	0.39	0.08
USe	Ref	0.05 (−0.04, 0.13)	−0.01 (−0.1, 0.09)	0.01 (−0.05, 0.08)	0.96	0.76
HDL-C						
WBSe	Ref	0.02 (−0.01, 0.05)	0.04 (0.00, 0.07)	0.08 (−0.02, 0.19)	0.07	0.12
USe	Ref	0.01 (−0.01, 0.04)	0.03 (0.00, 0.06)	0.01 (−0.01, 0.03)	0.06	0.18
LDL-C						
WBSe	Ref	**0.05 (0.01, 0.09)**	**0.10 (0.05, 0.15)**	**0.28 (0.13, 0.42)**	**<0.01**	**<0.01**
USe	Ref	0.00 (−0.04, 0.04)	0.01 (−0.04, 0.05)	0.00 (−0.03, 0.03)	0.80	0.84
Glucose						
WBSe	Ref	**0.03 (0.00, 0.06)**	**0.07 (0.02, 0.11)**	**0.22 (0.10, 0.34)**	**<0.01**	**<0.01**
USe	Ref	−0.01 (−0.04, 0.02)	−0.03 (−0.06, 0.01)	−0.01 (−0.04, 0.01)	0.17	0.28
Uric acid						
WBSe	Ref	0.02 (−0.02, 0.05)	**0.05 (0.01, 0.10)**	**0.16 (0.04, 0.28)**	**0.02**	**<0.01**
USe	Ref	0.01 (−0.02, 0.05)	−0.02 (−0.05, 0.02)	−0.01 (−0.04, 0.01)	0.48	0.37
SBP						
WBSe	Ref	1.51 (−1.84, 4.73)	−1.25 (−5.36, 2.92)	−7.40 (−17.93, 3.40)	0.63	0.19
USe	Ref	−1.94 (−5.24, 1.26)	−3.22 (−6.82, 0.36)	**−3.37 (−5.68, −1.01)**	0.08	**<0.01**
DBP						
WBSe	Ref	0.47 (−1.45, 2.25)	2.06 (−0.31, 4.28)	2.64 (−3.33, 8.30)	0.09	0.39
USe	Ref	−0.51 (−2.32, 1.30)	−1.56 (−3.59, 0.42)	**−1.69 (−3.00, −0.39)**	0.14	**0.01**
MAP						
WBSe	Ref	1.03 (−1.08, 3.00)	0.92 (−1.68, 3.45)	−1.35 (−7.97, 5.21)	0.47	0.70
USe	Ref	−0.80 (−2.83, 1.17)	−2.17 (−4.40, 0.03)	**−2.32 (−3.76, −0.88)**	0.06	**<0.01**
Waistline						
WBSe	Ref	0.32 (−1.19, 1.81)	0.33 (−1.50, 2.23)	−1.40 (−5.98, 3.66)	0.73	0.58
USe	Ref	0.20 (−1.32, 1.58)	0.34 (−1.39, 1.83)	0.30 (−0.81, 1.28)	0.67	0.58
Hipline						
WBSe	Ref	−1.02 (−2.12, 0.07)	−0.57 (−1.98, 0.79)	−0.68 (−4.33, 2.93)	0.38	0.72
USe	Ref	0.28 (−0.79, 1.31)	0.26 (−0.95, 1.37)	0.00 (−0.77, 0.74)	0.65	0.99
Waist-hip ratio						
WBSe	Ref	0.01 (0.00, 0.02)	0.01 (0.00, 0.03)	0.00 (−0.04, 0.04)	0.18	0.97
USe	Ref	0.00 (−0.02, 0.01)	0.00 (−0.02, 0.01)	0.00 (−0.01, 0.01)	0.87	0.69
MSS-sex						
WBSe	Ref	0.30 (−0.12, 0.74)	0.28 (−0.26, 0.87)	0.44 (−1.01, 2.05)	0.30	0.58
USe	Ref	0.00 (−0.42, 0.41)	−0.29 (−0.74, 0.16)	−0.28 (−0.57, 0.02)	0.23	0.06

95% CI, 95% confidence interval; T1, first tertile; T2, second tertile; T3, third tertile; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; MSS, metabolic syndrome score; MSS -sex, metabolic syndrome score (sex specific); WBSe, whole blood selenium; USe, urinary selenium. Boldface type indicates effect estimates were statistically significant, p-values < 0.05.

[a] *Per one-unit increase of natural log transformed urinary selenium concentration or per one-unit increase of natural log transformed whole blood selenium concentration.*

[b] *p-values were FDR corrected.*

Consistent with our study, a cross-sectional study of 8,198 Chinese participants from rural areas discovered that serum selenium (mean: 120 μg/L) was positively correlated with triglycerides (0.24, 95% CI: 0.17, 0.31), total cholesterol (0.57, 95% CI: 0.49, 0.65), LDL-C (0.37, 95% CI: 0.32, 0.42), and HDL-C (0.12, 95% CI: 0.08, 0.16) (24). A cross-sectional analysis using NHANES data (2011–2012, N = 2,287) revealed that serum selenium (mean: 192.99 μg/L) was positively associated with LDL-C (0.063, 95% CI: 0.016, 0.110) (5). However, a cross-sectional analysis using NHANES data (2007–2014, *N* = 7,597) revealed that dietary selenium intake was negatively associated with total cholesterol and HDL-C (71). Furthermore,

TABLE 5 | Changes and 95% confidence intervals of cardiometabolic risk factors associated with one-unit increments in VVV and IM of whole blood selenium.

	Total cholesterol	Triglyceride	HDL-C	LDL-C	Glucose
Mean-WBSe					
Model 1	**0.002 (0.001, 0.004)**	**0.007 (0.004, 0.011)**	0.001 (−0.001, 0.003)	**0.002 (0.000, 0.004)**	**0.002 (0.000, 0.004)**
Model 2					
+SD-USe	**0.003 (0.001, 0.004)**	**0.007 (0.003, 0.011)**	0.001 (−0.000, 0.003)	**0.003 (0.000, 0.005)**	**0.002 (0.000, 0.004)**
+CV-USe	**0.002 (0.001, 0.004)**	**0.007 (0.004, 0.011)**	0.001 (−0.001, 0.003)	**0.002 (0.000, 0.004)**	**0.002 (0.000, 0.004)**
+ARV-USe	**0.003 (0.001, 0.004)**	**0.007 (0.003, 0.011)**	0.001 (−0.000, 0.003)	**0.003 (0.001, 0.005)**	0.002 (−0.000, 0.004)
+VIM-USe	**0.002 (0.001, 0.004)**	**0.007 (0.004, 0.011)**	0.001 (−0.001, 0.003)	**0.002 (0.000, 0.004)**	**0.002 (0.000, 0.004)**
SD-WBSe					
Model 1	−0.001 (−0.004, 0.002)	**0.009 (0.000, 0.017)**	−0.002 (−0.006, 0.001)	−0.001 (−0.006, 0.003)	0.003 (−0.001, 0.007)
Model 2	−0.003 (−0.006, 0.001)	0.004 (−0.004, 0.012)	−0.003 (−0.007, 0.001)	−0.003 (−0.008, 0.002)	0.002 (−0.002, 0.006)
CV-WBSe					
Model 1	−0.264 (−0.744, 0.216)	0.726 (−0.484, 1.935)	−0.414 (−0.971, 0.143)	−0.306 (−0.984, 0.372)	0.218 (−0.355, 0.791)
Model 2	−0.328 (−0.798, 0.143)	0.518 (−0.649, 1.686)	−0.444 (−1.000, 0.112)	−0.370 (−1.044, 0.303)	0.157 (−0.410, 0.725)
ARV-WBSe					
Model 1	−0.002 (−0.004, 0.001)	0.006 (0.000, 0.011)	−0.002 (−0.004, 0.001)	−0.002 (−0.005, 0.001)	**0.003 (0.000, 0.005)**
Model 2	**−0.003 (−0.005, −0.000)**	0.003 (−0.002, 0.008)	−0.002 (−0.005, 0.000)	−0.003 (−0.006, −0.000)	0.002 (−0.001, 0.005)
VIM-WBSe					
Model 1	−0.002 (−0.005, 0.002)	0.005 (−0.003, 0.014)	−0.003 (−0.007, 0.001)	−0.002 (−0.007, 0.003)	0.002 (−0.003, 0.006)
Model 2	−0.002 (−0.006, 0.001)	0.004 (−0.005, 0.012)	−0.003 (−0.007, 0.001)	−0.003 (−0.008, 0.002)	0.001 (−0.003, 0.005)
	Uric acid	**SBP**	**DBP**	**MAP**	**Waistline**
Mean-WBSe					
Model 1	0.001 (−0.000, 0.003)	0.003 (−0.110, 0.114)	0.016 (−0.042, 0.072)	0.010 (−0.060, 0.078)	0.032 (−0.019, 0.079)
Model 2					
+SD-USe	0.001 (−0.001, 0.003)	−0.016 (−0.136, 0.099)	0.003 (−0.058, 0.063)	−0.003 (−0.077, 0.068)	0.032 (−0.022, 0.080)
+CV-USe	0.001 (−0.000, 0.003)	−0.002 (−0.116, 0.108)	0.013 (−0.046, 0.069)	0.007 (−0.063, 0.074)	0.032 (−0.020, 0.078)
+ARV-USe	0.001 (−0.001, 0.003)	−0.015 (−0.132, 0.100)	0.010 (−0.051, 0.068)	0.001 (−0.071, 0.071)	0.034 (−0.019, 0.082)
+VIM-USe	0.001 (−0.000, 0.003)	−0.003 (−0.117, 0.108)	0.012 (−0.046, 0.069)	0.006 (−0.064, 0.074)	0.032 (−0.020, 0.078)
SD-WBSe					
Model 1	0.003 (−0.000, 0.007)	0.125 (−0.121, 0.370)	0.090 (−0.037, 0.215)	0.093 (−0.058, 0.243)	0.023 (−0.085, 0.127)
Model 2	0.003 (−0.001, 0.006)	0.136 (−0.121, 0.395)	0.088 (−0.045, 0.220)	0.095 (−0.063, 0.254)	0.002 (−0.110, 0.113)
CV-WBSe					
Model 1	0.431 (−0.085, 0.945)	19.998 (−16.149, 56.358)	12.575 (−6.142, 31.069)	14.066 (−8.242, 36.300)	1.079 (−14.713, 16.644)
Model 2	0.395 (−0.120, 0.911)	20.056 (−16.163, 56.583)	12.248 (−6.513, 30.824)	13.890 (−8.468, 36.234)	0.201 (−15.602, 15.928)
ARV-WBSe					
Model 1	0.002 (−0.001, 0.004)	0.090 (−0.072, 0.252)	0.038 (−0.046, 0.121)	0.049 (−0.051, 0.149)	0.002 (−0.069, 0.070)
Model 2	0.001 (−0.001, 0.004)	0.096 (−0.071, 0.264)	0.035 (−0.052, 0.121)	0.049 (−0.054, 0.152)	−0.011 (−0.083, 0.061)
VIM-WBSe					
Model 1	0.003 (−0.001, 0.007)	0.146 (−0.118, 0.413)	0.093 (−0.044, 0.228)	0.103 (−0.060, 0.266)	0.009 (−0.107, 0.123)
Model 2	0.003 (−0.001, 0.007)	0.147 (−0.119, 0.415)	0.090 (−0.048, 0.226)	0.102 (−0.062, 0.266)	−0.000 (−0.001, 0.000)

(Continued)

TABLE 5 | Continued

	Hipline	Waist-hip ratio	MSS_sex
Mean-WBSe			
Model 1	0.013 (−0.029, 0.050)	0.000 (−0.000, 0.001)	**0.022 (0.002, 0.041)**
Model 2			
+SD-USe	0.007 (−0.037, 0.046)	0.000 (−0.000, 0.001)	0.017 (−0.004, 0.036)
+CV-USe	0.011 (−0.030, 0.048)	0.000 (−0.000, 0.001)	**0.021 (0.001, 0.039)**
+ARV-USe	0.008 (−0.035, 0.047)	0.000 (−0.000, 0.001)	0.017 (−0.003, 0.036)
+VIM-USe	0.011 (−0.031, 0.048)	0.000 (−0.000, 0.001)	**0.021 (0.001, 0.039)**
SD-WBSe			
Model 1	0.046 (−0.040, 0.131)	−0.000 (−0.001, 0.001)	**0.047 (0.005, 0.088)**
Model 2	0.042 (−0.048, 0.132)	−0.000 (−0.001, 0.000)	0.035 (−0.008, 0.079)
CV-WBSe			
Model 1	6.414 (−6.285, 18.984)	−0.043 (−0.158, 0.071)	5.099 (−1.121, 11.285)
Model 2	6.115 (−6.600, 18.806)	−0.049 (−0.164, 0.065)	4.511 (−1.674, 10.695)
ARV-WBSe			
Model 1	0.028 (−0.029, 0.084)	−0.000 (−0.001, 0.000)	**0.034 (0.006, 0.061)**
Model 2	0.025 (−0.033, 0.084)	−0.000 (−0.001, 0.000)	0.028 (−0.001, 0.056)
VIM-WBSe			
Model 1	0.047 (−0.046, 0.139)	−0.000 (−0.001, 0.001)	0.038 (−0.008, 0.083)
Model 2	0.045 (−0.048, 0.138)	0.002 (−0.114, 0.117)	0.033 (−0.012, 0.079)

Boldface type indicates effect estimates were statistically significant, p-values < 0.05.

few longitudinal studies have been conducted, and those that have been conducted have reported controversial results. A 21-year follow-up analysis of the Young Finns Study and an 8-year follow-up analysis of the Olivetti Heart Study discovered positive cross-sectional associations between selenium and lipid concentrations but no such longitudinal relations (72, 73). The inconsistency between the results of our study and those of the two longitudinal studies might be partly attributable to the different population characteristics, substantial disparity in selenium levels, and potential non-linearity of the associations of selenium with lipid profiles. First, the participants of the Young Finns Study and the Olivetti Heart Study were children (aged 3–18 years) and adult males, whose physiological characteristics differ from those of a population of older adults. Second, the mean (SD) selenium concentrations reported in those two studies were 74.3 (14.0) and 77.5 (18.4) μg/L, respectively, which differed from the 134.30 (1.19) μg/L observed in our study. Therefore, large repeated-measures studies of participants with different selenium statuses and demographic characteristics are warranted to further investigate the associations of selenium and lipid profiles and the exposure–response curves.

Whole blood selenium and glucose were positively associated in older adults with high selenium. A systematic review of 15 observational studies enrolling 32,728 participants observed a positive association between selenium concentration and the odds ratio (OR) for diabetes, with a summary OR of 2.03 (95% CI: 1.51, 2.72) (74). Specifically, a cross-sectional analysis of 8,142 middle-aged adults (mean serum selenium: 121.5 μg/L) residing in Linyi, China, revealed that, compared with a low-selenium group (<124.9 μg/L), the ORs for elevated fasting serum glucose for two high-selenium groups (124.9–143.9 and >143.9 μg/L) were 2.31 (95% CI: 1.37, 3.90) and 2.67 (95% CI: 1.59, 4.48) (75). Moreover, a cross-sectional study of NHANES data (1999–2006, $N = 41{,}474$) reported that serum selenium concentration (mean: 129 μg/L) was positively associated with plasma glucose (12.454, 95 % CI: 4.122, 20.786) (76). Another NHANES-based (2003–2004, $N = 917$) cross-sectional analysis of adults aged over 40 years revealed a higher OR of serum selenium–related diabetes (mean: 137.1 mg/L) in women (OR: 5.99) than in men (OR: 2.30) (9). In combination with these results, our study suggests that an individual's sex may affect the selenium–glucose association.

The associations between selenium and other cardiovascular risk factors such as uric acid have not been widely studied. A positive association between whole blood selenium and uric acid among older adults with high selenium levels was observed in the present study. Consistent with our findings, a cross-sectional study of 1,406 Han Chinese adults revealed a positive association between serum selenium and the odds of hyperuricaemia, with an OR of 1.50 (95% CI: 1.01, 2.23) (77). In addition, selenium intake exceeding the recommended amount was positively correlated with the uric acid levels in the first (mean: 53.99 μg/day) and second (mean: 58.93 μg/day) trimesters of pregnancy for 95 Polish women (78). Previous findings suggest that the relationships of selenium biomarkers with uric acid may be confounded by diet and physical activity (79). All three visits in our repeated-measures study were conducted in winter to minimize the effect of seasonally relevant confounding factors, including diet and physical activity which could vary by seasons. High uric acid has been identified as an independent risk

TABLE 6 | Changes and 95% confidence intervals of cardiometabolic risk factors associated with one-unit increments in VVV and IM of urinary selenium.

	Total cholesterol	Triglyceride	HDL-C	LDL-C	Glucose
Mean-USe					
Model 1	0.001 (−0.002, 0.004)	0.006 (−0.001, 0.014)	0.000 (−0.003, 0.004)	0.000 (−0.004, 0.004)	0.003 (−0.000, 0.007)
Model 2					
+SD-USe	0.005 (−0.000, 0.009)	**0.014 (0.002, 0.025)**	0.002 (−0.003, 0.007)	0.003 (−0.003, 0.010)	**0.006 (0.000, 0.011)**
+CV-USe	0.002 (−0.001, 0.005)	0.008 (−0.000, 0.016)	0.001 (−0.002, 0.005)	0.000 (−0.004, 0.005)	0.004 (−0.000, 0.008)
+ARV-USe	0.004 (−0.001, 0.008)	0.010 (−0.000, 0.021)	0.002 (−0.003, 0.007)	0.002 (−0.004, 0.008)	0.005 (−0.000, 0.010)
+VIM-Use	0.001 (−0.002, 0.004)	0.006 (−0.002, 0.014)	0.001 (−0.003, 0.005)	−0.000 (−0.005, 0.004)	0.003 (−0.001, 0.007)
SD-USe					
Model 1	−0.001 (−0.004, 0.001)	−0.001 (−0.006, 0.005)	0.000 (−0.003, 0.003)	−0.002 (−0.005, 0.001)	0.000 (−0.003, 0.003)
Model 2	**−0.004 (−0.007, −0.000)**	−0.008 (−0.016, 0.000)	−0.001 (−0.005, 0.003)	−0.004 (−0.009, 0.001)	−0.003 (−0.007, 0.001)
CV-USe					
Model 1	−0.091 (−0.204, 0.021)	−0.180 (−0.459, 0.101)	−0.044 (−0.173, 0.084)	−0.081 (−0.242, 0.079)	−0.061 (−0.198, 0.077)
Model 2	−0.108 (−0.224, 0.009)	−0.250 (−0.537, 0.037)	−0.057 (−0.190, 0.076)	−0.084 (−0.250, 0.082)	−0.094 (−0.234, 0.047)
ARV-USe					
Model 1	−0.001 (−0.003, 0.001)	0.000 (−0.005, 0.005)	−0.000 (−0.003, 0.002)	−0.002 (−0.004, 0.001)	0.000 (−0.002, 0.003)
Model 2	−0.002 (−0.005, 0.000)	−0.004 (−0.010, 0.003)	−0.001 (−0.004, 0.002)	−0.002 (−0.006, 0.001)	−0.002 (−0.005, 0.001)
VIM-USe					
Model 1	−0.005 (−0.010, 0.001)	−0.010 (−0.024, 0.003)	−0.003 (−0.009, 0.003)	−0.003 (−0.011, 0.004)	−0.005 (−0.011, 0.002)
Model 2	−0.005 (−0.010, 0.001)	−0.010 (−0.024, 0.003)	−0.003 (−0.009, 0.003)	−0.003 (−0.011, 0.004)	−0.005 (−0.011, 0.002)
	Uric acid	**SBP**	**DBP**	**MAP**	**Waistline**
Mean-USe					
Model 1	0.000 (−0.003, 0.004)	−0.024 (−0.246, 0.197)	−0.050 (−0.164, 0.065)	−0.036 (−0.172, 0.101)	**−0.105 (−0.195, −0.010)**
Model 2					
+SD-USe	−0.001 (−0.006, 0.004)	−0.005 (−0.352, 0.341)	−0.094 (−0.276, 0.088)	−0.072 (−0.287, 0.143)	−0.128 (−0.271, 0.014)
+CV-USe	−0.002 (−0.005, 0.002)	−0.005 (−0.252, 0.241)	−0.050 (−0.180, 0.080)	−0.035 (−0.188, 0.119)	**−0.135 (−0.234, −0.031)**
+ARV-USe	−0.002 (−0.007, 0.002)	−0.083 (−0.399, 0.232)	−0.138 (−0.303, 0.027)	−0.125 (−0.320, 0.070)	**−0.149 (−0.278, −0.019)**
+VIM-USe	−0.002 (−0.005, 0.002)	−0.029 (−0.267, 0.208)	−0.050 (−0.175, 0.076)	−0.040 (−0.188, 0.109)	**−0.133 (−0.228, −0.033)**
SD-USe					
Model 1	−0.001 (−0.004, 0.001)	−0.029 (−0.204, 0.148)	−0.005 (−0.098, 0.088)	−0.006 (−0.115, 0.104)	−0.074 (−0.144, 0.002)
Model 2	−0.001 (−0.004, 0.003)	−0.026 (−0.281, 0.230)	0.045 (−0.089, 0.180)	0.032 (−0.126, 0.192)	−0.005 (−0.107, 0.103)
CV-USe					
Model 1	−0.006 (−0.126, 0.115)	−3.498 (−11.924, 4.930)	−0.389 (−4.838, 4.056)	−1.080 (−6.324, 4.171)	−0.984 (−4.481, 2.563)
Model 2	0.009 (−0.114, 0.134)	−3.451 (−12.177, 5.278)	0.076 (−4.529, 4.673)	−0.763 (−6.194, 4.671)	0.283 (−3.302, 3.891)
ARV-USe					
Model 1	−0.000 (−0.003, 0.002)	0.016 (−0.133, 0.166)	0.028 (−0.051, 0.106)	0.030 (−0.062, 0.123)	−0.045 (−0.106, 0.018)
Model 2	0.000 (−0.002, 0.003)	0.050 (−0.147, 0.248)	0.085 (−0.019, 0.188)	0.081 (−0.040, 0.204)	0.016 (−0.064, 0.098)
VIM-USe					
Model 1	0.000 (−0.005, 0.006)	−0.192 (−0.594, 0.210)	−0.020 (−0.233, 0.192)	−0.062 (−0.313, 0.188)	0.018 (−0.151, 0.187)
Model 2	0.000 (−0.005, 0.006)	−0.192 (−0.593, 0.211)	−0.019 (−0.232, 0.192)	−0.062 (−0.312, 0.189)	0.021 (−0.146, 0.187)

(Continued)

TABLE 6 | Continued

	Hipline	Waist-hip ratio	MSS_sex
Mean-USe			
Model 1	−0.075 (−0.147, 0.001)	−0.000 (−0.001, 0.000)	0.009 (−0.028, 0.047)
Model 2			
+SD-USe	−0.073 (−0.186, 0.041)	−0.001 (−0.002, 0.000)	0.017 (−0.042, 0.077)
+CV-USe	**−0.085 (−0.164, −0.003)**	−0.001 (−0.001, 0.000)	0.002 (−0.040, 0.045)
+ARV-USe	−0.085 (−0.186, 0.019)	−0.001 (−0.002, 0.000)	0.000 (−0.053, 0.055)
+VIM-USe	**−0.085 (−0.161, −0.005)**	−0.001 (−0.001, 0.000)	−0.001 (−0.041, 0.041)
SD-USe			
Model 1	−0.051 (−0.107, 0.008)	−0.000 (−0.001, 0.000)	−0.009 (−0.039, 0.021)
Model 2	−0.012 (−0.094, 0.073)	0.000 (−0.001, 0.001)	−0.019 (−0.062, 0.026)
CV-USe			
Model 1	−0.768 (−3.527, 2.019)	−0.003 (−0.029, 0.023)	−0.410 (−1.852, 1.040)
Model 2	0.024 (−2.827, 2.876)	0.003 (−0.024, 0.030)	−0.433 (−1.929, 1.064)
ARV-USe			
Model 1	−0.035 (−0.083, 0.015)	−0.000 (−0.001, 0.000)	−0.001 (−0.026, 0.025)
Model 2	−0.000 (−0.064, 0.064)	0.000 (−0.000, 0.001)	−0.001 (−0.035, 0.033)
VIM-USe			
Model 1	0.006 (−0.128, 0.138)	0.000 (−0.001, 0.001)	−0.019 (−0.089, 0.050)
Model 2	0.007 (−0.125, 0.139)	0.000 (−0.001, 0.001)	−0.019 (−0.089, 0.050)

Boldface type indicates effect estimates were statistically significant, p-values < 0.05.

factor for long-term CVD events, CVD-related death, and all-cause mortality (80, 81). Furthermore, the positive relationship between selenium and uric acid observed in this study should be interpreted with caution, and additional comprehensive studies are required.

Few studies have explored the relationship between urinary selenium and BP. However, in the present study, we observed that urinary selenium as a continuous variable was negatively associated with SBP, DBP, and MAP among older adults with high selenium levels; however, these negative associations were non-significant when urinary selenium was treated as a categorical variable. A cross-sectional study conducted in Wuhan, China ($N = 823$), revealed a positive association between increased ORs for hypertension and urinary selenium quartiles (geometric mean: 19.8 μg/g creatinine) by using a single-metal regression model (p trend < 0.05) (26). However, another case–control study conducted in Wuhan ($N = 1{,}004$) reported that urinary selenium (geometric mean: 20.47 μg/g creatinine) was not associated with hypertension (82). This disparity can be partly attributed to the different study design and the fact that urinary selenium accounts for the majority of absorbed selenium that is not retained and is easily influenced by diet and physical activity (83).

Associations of VVV and IM of Selenium Biomarkers With Cardiometabolic Risk Factors

In this three-wave repeated-measures study of older adults with high selenium status, the reproducibility of the whole blood selenium measurements was high, and that of urinary selenium was poor. Because the collection of blood samples is invasive, the reproducibility of whole blood selenium measurement has rarely been studied. A longitudinal analysis of urine samples collected from 11 Chinese men at days 0, 1, 2, 3, 4, 30, 60, and 90 suggested poor reproducibility of urinary selenium (ICC = 0.03) (84). The reproducibility of whole blood and urinary selenium measurements may be affected by the kinetics of selenium absorption, distribution, metabolism, and elimination (85). Selenium is absorbed and accumulates in the human body, mainly in the liver, kidneys, and blood. The elimination of selenium in urine is also influenced by diet, lifestyle, and activity (83). Therefore, the reproducibility of whole blood selenium measurement is superior to that of urinary selenium. The reproducibility of the selenium biomarkers observed in the present study suggests that single measurements may not accurately reflect individual exposure to selenium over time, at least among older adults with high selenium, and repeated-measures studies with even more waves should be employed to minimize exposure misclassification.

We explored the relations of the VVV and IM of selenium biomarkers with cardiometabolic risk factors in the present study. Among the older adults with high selenium levels, the VVV of the selenium biomarkers was not significantly associated with cardiometabolic risk factors after adjustments for IM. The VVV of whole blood selenium, including SD-WBSe and ARV-WBSe, was associated with cardiometabolic factors, including glucose, triglycerides, and MSS-sex, but these associations were non-significant after adjustment for IM. VIM-WBSe was also not associated with any cardiometabolic risk factors. Four indicators were calculated to comprehensively evaluate the VVV of the selenium biomarkers. However, in whole blood selenium, these four indicators of VVV were not all associated with any particular cardiometabolic risk factors. Moreover, no stable association between the VVV of urinary selenium and any

cardiometabolic risk factor was discovered. Although we did not observe associations between the VVV of the selenium biomarkers and cardiometabolic risk factors among older adults with high selenium levels, future research is suggested. This three-wave repeated-measures study was conducted from November 2016 through January 2018 and captured the long-term trends of the selenium biomarkers. However, repeated-measures studies conducted in different seasons are warranted to characterize seasonal biomarker variability. The VVV of the selenium biomarkers remains an informative measure for selenium biomarkers, and the potential relations of this measure with cardiometabolic risk factors should not be ignored.

We also observed that the IM of whole blood selenium was positively correlated with cardiometabolic factors, including total cholesterol, triglycerides, LDL-C, and MSS-sex, and these associations remained significant after adjustment for VVV. The IM of urinary selenium was also significantly associated with cardiometabolic risk factors after adjustment for VVV. Specifically, the IM of urinary selenium was positively related with triglycerides and glucose and negatively associated with waistline and hipline. In short, the IM of selenium biomarkers can be used in conjunction with the absolute levels for prediction of cardiometabolic risk factors among older adults with high selenium levels. The IM of the selenium biomarkers reflected the centralized levels across the study period. In sum, cardiometabolic risk is associated with exposure to selenium over time among older adults with high selenium levels. Furthermore, a pooled analysis of two prospective population-based cohort studies (i.e., the Health Retirement Study and the English Longitudinal Study of Aging, $n = 6{,}237$) revealed that a 1% increase in mean glycosylated hemoglobin A1c was associated with a faster rate of memory function decline (−0.041, 95% CI: −0.071, −0.012) (40). These and our findings suggest that IM in biomarkers and physiological parameters may have an impact on health end points and other studies exploring the relationship between nutrition and disease should consider the impact of IM.

In conclusion, the associations between whole blood and urinary selenium and cardiometabolic risk factors are inconsistent. Except for VVV, concentrations and IM of whole blood selenium were associated with cardiometabolic risk factors. In contrast, only VVV of urinary selenium was significantly associated with cardiometabolic risk factors. These differences could be explained for several reasons. First, selenium enters into the human body mainly by three routes (i.e., digestion, inhalation and skin absorption) (86). Whole body retention studies following oral administration of sodium selenite have indicated that human bodies absorb and accumulate selenium, primarily in the liver, kidneys, and blood (half-life: almost 9 months). The urinary excretion of selenium lasted ∼1 week, with a half-life of 8–9 days (87). Hence, whole blood selenium reflected internal burden of selenium and urinary selenium reflected absorbed but unutilized selenium. This selenium metabolism may partly explain the differences in the associations observed in urinary and whole blood selenium. Second, the elimination of urinary selenium is also influenced by diet, lifestyle, and activity (83). Our data also found that the reproducibility of urinary selenium measurement was lower to that of whole blood selenium. Tough we observed negative associations of urinary selenium and SBP, DBP, and MAP, these associations were non-significant when urinary selenium concentration was treated as a categorical variable. Finally, single measurements may not accurately reflect selenium status. IM can capture the centralizing trend of multiple repeated measurements. We did not observe negative association between IM of urinary selenium and BP-related factors. However, both whole blood selenium concentration and IM of whole blood selenium were positively associated with total cholesterol, LDL-C, glucose, and uric acid. Hence, association of whole blood selenium are more stable than that of urinary selenium. Even though the available evidence can partially explain the inconsistent results in the associations observed in urinary and whole blood selenium, experimental animal studies exploring the underlying mechanisms are warrant.

As an essential trace element, selenium is incorporated into selenoproteins that have a wide range of dose-dependent effects. The *U*-shaped associations between selenium levels and cardiometabolic risk factors must be emphasized (10). A case–control study conducted in Wuhan demonstrated a *U*-shaped association between plasma selenium (median: 92.66 μg/L) with central obesity and high blood pressure (12). As above mentioned, another cross-sectional analysis using data from NHANES reported a *U*-shaped association between plasma selenium and the likelihood of diabetes, with the lowest risk noted for a concentration of ∼122 μg/L (9). Due to the high blood selenium status (GM:134.30 μg/L), the positive association between whole blood selenium and blood glucose was observed in our study. Additionally, the *U*-shaped association was observed between selenium status and all-cause and cancer mortality among 13,887 adult participants in the NHANES (1988-1994), with the lowest risk noted for a concentration of ∼135 μg/L (88). In order to analyse the two-way effect of selenium on health, especially cardiometabolic risk factors, more epidemiological studies of participants with wide selenium concentration statuses and demographic characteristics were warranted. The exposure response curve of selenium biomarkers and cardiometabolic risk factors among both deficient and excessive selenium levels were needed to be profiled.

Potential Mechanisms

The biological mechanisms explaining selenium's involvement in the pathogenesis of cardiometabolic diseases are not well-understood. High levels of selenium are incorporated into selenoproteins, damaging cardiometabolic health mainly by initiating oxidative stress, promoting insulin resistance, and regulating gluconeogenesis and lipid metabolism. Excess selenium increases the production of reactive oxygen species (ROS) by promoting the overexpression of selenocysteine transfer RNA and reducing selenoproteins synthesis. Increased ROS levels cause insulin resistance in the peripheral tissues by affecting insulin receptor signal transduction, ultimately resulting in hyperinsulinemia and cell glucose desensitization (89). In an experimental study, C57BL/6J mice ($n = 6$ or 7 per group) on a selenium-supplemented diet (0.1 and 0.4 ppm selenium) developed hyperinsulinemia and reduced insulin sensitivity (90).

In addition, excessive ROS production can reduce nitric oxide; accelerate endothelial cell apoptosis; induce upregulation of NF-kB; activate intercellular adhesion molecule-1, monocyte chemotactic protein-1, and vascular cell adhesion molecule-1; and trigger diabetic vascular complications and cardiometabolic disorders (91).

Meanwhile, high dietary selenium intake can increase the expression or activity of key proteins related to gluconeogenesis, glycolysis, and lipogenesis by upregulating the selenoproteins glutathione peroxidase family. First, high glutathione peroxidase-1 production due to high selenium intake results in the upregulation of phosphoenolpyruvate carboxykinase and upregulation of fatty acid synthesis. In an experimental study, prolonged high dietary intake of selenium was demonstrated to induce gestational diabetes in rats and hyperinsulinemia in pigs (92). Second, high selenium upregulates protein tyrosine phosphatase 1B (PTP1B), a key enzyme in triggering fatty acid synthesis and in reverse regulation of insulin signaling, eventually inducing lipid disorders and insulin resistance. In one animal study, PTP1B expression were elevated in rats with fructose-rich diets, resulting in the induction of fatty acid synthesis and an increase in liver triglycerides (93). Further studies are warranted to clarify the mechanisms underlying the complex associations between selenium and cardiometabolic risk.

Limitations and Strengths

This study had several limitations. First, we selected whole blood selenium and urinary selenium as the selenium biomarkers to investigate, but serum or plasma selenium may have been more favorable alternatives. Nevertheless, in our study, whole blood selenium and urinary selenium were preferable because of the consistency of concentrations and determinants between the whole blood and serum selenium; in addition, whole blood selenium is reportedly a suitable indicator of medium- to long-term selenium status (94). Moreover, measuring both whole blood and urinary selenium provides an accurate picture of the function and excretion characteristics of selenium (83). Second, information regarding the participants' alcohol consumption, cigarette smoking habits, and disease history was collected using questionnaires. Consequently, unmeasured confounding factors and recall bias likely limit the generalisability of our findings. Third, the modest number of repeat measurements and their short time intervals might have hampered our ability to characterize selenium variability. Despite the limited data, we provide a fresh perspective on the relationships between selenium and cardiometabolic risk factors. Finally, the participants in our study were older adults (age $\geq$50 years) with high selenium levels; thus, the generalisability of the results to more general populations may be limited.

Despite these limitations, several strengths should also be considered. The three-wave repeated-measures design enabled repeated data collection and inference of causal relationships. Additionally, all three measurements were conducted in winter, minimizing the effect of seasonally relevant confounding factors. To our knowledge, this is the first study to comprehensively evaluate the associations of selenium concentration, VVV, IM with cardiometabolic risk factors. Finally, our findings are derived from observations of older adults with high blood selenium in the Beijing region, elucidating the health effects of high selenium and providing science-based evidence for nutritional guidelines in the region.

CONCLUSION

We discovered that selenium levels and their IMs were significantly associated with several cardiometabolic factors, namely total cholesterol, LDL-C, and glucose, in older adults with high selenium in the Beijing area. This indicates that selenium affects cardiometabolic risk. However, we do not observe any robust associations between the VVV of the selenium biomarkers and cardiometabolic risk factors after adjustment for IM. The findings suggest that older adults with high selenium should not take dietary selenium supplements to prevent cardiometabolic risk. In the future, longer repeated-measures studies of the general population are warranted to minimize selenium exposure misclassification, explore the associations of the VVV and IM of selenium biomarkers with cardiometabolic risk factors, and determine the relevant underlying mechanisms.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AL: conceptualization, methodology, data-analysis and interpretation, writing-original draft, writing-review and editing, and funding acquisition. QZ: data-cleaning and interpretation, writing-original draft, and writing-review and editing. YM and JZ: investigation, data cleaning, and writing-review and editing. MZ, JX, and XG: investigation and writing-review and editing. QX: funding acquisition, writing-review and editing, and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the China Medical Board (Grant No. 15-230), the Fundamental Research Funds for the Central Universities (Grant No. 3332019147), Peking Union Medical College Graduate Innovation Fund (No. 2019-1004-02), the China Prospective cohort study of Air pollution and health effects in Typical areas (C-PAT; Grant No. MEE-EH-20190802), and the Chinese Academy of Medical Science Innovation Fund for Medical Sciences (Grant No. 2017-I2M-1-009).

ACKNOWLEDGMENTS

We are grateful for the kind cooperation of all participants in this study. Also, we would like to thank all the investigators involved in our study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.838613/full#supplementary-material

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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.

GLOSSARY

ARV, average real variability; ARV-Use, average real variability of urinary selenium; ARV-WBSe, average real variability of whole blood selenium; BMI, body mass index; CI, confidence interval; CV, coefficient of variation; CVD, cardiovascular disease; CV-Use, coefficient of variation of urinary selenium; CV-WBSe, coefficient of variation of whole blood selenium; DBP, diastolic blood pressure; EDF, estimated degrees of freedom; FAS, fatty acid synthesis; FDR, false discovery rate; FFQ, food frequency questionnaire; GAMMs, generalized additive mixed models; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; ICCs, intraclass correlation coefficients; ICP-MS, inductively coupled plasma mass spectrometer; LDL-C, low-density lipoprotein cholesterol; LOD, limit of detection; MAP, mean arterial pressure; MSS, metabolic syndrome score; MSS-sex, sex-specific metabolic syndrome score; NHANES, the National Health and Nutrition Examination Survey; OR, odds ratio; PTP1B, protein tyrosine phosphatase 1B; ROS, reactive oxygen species; SBP, systolic blood pressure; SD, standard deviation; SD-USe, standard deviation of urinary selenium; SD-WBSe, standard deviation of whole blood selenium; Use, urinary selenium; VIM, variability independent of the mean; VIM-USe, variability independent of the mean of urinary selenium; VIM-WBSe, variability independent of the mean of whole blood selenium; WBSe, whole blood selenium.

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 15 June 2022
doi: 10.3389/fnut.2022.899780

Blood Lead and High-Density Lipoprotein Concentrations in Relation to Human Blood Pressure: A Cross Sectional Study

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

Received: 19 March 2022
Accepted: 16 May 2022
Published: 15 June 2022

Citation:
Hu B, He P-y, Zhong N-n, Gao Z-m, Guo J-l, Feng J-t, Huang C-q, Yang J-b and Sun D-l (2022) Blood Lead and High-Density Lipoprotein Concentrations in Relation to Human Blood Pressure: A Cross Sectional Study. Front. Nutr. 9:899780. doi: 10.3389/fnut.2022.899780

Background: While the relationship between blood pressure and blood lead has been studied more extensively, the effect of high-density lipoprotein (HDL) concentration on this relationship remains uncertain. Therefore, this study aimed to determine the effect of HDL concentration on the relationship between blood lead and blood pressure.

Methods: The research used cross-sectional data from the 2005 to 2014 National Health and Nutrition Examination Survey (NHANES), which included 16,451 participants aged 20–60 years. Multivariable linear regression was used to evaluate the correlation among blood lead, systolic blood pressure (SBP), and diastolic blood pressure (DBP). HDL concentration was determined by low HDL concentration (≤49 mg/dl) and high HDL concentration (>49 mg/dl) stratified. The effect of HDL concentration was assessed by an interaction test between blood lead and SBP in multivariable linear regression.

Results: In this cross-sectional research, we identified a positive correlation between blood lead and SBP, but not DBP. The relationship between blood lead and SBP was different in the group with low and high HDL concentrations (β: 0.21 95% CI:−0.05-0.46 vs. β:0.47 95% CI: 0.15-0.79). In addition, high HDL significantly altered the positive correlation between blood lead and SBP (*P*-value of interaction < 0.001).

Conclusion: The study suggests an interaction between HDL and blood lead in elevating SBP, which may have important clinical implications.

Keywords: blood pressure, blood lead, HDL, interaction, cross sectional study

INTRODUCTION

Hypertension is the most common chronic non-communicable disease, second only to smoking among preventable causes of death from any cause (1), and is an important global health problem. Hypertension is also the most important risk factor for cardiovascular disease, leading to half of the coronary heart disease and about two-thirds of the cerebrovascular disease burden (2).

Lead is a relatively common environmental toxin that can cause many acute and chronic diseases. And lead is absorbed and distributed in the blood, bone and soft tissue (3). Blood lead levels were measured as an indicator of exposure and toxicity, and studies have shown that lead has acute effects on blood pressure through recent doses and chronic effects on the risk of hypertension through cumulative doses (4). Other studies have found that high blood pressure is associated with high blood lead levels in the west of Scotland, which may explain the high incidence of cardiovascular disease in the area (5). Similar cardiovascular complications have been observed after excessive lead exposure in laboratory animals (6). Several candidates have been identified for the mechanisms of lead-induced hypertension, including oxidative stress, inflammation, dysregulation of vasoactive hormones, impaired nitric oxide (NO) systems, and altered cellular Ca^{2+} transport and intracellular Ca^{2+} distribution (7, 8).

In various models, and even in human studies, HDL has been shown to have multiple properties that can reasonably provide cardiovascular protection (9). It has many beneficial effects, including reverse transport of cholesterol, antioxidant, anti-inflammatory, anti-apoptotic, and as a vasodilator (10). Many studies have indicated that HDL can protect endothelial cells (11), Specifically, HDL exerts its vasodilator effect by activating (phosphorylating) the endothelial nitric oxide synthase, which increases the level of NO, the most active vasodilator in the body (12). And studies have found that blood lead has a certain influence on HDL production (13). However, clinical studies on the effect of HDL on the relationship between blood lead and hypertension risk are limited. So we hypothesized that blood lead and HDL interact with the risk of hypertension. We aimed to explore the relationship between blood lead and blood pressure and the effect of HDL on this relationship.

METHODS

Data Selection

We used data collected by the NHANES conducted by the U.S. National Center for Health Statistics to assess the health and nutrition status of a representative sample of the non-institutionalized U.S. civilian population. All procedures in the NHANES survey cycle used in this study were approved by the Ethics Review Board of the National Center for Health Statistics Research and informed consent was given by all participants.

We included adult participants aged 20–60 years who underwent blood lead and blood pressure and HDL measurements in NHANES from 2005 to 2014. Participants with missing data on blood lead concentration, blood HDL concentration, blood pressure, and covariates were excluded, and the final study population comprised 16,451 participants.

Measurement of Blood Pressure

BP was measured by trained inspectors using standardized protocols, and three consecutive blood pressure readings were taken after sitting quietly for 5 min and determining the maximum inflation level (MIL) of the participant. If the blood pressure measurement was interrupted or performed completely, a fourth attempt can be made. All blood pressure measurements (systolic and diastolic) were performed at a mobile screening center (MEC) (14). We calculated the mean of the first 3 systolic and diastolic readings for further analysis.

Blood Lead Measurement

The whole blood samples were processed, stored, and shipped to the National Center for Environmental Health and centers for Disease Control and Prevention for analysis, following a simple dilution sample preparation step, using an inductively coupled Plasma Dynamic Reaction Cell mass spectrometer (ELAN DRC II, PerkinElmer, Norwalk), blood lead content in whole blood samples was directly measured by mass spectrometry (15).

Measurement of HDL Concentration

Serum samples were stored at appropriate freezing conditions (−30°C) and then transported to the laboratory for testing. Magnesium sulfate/dextran solution was first added to the sample, followed by reagent 2, and finally measured on Roche modular P and Roche Cobas 6000 chemical analyzers (16). Blood HDL concentration was defined as low HDL (≤49 mg/dl) and high HDL (>49 mg/dl).

Covariates

Several factors could influence the outcome, age, gender (male or female), race (Mexican American, non-Hispanic Black, non-Hispanic White, other Hispanic, other race-including multi-racial), Marital status (married and unmarried), family PIR, BMI (<25, 25–29.9, ≥30 kg/m^2), Educational level (less than high school, high school graduation, college or above), diabetes, hypertension, smoking status (never = smoked <100 cigarettes in life, former = smoked <100 cigarettes in life and smoke not at all now, now = smoked moth than 100 cigarettes in life and smoke some days or every day), Work activity (non-work activity, moderate work activity, and vigorous work activity), Alcohol consumption (yes = at least 12 alcohol drinks per year vs. no = <12 alcohol drinks per year), cardiovascular disease and albumin/urine (ug/ml) were included as covariables.

The definition criteria of diabetes are as follows: (1) doctor told you to have diabetes (2) Self-reported diabetes for a long time (3) glycohemoglobin HbA1c (%) >6.5 (4) the fasting glucose (mmol/L) ≥7.0 (5) random blood glucose (mmol/L) ≥11.1 (6) 2-h OGTT blood glucose (mmol/L) ≥11.1 (7) Use of diabetes medication or insulin (8) diabetes at birth is considered type 1 diabetes. Hypertension case definitions are based on the International Society of High Blood Pressure standards and a self-reported questionnaire. Participants were identified as hypertensive if they met the following criteria: (1) current use of hypertension medication (2) based on accurate diagnosis by the physician (3) based on blood pressure measured in real-time ≥140/90 mmHg (4) self-reported questionnaire data showing physician's prior diagnosis of hypertension and current use of medication to lower blood pressure. (5) The diagnostic criteria for hypertension by ambulatory blood pressure monitoring (ABPM) were: mean blood pressure ≥130/80 mmHg within 24 h, daytime ≥135/85 mmHg, at night ≥120/70 mmhg. In addition, CVD is determined by any reported diagnosis of

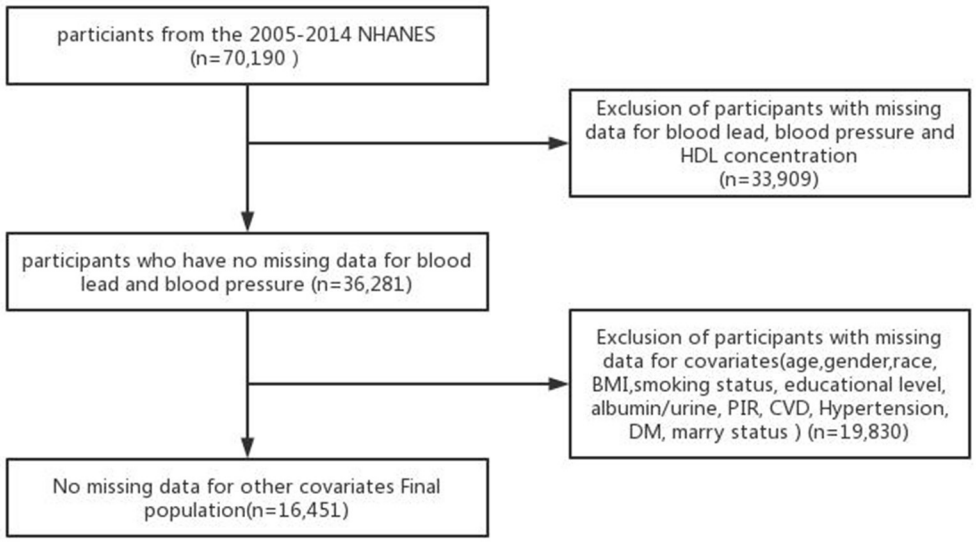

FIGURE 1 | Flowchart for exclusion criteria.

congestive heart failure, coronary heart disease, angina, heart attack, or stroke (17). Residents were asked "Has a doctor or other health professional ever told you that you have congestive heart failure/coronary heart disease/angina/heart attack/stroke?" and participants who answered "yes" to either question were included in our study's general cardiovascular disease group.

Statistical Analysis

Data analysis was performed using a statistical software package R (http://www.R-project.org, R Foundation). During statistical analysis, complex multistage stratified sampling was analyzed using appropriate stratification, clustering, and weights. Multiple linear regression analysis was used to study the relationship between blood lead and blood pressure. SBP and DBP mean values were assessed at different HDL concentrations. The likelihood ratio test was used to examine the interactions between subgroups.

In the descriptive analysis, continuous variables are represented as mean and standard deviation (SD) or median and quartile range (QR), and categorical variables as weighted percentages (%). Calculate 95% confidence intervals (Cls). The statistical significance level was set at $p < 0.05$.

The continuous variables were evaluated by chi-square test, *T*-test (normal distribution), and Kruskal-Wallis test (skewness distribution).

RESULTS

Baseline Characteristics of the Study Population

Six NHANES cycles (2005–2006, 2006–2007, 2007–2008, 2009–2010, 2011–2012, and 2013–2014) were used in this study. After several screenings to exclude participants with missing covariate data, the remaining 16,451 participants were included in our analysis. A flowchart for exclusion criteria is shown in **Figure 1**.

Table 1 shows the study population according to the high and low blood concentrations of HDL (HDL $\leq$ 49 mg/dl for low concentration, HDL $>$ 49 mg/dl for high concentration). Compared with low HDL concentration, people with high HDL concentration may be female, non-Hispanic White, married, higher PIR, lower BMI, well-educated, no diabetes, no hypertension, non-work activity, no cardiovascular disease, no smoking, and low albumin/urine. No statistically significant differences were detected in age and alcohol use (all *p*-values were $>$0.05).

The Connection Between Blood Lead and Blood Pressure

In DBP, in the second model, β was negative, but by model 5, β was positive and the *p*-value had been $>$ 0.05 (**Table 2**). It is not statistically significant, so the relationship between blood lead obtained in DBP and blood pressure is not reliable. In contrast, in SBP, fully adjusted β $>$ 0, *p* change little and both $<$0.05, so the Connection between blood lead obtained in SBP and blood pressure is reliable, and blood lead is positively correlated with blood pressure (β: 0.35, 95 Cl: 0.16–0.55).

Effect of HDL Concentration and Blood Lead on SBP

In model 1, we did not add any covariates to control confounding factors. At this time, HDL $\leq$ 49 (mg / dl) group, β = 0.94 and $p < 0.001$; HDL $>$ 49 (mg / dl) group, β = 2.95 and $p < 0.001$. In model 2, we adjusted three confounding factors: age, gender, and race, which belong to an important part of population characteristics. At this time, HDL $\leq$ 49 (mg / dl) group, β = 0.06 and $p = 0.661$; HDL $>$ 49 (mg / dl) group, β = 0.55 and $p = 0.002$ ($p < 0.05$). The results show that in HDL

TABLE 1 | Baseline characteristics of the study population.

Variables	Total (n = 16,451)	High density lipoprotein (mg/dl)		p
		HDL ≤ 49 (mg/dl) (n = 8,015)	HDL > 49 (mg/dl) (n = 8,436)	
Age, Median (IQR)	40.0 (30.0, 50.0)	40.0 (30.0, 50.0)	40.0 (29.0, 50.0)	0.359
Gender, *n* (%)				<0.001
Female	8,472 (51.5)	2,964 (37)	5,508 (65.3)	
Male	7,979 (48.5)	5,051 (63)	2,928 (34.7)	
Race, *n* (%)				<0.001
Mexican American	2,812 (17.1)	1,597 (19.9)	1,215 (14.4)	
Non—hispanic black	3,388 (20.6)	1,352 (16.9)	2,036 (24.1)	
Non—hispanic white	6,769 (41.1)	3,342 (41.7)	3,427 (40.6)	
Other hispanic	1,541 (9.4)	818 (10.2)	723 (8.6)	
Other race- including multi-racial	1,941 (11.8)	906 (11.3)	1,035 (12.3)	
Marital status, *n* (%)				<0.001
No	3,826 (23.3)	1,724 (21.5)	2,102 (24.9)	
Yes	12,625 (76.7)	6,291 (78.5)	6,334 (75.1)	
PIR, Mean ± SD	2.5 ± 1.7	2.4 ± 1.6	2.7 ± 1.7	<0.001
BMI, *n* (%)				<0.001
<25	4,975 (30.2)	1,420 (17.7)	3,555 (42.1)	
25–29.9	5,327 (32.4)	2,720 (33.9)	2,607 (30.9)	
≥30	6,149 (37.4)	3,875 (48.3)	2,274 (27)	
Educational level, *n* (%)				<0.001
Less than high school	3,548 (21.6)	1,961 (24.5)	1,587 (18.8)	
High school graduation	3,676 (22.3)	1,963 (24.5)	1,713 (20.3)	
College or above	9,227 (56.1)	4,091 (51)	5,136 (60.9)	
DM, *n* (%)				<0.001
No	13,445 (81.7)	6,146 (76.7)	7,299 (86.5)	
Yes	3,006 (18.3)	1,869 (23.3)	1,137 (13.5)	
Hypertension, *n* (%)				<0.001
No	11,993 (72.9)	5,545 (69.2)	6,448 (76.4)	
Yes	4,458 (27.1)	2,470 (30.8)	1,988 (23.6)	
Marital status, *n* (%)				<0.001
Never	9,451 (57.4)	4,311 (53.8)	5,140 (60.9)	
Former	2,967 (18.0)	1,500 (18.7)	1,467 (17.4)	
Now	4,033 (24.5)	2,204 (27.5)	1,829 (21.7)	
Work activity, *n* (%)				<0.001
Non-work activity	7,708 (46.9)	3,669 (45.8)	4,039 (47.9)	
Moderate work activity	3,224 (19.6)	1,558 (19.4)	1,666 (19.7)	
Vigorous work activity	3,348 (20.4)	1,862 (23.2)	1,486 (17.6)	
Unknown	2,171 (13.2)	926 (11.6)	1,245 (14.8)	
Alcohol, *n* (%)				0.388
Yes	3,113 (18.9)	1,524 (19)	1,589 (18.8)	
No	9,950 (60.5)	4,876 (60.8)	5,074 (60.1)	
Unknown	3,388 (20.6)	1,615 (20.1)	1,773 (21)	
CVD, *n* (%)				<0.001
No	15,697 (95.7)	7,560 (94.7)	8,137 (96.6)	
Yes	706 (4.3)	423 (5.3)	283 (3.4)	
Albumin/urine ug/ml., Median (IQR)	7.2 (3.9, 14.1)	7.9 (4.3, 15.4)	6.8 (3.5, 12.9)	<0.001
lead, Median (IQR)	1.0 (0.6, 1.6)	1.0 (0.7, 1.6)	1.0 (0.6, 1.6)	<0.001

≤49 (mg / dl) group, the effect value β has become unstable, with $P > 0.05$, which is not statistically significant. In model 3, we continued to adjust the remaining socio-economic demographic characteristics and BMI: marital status, BMI, PIR, education level at that time, HDL ≤ 49 (mg/dl) group, $\beta = 0.13$, $p = 0.347$; HDL > 49 (mg / dl) group, $\beta = 0.57$, $p = 0.001$ ($p < 0.05$). In the

TABLE 2 | Association between blood lead and blood pressure.

Models	*n*	DBP		SBP	
		β_95 CI	*P*_value	β_95 CI	*P*_value
Model1	16,451	0.8 (0.62–0.97)	<0.001	1.8 (1.57–2.02)	<0.001
Model2	16,451	−0.16 (−0.33–0.01)	0.066	0.25 (0.03–0.46)	0.026
Model3	16,451	0.01 (−0.17–0.18)	0.93	0.33 (0.12–0.55)	0.003
Model4	16,451	0.12 (−0.05–0.29)	0.157	0.37 (0.17–0.57)	<0.001
Model5	16,451	0.11 (−0.06–0.28)	0.186	0.35 (0.16–0.55)	<0.001

Model1: not adjusted.
Model2: adjusted for age, gender, and race.
Model3: model2+ Marital status, BMI, PIR, and education level.
Model4: model3+DM, Hypertension, smoking status, work activity, alcohol consumption, and CVD.
Model5: model4+ albumin/urine.
DBP, diastolic blood pressure; SBP, systolic blood pressure; PIR, poverty income ratio; BMI, body mass index; DM, diabetes mellitus.

TABLE 3 | Interactive effect of blood lead and HDL on SBP.

Models	HDL ≤ 49 (mg/dl) (*n* = 8,015)		HDL > 49 (mg/dl) (*n* = 8,436)		*p* for interaction
	β_95 CI	*P*_value	β_95 CI	*P*_value	
Model1	0.94 (0.65–1.23)	<0.001	2.95 (2.6–3.31)	<0.001	<0.001
Model2	0.06 (-0.22–0.34)	0.661	0.55 (0.2–0.89)	0.002	<0.001
Model3	0.13 (-0.14–0.41)	0.347	0.57 (0.23–0.92)	0.001	<0.001
Model4	0.23 (-0.02–0.49)	0.073	0.48 (0.16–0.8)	0.003	<0.001
Model5	0.21 (-0.05–0.46)	0.109	0.47 (0.15–0.79)	0.004	<0.001

Model1: not adjusted.
Model2: adjusted for age, gender, and race.
Model3: model2+marry status, BMI, PIR, and education level.
Model4: model3+DM, Hypertension, smoking status, work activity, alcohol consumption, and CVD.
Model5: model4+ albumin/urine.
HDL, high-density lipoprotein; DBP, diastolic blood pressure; SBP, systolic blood pressure; PIR, poverty income ratio; BMI, body mass index; DM, diabetes mellitus.

low HDL group, the *p*-value was still not statistically significant. In model 4 and model 5, we continued to adjust some diseases and risk or protective factors that may lead to vascular diseases and changes in blood pressure. The final result: HDL $\leq$ 49 (mg / dl) group, $\beta = 0.21$ and $p = 0.109$; HDL $>$ 49 (mg / dl) group, $\beta = 0.47$ and $p = 0.004$ ($p < 0.05$). In the high HDL group, the *p*-value of β remained stable, while in the low HDL group, most of the *P*-values of β are not statistically significant (**Table 3**).

It can be clearly seen that in low concentrations of HDL (HDL $\leq$ 49 mg/dl), β varied from negative to positive, with a large range of data, *p* was variable, and most of *p* was >0.05, not statistically significant. In high concentrations of HDL (HDL $>$ 49 mg/dl), the β values of the five models were positive, and the changes of *p* were small and all *P* was <0.05, which was statistically significant, indicating that when HDL was high, the relationship between blood lead and HDL on SBP was reliable and showed a positive correlation. In the fully adjusted model (Model 5), there was a significant interaction between high HDL concentration and blood lead and SBP (*P*-value of interaction likelihood ratio test < 0.05).

DISCUSSION

In our study, both blood lead and high HDL concentration were significantly correlated with SBP. After adjustment, the association remained significant. In NHANES, the sample size is large and the level can represent the general population. And the measurement of these data was carried out with strict quality control and strict protocol. Therefore, the relationship we obtained among blood lead, HDL, and blood pressure was applied to the whole population.

In some previous studies, a link has been found among lead exposure and subsequent hypertension (HTN) and cardiovascular disease. Lead can induce hypertension in rats, and lead acts on many parts of the cardiovascular system, possibly affecting blood pressure through the renin-angiotensin system (18). A large number of animal experiments showed that long-term exposure to low concentration lead can lead to arterial hypertension, but explain low long-term environmental lead exposure to the influence of high blood pressure, the exact mechanism is unclear, underlying mechanisms including increased oxidative stress, the stimulation of renin-angiotensin system, as well as cut and nitric oxide cyclase guanylic acid (19). In humans, lead toxicity has been associated with increased cardiovascular risk and may be associated with impaired antioxidant metabolism and oxidative stress, but the shape of the dose-response relationship still needs to be explored (20, 21).

Potential mechanisms of HDL action in humans include stimulation of reverse cholesterol transport, antioxidant and anti-inflammatory properties, reduction of endothelial dysfunction, anticoagulation, and prostacyclin half-life (22). HDL has classically been thought to have a protective effect on atherosclerosis because of an inverse relationship between HDL-C concentration and coronary artery disease: each 1 mg/dL increase in HDL-C reduces the risk of cardiovascular disease by 2–3% (23). However, new data cast doubt on this, for example, HDL-elevating drugs do not reduce CV risk (24). This is due to the fact that only 5% of HDL-C comes from macrophage cholesterol efflux, and HDL-C does not represent many important anti-atherosclerotic HDL properties (for example, anti-inflammatory or vascular relaxants) (10). Therefore, HDL-C may be an insensitive method to quantify the anti-atherosclerosis properties of HDL. This is why HDL function is considered more important than HDL-C levels (25). In fact, HDL function and cholesterol outflow predict CAD events while HDL-C does not (26), confirming the fact that HDL function (quality) is more important than HDL-C level (quantity).

S1P is a bioactive lysophospholipid that is derived from the ubiquitous membrane lipid sphingomyelin. Only S1P-bound HDL, which accounts for 60% of total plasma S1P,

has functional activity. Apolipoprotein (apo) M has been identified as a S1P-binding protein in HDL. S1P is the main substance responsible for the vasodilatory properties of HDL (27). The level of HDL-bound S1P in patients with CHD was lower than that in healthy volunteers, and the concentration of HDL-bound S1P was negatively correlated with the severity of CHD (28). The results of *in vitro* experiments explain the protective effect of S1P on atherosclerosis. S1P has the vasodilation effect of HIGH-DENSITY lipoprotein because S1P activates endothelial nitric oxide synthase, stimulates endothelial nitric oxide release, and induces vasodilation (27). S1P also shows endothelial protection because HDL-bound S1P enhances endothelial cell survival and migration (29).

Studies have shown that S1P improves HDL function (30). HDL-induced endothelial signaling is mediated by the S1P load because it is completely eliminated in the presence of S1P receptor antagonists and S1P neutralizing antibodies. Most importantly, S1P-loaded HDL particles significantly improved CAD-HDL function, as demonstrated by HDL-mediated signal transduction and enhanced HDL vasodilation *in vitro*.

However, some studies have found a relationship between blood lead and HDL. Women with MetS had higher HDL, and although blood lead in both groups was below the threshold range, blood lead in the experimental group (MetS group) was lower (31), the population had low blood lead and high HDL concentration. HDL concentrations were significantly higher in occupations with less lead exposure, suggesting that lead may block HDL production, but the initial dose is not clear, or the reduced effect of lead on HDL may be at higher doses (13). However, some researchers have found that with the increase of blood lead level, HDL also increases significantly, which may be related to age, gender, and sample size (32).

These correlations can demonstrate and explain the interaction effect of blood lead and HDL concentration on blood pressure to a certain extent. It makes sense that lead at low HDL-C levels can cause hypertension, but high HDL-C levels can reduce the hypertensive effect of lead through THE S1P carried by HDL particles. These studies are still very limited, but we can be a director of the research in the future, lead to high blood pressure of the threshold value has not been fully elucidated, with blood lead levels and high levels of HDL may lead to high blood pressure, the findings of the study helps to increase blood lead levels and high levels of HDL alert, and may have significance for clinical prevention and treatment. Of course, this aspect also needs more research to demonstrate.

This study still has limitations, which are reflected in the defects of the cross-sectional study itself, the deviation of the return visit, the accuracy of the questionnaire answer collection, confounding factors affecting the experimental data, possible errors in the measured samples, and even errors in the database. If there are different articles with different results, the results may be different due to different covariables considered or different database use, and different characteristics such as the age of the selected population, which will lead to different results. In addition, we did not assess HDL function, and we cannot prove whether lead causes HDL dysfunction; We did not measure S1P levels in HDL particles. It is easy to speculate that lead reduces S1P in HDL and thus reduces HDL function (33). There are several metals that can affect HDL and cause atherosclerosis, such as cadmium (34). Many patients have elevated levels of several metals at once, not just lead. Thus, HDL may be affected by lead, but it may also be affected by other metals such as cadmium.

CONCLUSION

In general, through this study, we found that blood lead and high HDL concentration together affect SBP. While we have provided some clinical clues, further research is needed to provide more evidence.

DATA AVAILABILITY STATEMENT

NHANES has developed a public use dataset, available at: https://www.cdc.gov/nchs/nhanes/index.htm. Users can download relevant data for free for research and publish relevant articles. Our study is based on open source data, so there are no ethical issues and other conflicts of interest.

ETHICS STATEMENT

The authors are responsible for all aspects of the work to ensure that issues related to the accuracy or completeness of any part of the work are properly investigated and resolved. This research was conducted following the Declaration of Helsinki (revised 2013).

AUTHOR CONTRIBUTIONS

BH and P-yH: conception and design. N-nZ, Z-mG, and J-tF: collection and assembly of data. J-lG: drafting the work or revising it critically for important intellectual content. C-qH: substantial contributions to the conception or design of the work. All authors: data analysis and interpretation, manuscript writing, and final approval of manuscript.

FUNDING

This study was supported by grant from Zhongnanshan Medical Foundation of Guangdong Province (ZNSA-2020013) and grant from Guangzhou Municipal Science and Technology Project (202102010168).

ACKNOWLEDGMENTS

We acknowledge the NHANES database for providing their platforms and contributors for uploading their meaningful datasets sincerely. We thank to ZY Lin's administrative support.

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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 Nutrition

ORIGINAL RESEARCH
published: 23 June 2022
doi: 10.3389/fnut.2022.919124

Impact of Eating Speed on Muscle Mass in Older Patients With Type 2 Diabetes: A Prospective Study of KAMOGAWA–DM Cohort

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

Received: 13 April 2022
Accepted: 30 May 2022
Published: 23 June 2022

Citation:
Kobayashi G, Hashimoto Y, Takahashi F, Kaji A, Sakai R, Okamura T, Okada H, Kitagawa N, Nakanishi N, Majima S, Osaka T, Senmaru T, Ushigome E, Asano M, Hamaguchi M, Yamazaki M and Fukui M (2022) Impact of Eating Speed on Muscle Mass in Older Patients With Type 2 Diabetes: A Prospective Study of KAMOGAWA–DM Cohort. Front. Nutr. 9:919124. doi: 10.3389/fnut.2022.919124

Background and Aims: Maintenance of muscle mass is important for sarcopenia prevention. However, the effect of eating speed, especially fast, normal, or slow speed, on muscle mass changes remains unclear. Therefore, the purpose of this prospective study was to investigate the effect of eating speed on muscle mass changes in patients with type 2 diabetes (T2DM).

Methods: This study included 284 patients with T2DM. Based on a self–reported questionnaire, participants were classified into three groups: fast–, normal–, and slow–speed eating. Muscle mass was assessed using a multifrequency impedance body composition analyzer, and skeletal muscle mass (SMI) decrease (kg/m²/year) was defined as [baseline SMI (kg/m²)–follow–up SMI (kg/m²)] ÷ follow–up duration (year). The rate of SMI decrease (%) was defined as [SMI decrease (kg/m²/year) ÷ baseline SMI (kg/m²)] × 100.

Results: The proportions of patients with fast–, normal–, and slow–speed eating were, respectively, 50.5%, 42.9%, and 6.6% among those aged <65 years and 40.4%, 38.3%, and 21.3% among those aged ≥65 years. In patients aged ≥65 years, the rate of SMI decrease in the normal (0.85 [95% confidence interval, CI: −0.66 to 2.35]) and slow (0.93 [95% CI −0.61 to 2.46]) speed eating groups was higher than that in the fast speed eating group (−1.08 [95% CI −2.52 to 0.36]). On the contrary, there was no difference in the rate of SMI decrease among the groups in patients aged <65 years. Compared with slow speed eating, the adjusted odds ratios of incident muscle loss [defined as rate of SMI decrease (%) ≥0.5%] due to fast– and normal–speed eating were 0.42 (95% CI 0.18 to 0.98) and 0.82 (95% CI 0.36 to 2.03), respectively.

Conclusion: Slow–speed eating is associated with a higher risk of muscle mass loss in older patients with T2DM.

Keywords: eating speed, diet, muscle mass, diabetes, sarcopenia, older patients

INTRODUCTION

With increase in the number of patients with type 2 diabetes mellitus (T2DM), the number of elderly patients with T2DM has also increased worldwide (1). In elderly patients with DM, insulin signals are reduced because of insulin resistance and reduced insulin secretion, leading to increased protein degradation and decreased protein synthesis, ultimately reducing muscle mass (2). Because muscle is a major organ that accounts for 20% of the body's total glucose metabolism (3); muscle mass is very important for patients with T2DM. Loss of muscle mass leads to sarcopenia, which is known to increase the risk of not only cardiovascular disease (4, 5) but also mortality (6–8). Moreover, the prevalence of sarcopenia is high in patients with T2DM (9, 10).

Sarcopenia can be prevented through modifying eating habits. Previous studies have shown that adequate macro– and micronutrients are important for maintaining muscle mass in patients with T2DM (11–13). Eating speed is also important because fast eating is associated with obesity (14), non–alcoholic fatty liver disease (NAFLD) (15), and T2DM (16); therefore, slow eating speed may be effective for lowering obesity risk. On the contrary, slow eating increases the risk of undernutrition (17). It is because slow speed eating increases anorexigenic gut hormones such as peptide YY and glucagon–like peptide−1 (GLP−1) (18, 19) and diet–induced thermogenesis (20, 21). Additionally, we recently conducted a cross–sectional study and reported that there is an association between slow eating speed and a risk of sarcopenia in elderly patients with T2DM (22). However, it is not clear which eating speed (fast, normal, or slow) is effective for maintaining muscle mass in patients with T2DM. Therefore, we conducted a prospective cohort study with an aim to clarify the effect of various eating speed and changes in muscle mass in older patients with T2DM.

MATERIALS AND METHODS

Study Participants

The KAMOGAWA–DM cohort study is an ongoing prospective cohort study that began in 2014 (23). This study aimed to clarify the natural clinical history of patients with T2DM. Patients also have chronic diseases other than diabetes such as hypertension and dyslipidemia. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg and/or taking anti–hypertensive drugs, and dyslipidemia was defined as low–density lipoprotein cholesterol ≥140 mg/dL, triglycerides ≥150 mg/dL and/or taking anti–dyslipidemia drug. This study enrolled diabetes specialty outpatients of university hospital (Kyoto Prefectural University of Medicine (KPUM) Hospital, Kyoto, Japan) and city hospital (Kameoka Municipal Hospital, Kameoka, Japan) with written informed consent. This study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of KPUM (No. RBMR–E−466–5). Patients who correctly answered the Brief–Type Self Diet History Questionnaire (BDHQ) between January 2016 and April 2018 were extracted to this study. Patients who had the following criteria were excluded: no T2DM, missing data of eating speed or body impedance analyzer (BIA) parameters, extremely high or low energy intake (>4000 or <600 kcal/day) because of unreliability (24), follow–up duration of <6 months, steroid usage, and no follow–up.

Data Collection

We used a standardized questionnaire to collect data on the duration of T2DM, exercise, and smoking. We defined exercisers as those who performed physical activities at least once a week regularly, and we divided the participants into exercisers and non–exercisers. The questionaries about smoking consisted of a question " do you smoke?" and the 2 choices, "yes" or "no". Then, we classified "yes" and "no" as "smoker", and "non–smoker". Medication data, including information on steroids, insulin and sodium glucose cotransporter−2 (SGLT2) inhibitors, were collected from the medical records.

Samples of venous blood were collected from participants after overnight fasting, and levels of fasting plasma glucose (FPG) and hemoglobin A1c (HbA1c) were measured from these blood samples.

Body Composition Measurement

Evaluation of the body composition of patients was performed by a multifrequency BIA (InBody 720; InBody Japan, Tokyo, Japan), which was reported to correlate well with the results of dual–energy X–ray absorptiometry (DEXA) (25). Body weight (kg) and appendicular muscle mass (kg) were measured, and the body mass index (BMI, kg/m^2) and skeletal muscle mass index (SMI, kg/m^2) were calculated, which were defined, respectively, as body weight (kg) ÷ height–squared (m^2) and appendicular muscle mass (kg) ÷ height–squared (m^2). Next, ideal body weight (IBW) was estimated using the following formula: 22 × height–squared (m^2) (26). Furthermore, definition of SMI decrease (kg/m^2/year), the rate of SMI decrease (%) were follows; [baseline SMI (kg/m^2)–follow–up SMI (kg/m^2)] ÷ follow–up duration (year) and [SMI decrease (kg/m^2/year) ÷ baseline SMI (kg/m^2)] ×100. Loss of muscle mass was defined as the rate of SMI decrease (%) ≥0.5%, because it was reported that muscle mass decreases with aging; in particular, the average rate of loss in aged people was 0.5%−1% per year (27).

Assessment of Habitual Food, Nutrient Intake, and Eating Speed

Habitual food intake, nutrient intake, and eating speed were assessed using the BDHQ, which estimated the dietary intake of 58 food and beverage items during the past month. BDHQ is a dietary intake questionnaire for Japanese and has been validated. Previous studies showed that the correlation between BDHQ and semi–weighed dietary records was $r = 0.44$ to 0.56 (28–31). From the BDHQ, we gathered data on total energy intake (kcal/day), protein intake (g/day), fat intake (g/day), and carbohydrate intake (g/day). Total energy intake (kcal/IBW/day) was calculated as total energy intake (kcal/day) ÷ IBW. Protein intake (% energy), fat intake (% energy), and carbohydrate intake (% energy) were calculated as [protein intake (g/day) ×4 (kcal/g) ÷ total energy intake (kcal/day)] ×100, [fat intake (g/day) ×9 (kcal/g) ÷ total energy intake (kcal/day)] ×100, and

[carbohydrate intake (g/day) ×4 (kcal/g) ÷ total energy intake (kcal/day)] ×100, respectively. Alcohol intake was also estimated, and habitual alcohol intake was defined as alcohol intake of >30 g/day for men and >20 g/day for women (32).

The questionaries about eating speed consisted of a question "how fast do you eat?" and the 5 choices, "very fast" "a little fast" "normal" "a little slow" or "very slow". Then, "very fast", or "a little fast", "normal", and "a little slow" or "very slow" were classified as "fast speed eating", "normal speed eating", and "slow speed eating"(15).

Statistical Analysis

Continuous variables are presented as mean (standard deviation [SD]), and categorical variables are presented as number (%). *P*–value of <0.05 was considered to indicate statistical significance. The between–group differences were evaluated using one–way analysis of variance and the Tukey–Kramer test or chi–square test. We analyzed patients aged <65 years and patients aged ≥65 years separately because the recommended dietary energy intake varied by age (33).

Multiple regression analyses were performed to calculate the odds ratio (OR) and 95% confidence interval (CI) and assess the effects of eating speed on SMI decrease (kg/m²/year), rate of SMI decrease (%), and logistic regression analyses was also carried out to calculate the OR and 95% CI for eating speed on incident muscle mass loss. Factors of age, sex, HbA1c (%), insulin usage, SGLT2 inhibitor use, smoking, exercise, alcohol consumption, total energy intake (kcal/IBW/day), protein intake (% energy), and BMI, were considered to be independent variables.

In addition, we used the other cutoff levels for loss of muscle mass: SMI decrease of ≥1.2% (34) and 2.0% (35).

All statistical analyses were performed using JMP ver13.2 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

A total of 523 patients participated in the study; however, 239 participants were excluded for various reasons and 284 participants were finally included in the study (**Figure 1**).

The participants' characteristics are shown in **Table 1**. In patients aged <65 years, mean BMI and SMI were 26.6 kg/m² and 7.3 kg/m², respectively. Furthermore, the percentages of patients with fast, normal, and slow eating were 50.5%, 42.9%, and 6.6%, respectively. In patients aged ≥65 years, mean BMI and SMI were 23.8 kg/m² and 6.9 kg/m², respectively, and the proportions of patients with fast, normal, and slow speed eating were 40.4%, 38.3%, and 21.3%.

In patients aged <65 years, the follow–up duration of the slow–speed eating group was significantly longer than that of the fast– and normal–speed eating groups ($p < 0.05$), and there were no significant differences in total energy intake and protein intake among the groups. In patients aged ≥65 years, the SMI decrease and the rate of SMI decrease in the slow– and normal–speed eating groups were lower than those in the fast–speed eating group, with significant differences ($p < 0.05$) (**Table 1**).

The relationship between eating speed and SMI decrease (kg/m²/year) or rate of SMI decrease (%) adjusted for age, sex, smoking, exercise, alcohol, use of insulin and use of SGLT2 inhibitors is shown in **Table 2**. In patients aged <65 years, there were no statistically significant differences in SMI decrease or rate of SMI decrease among the groups. On the other hand, in patients aged ≥65 years, SMI decrease of fast eating groups (−0.006 [95% CI −0,014 to 0.003] and rate of SMI decrease of fast eating groups (−1.08 [95% CI −2.52 to 0.36]) were lower than those of slow speed eating after adjusting for covariates, and there were statistically significant differences.

The relationship between eating speed and incident muscle mass loss is presented in **Table 3** and **Figure 2**. In patients aged

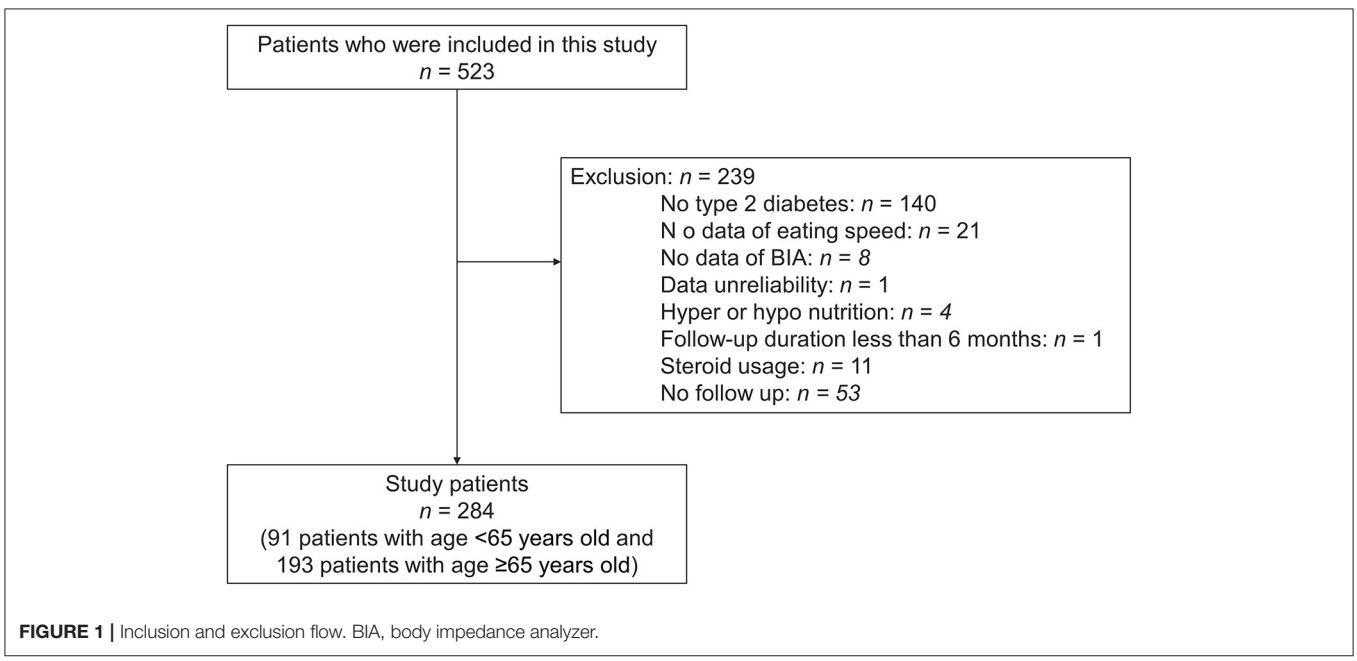

FIGURE 1 | Inclusion and exclusion flow. BIA, body impedance analyzer.

TABLE 1 | Clinical characteristics of study participants.

Age <65 years old	**All** ***n*** **= 91**	**Fast,** ***n*** **= 46**	**Normal,** ***n*** **= 39**	**Slow,** ***n*** **= 6**	***p***
Age, years	54.0 (8.7)	53.4 (10.0)	54.4 (7.4)	55.3 (6.3)	0.806
Men, *n* (%)	44 (48.4)	23 (50.0)	19 (48.7)	2 (33.3)	0.743
Duration of diabetes, years	9.2 (6.8)	9.8 (7.0)	8.7 (6.7)	7.3 (6.6)	0.615
Family history of diabetes, *n* (%)	52 (57.1)	25 (54.4)	22 (56.4)	5 (83.3)	0.399
Body mass index, kg/m^2	26.6 (5.3)	27.4 (5.7)	26.1 (5.0)	23.6 (3.3)	0.192
Appendicular muscle mass, kg	19.5 (4.4)	19.8 (4.4)	19.4 (3.8)	18.5 (7.4)	0.776
Skeletal muscle mass index, kg/m^2	7.3 (1.0)	7.4 (1.0)	7.3 (0.9)	6.8 (1.3)	0.375
Insulin, *n* (%)	18 (19.8)	8 (17.4)	8 (20.5)	2 (33.3)	0.646
SGLT2 inhibitor, *n* (%)	26 (28.6)	14 (30.4)	11 (28.2)	1 (16.7)	0.780
Hypertension, *n* (%)	57 (62.6)	31 (67.4)	23 (59.0)	3 (50)	0.584
Dyslipidemia, *n* (%)	63 (69.2)	36 (78.3)	23 (59.0)	4 (66.7)	0.157
Smoker, *n* (%)	17 (18.7)	7 (15.2)	9 (23.1)	1 (16.7)	0.646
Exerciser, *n* (%)	35 (38.5)	16 (34.8)	17 (43.6)	2 (33.3)	0.683
Alcohol intake, *n* (%)	12 (13.2)	6 (13.0)	5 (12.8)	1 (16.7)	0.966
HbA1c, mmol/mol	61.4 (19.1)	60.9 (14.8)	63.0 (23.9)	54.6 (12.9)	0.600
HbA1c, %	7.8 (1.7)	7.7 (1.4)	7.9 (2.2)	7.2 (1.2)	0.600
Plasma glucose, mmol/L	8.6 (2.9)	8.4 (2.4)	9.1 (3.5)	7.5 (2.4)	0.302
Total energy intake, kcal/day	1761 (571)	1810 (6584)	1726 (576)	1608 (450)	0.637
Total energy intake, kcal/kg IBW/day	30.4 (9.5)	31.2 (9.8)	29.8 (9.4)	28.2 (10.0)	0.691
Protein intake, g/ day	67.3 (20.8)	69.1 (22.3)	66.2 (19.6)	61.4 (17.6)	0.632
Protein intake, % Energy	15.6 (2.9)	15.5 (2.9)	15.7 (3.0)	15.4 (1.4)	0.935
Fat intake, g/ day	54.7 (18.8)	57.1 (20.0)	53.3 (17.9)	45.1 (12.3)	0.290
Fat intake, % Energy	28.2 (5.9)	28.5 (5.8)	28.3 (6.2)	25.7 (4.2)	0.554
Carbohydrate intake, g/ day	226.6 (83.1)	235.8 (95.2)	217.3 (68.2)	215.6 (75.1)	0.565
Carbohydrate intake, % Energy	51.6 (8.2)	51.9 (8.2)	50.9 (8.1)	53.4 (8.6)	0.733
SMI decrease, kg/m^2/year	0.004 (0.023)	0.005 (0.022)	0.005 (0.023)	−0.008 (0.024)	0.385
Rate of SMI decrease, %	0.60 (3.72)	0.81 (3.38)	0.69 (3.96)	−1.66 (4.55)	0.306
Follow up duration, years	1.6 (0.6)	1.5 (0.5)	1.6 (0.5)	2.3 (0.9)†‡	0.008
Age ≥65 years old	**All** ***n*** **= 193**	**Fast,** ***n*** **= 78**	**Normal,** ***n*** **= 74**	**Slow,** ***n*** **= 41**	***p***
Age, years	72.2 (5.2)	72.1 (5.2)	71.4 (5.0)	74.0 (5.2)	0.037
Men, *n* (%)	109 (56.5)	45 (57.7)	36 (48.7)	28 (68.3)	0.121
Duration of diabetes, years	15.9 (10.0)	16.5 (10.3)	15.0 (10.4)	16.4 (8.8)	0.623
Family history of diabetes, *n* (%)	81 (42.0)	37 (47.4)	24 (32.4)	20 (48.8)	0.105
Body mass index, kg/m^2	23.8 (3.9)	24.1 (3.4)	24.2 (4.4)	22.5 (3.6)	0.046
Appendicular muscle mass, kg	17.7 (3.9)	18.1 (3.9)	17.3 (3.9)	17.8 (3.6)	0.375
Skeletal muscle mass index, kg/m^2	6.9 (1.0)	6.9 (1.0)	6.8 (1.0)	6.8 (0.9)	0.679
Insulin, *n* (%)	51 (26.4)	23 (29.5)	17 (23.0)	11 (26.8)	0.659
SGLT2 inhibitor, *n* (%)	25 (13.0)	13 (16.7)	7 (9.5)	5 (12.2)	0.412
Hypertension, *n* (%)	137 (71.0)	55 (70.5)	53 (71.6)	29 (70.7)	0.988
Dyslipidemia, *n* (%)	134 (69.4)	51 (65.4)	53 (71.6)	30 (73.2)	0.595
Smoker, *n* (%)	27 (14.0)	14 (18.0)	7 (9.5)	6 (14.6)	0.318
Exerciser, *n* (%)	109 (56.5)	48 (61.5)	38 (51.4)	23 (56.1)	0.448
Alcohol intake, *n* (%)	19 (9.8)	4 (5.1)	6 (8.1)	9 (22.0)	0.011
HbA1c, mmol/mol	55.3 (10.8)	55.5 (11.3)	55.0 (11.2)	55.2 (9.0)	0.953
HbA1c, %	7.2 (1.0)	7.2 (1.0)	7.2 (1.0)	7.2 (0.8)	0.953
Plasma glucose, mmol/L	8.2 (2.9)	8.3 (3.5)	8.0 (2.1)	8.6 (2.8)	0.585
Total energy intake, kcal/day	1748 (602)	1799 (672)	1719 (602)	1702 (447)	0.619
Total energy intake, kcal/kg IBW/day	30.9 (10.1)	31.3 (10.9)	31.1 (10.4)	29.8 (8.0)	0.699
Protein intake, g/ day	75.6 (30.5)	75.2 (29.8)	78.2 (34.0)	71.8 (25.0)	0.549

(Continued)

TABLE 1 | Continued

Age <65 years old	All $n = 91$	Fast, $n = 46$	Normal, $n = 39$	Slow, $n = 6$	*p*
Protein intake, % Energy	17.4 (3.6)	17.0 (3.4)	18.1 (3.9)	16.7 (3.3)	0.076
Fat intake, g/ day	55.9 (22.2)	55.5 (19.5)	57.5 (26.3)	53.6 (19.1)	0.664
Fat intake, % Energy	29.0 (6.5)	28.6 (6.5)	29.8 (6.5)	28.1 (6.4)	0.359
Carbohydrate intake, g/ day	217.7 (82.9)	234.2 (103.5)	206.4 (65.6)	206.6 (60.4)	0.075
Carbohydrate intake, % Energy	50.1 (9.1)	51.4 (8.7)	49.0 (9.2)	49.5 (9.6)	0.246
SMI decrease, kg/m^2/year	0.005 (0.021)	−0.001 (0.020)	0.010 (0.023)†	0.009 (0.017)†	0.003
Rate of SMI decrease, %	0.88 (3.75)	−0.24 (3.79)	1.69 (3.94)†	1.55 (2.73)†	0.003
Follow up duration, years	1.7 (0.7)	1.8 (0.7)	1.7 (0.7)	1.8 (0.6)	0.410

Data was expressed as mean (standard deviation) or number (%). The difference between group was evaluated by one–way analysis of variance and Tukey–Kramer test, or chi–squared test. SMI, skeletal muscle mass; SGLT, sodium–glucose cotransporter; IBW, ideal body weight. $^{\dagger}p < 0.05$ *vs. fast speed eating and* ‡, $p < 0.05$ *vs. normal speed eating by Tukey–Kramer test.*

TABLE 2 | Relationship between eating speed and SMI decrease (kg/m^2/year) or rate of SMI decrease (%).

Age <65 years old	SMI decrease (kg/m^2/year)	Rate of SMI decrease (%)
Fast	0.005 (−0.005–0.015)	0.67 (−0.96–2.31)
Normal	0.002 (−0.007–0.012)	0.58 (−1.34–1.87)
Slow	−0.009 (−0.027–0.009)	−1.84 (−4.85–1.17)
Age ≥65 years old	**SMI decrease (kg/m^2/year)**	**Rate of SMI decrease (%)**
Fast	−0.006 (−0.014–0.003)	−1.08 (−2.52–0.36)
Normal	0.005 (−0.003–0.014)†	0.85 (−0.66–2.35)†
Slow	0.006 (−0.003–0.014)†	0.93 (−0.61–2.46)†

Values for outcome variables are geometric means and 95% CI. SMI, Skeletal muscle mass index. Adjusted for age, sex, smoking, exercise, alcohol, insulin, sodium–glucose cotransporter inhibitor, total energy intake (kcal/kg ideal body weight/day), and protein intake (% Energy). $^{\dagger}p < 0.05$ *vs. fast speed eating by Tukey–Kramer test.*

<65 years, alcohol intake (odds ratio 0.11, [95% CI 0.02 to 0.67], $p = 0.016$), total energy intake (odds ratio 0.90, [95% CI 0.85 to 0.96], $p = 0.002$), and protein intake (odds ratio 0.77, [95% CI 0.62 to 0.96], $p = 0.019$) were considered to have a lower risk of incident muscle mass loss but not in patients aged ≥65 years. Furthermore, the adjusted odds ratios of the incident muscle loss of fast speed eating were 0.42 (95% CI 0.18 to 0.98, $p = 0.044$) compared with slow speed eating, in patients aged ≥65 years.

In addition, the analysis results using different cutoff levels for muscle mass loss for the rates of SMI decrease of ≥1.2% and ≥2.0% in patients aged ≥65 years are shown in **Supplementary Table 1** and **Figure 2**. The risk of the incident muscle mass loss due to fast speed eating (cutoff point: rate of SMI decrease of ≥1.2%, odds ratio 0.38 [95% CI 0.16 to 0.88], $p = 0.024$, and cutoff point: rate of SMI decrease of ≥2.0%, odds ratio 0.32 [95% CI 0.13 to 0.81], $p = 0.015$) was lower than that due to slow speed eating.

DISCUSSION

This prospective study showed that slow–speed eating had the potential to promote SMI decrease and incident muscle mass loss in patients aged ≥65 years with T2DM. Furthermore, alcohol consumption, protein intake and total energy intake were considered low risk factors for muscle mass loss in patients aged <65 years with T2DM.

In general, muscle mass decreases with age (27), and adequate energy and protein intake are important for maintaining muscle mass (12, 13), which aids in sarcopenia prevention. Furthermore, lack of energy leads to a decrease in muscle mass, especially in elderly patients with T2DM (12). In this study, there was a significant difference in muscle mass loss depending on eating speed in patients aged ≥65 years with T2DM, even after adjusting for total energy intake and total protein intake.

Insulin stimulates protein synthesis, and defects in insulin signaling lead to decreased muscle mass (27). By contrast, fast–speed eating can cause elevation in the levels of postprandial blood glucose and FPG (18), which leads to increased insulin secretion and stimulation of protein synthesis. Additionally, slower eating speed and longer chewing time increase diet–induced thermogenesis (20, 21). This can lead to loss of muscle mass while preventing obesity. Furthermore, eating slowly increases anorexigenic gut hormones such as peptide YY and GLP−1 (18, 19). GLP−1 is a hormone that not only decreases appetite and body weight (19) but also maintains muscle mass (36). The reasons for this are thought to be GLP−1's various

TABLE 3 | Relationship between eating speed and the incident muscle mass loss.

Age <65 years old	Model 1		Model 2		Model 3	
	Odds ratio (95% CI)	*p*–value	Odds ratio (95% CI)	*p*–value	Odds ratio (95% CI)	*p*–value
Age (year)	—	—	0.96 (0.90–1.02)	0.204	0.97 (0.90–1.03)	0.310
Men	—	—	0.69 (0.23–2.06)	0.502	0.78 (0.25–2.42)	0.671
HbA1c (%)	—	—	0.70 (0.50–0.98)	0.038	0.68 (0.47–0.97)	0.032
Insulin usage (yes)	—	—	1.14 (0.31–4.20)	0.846	1.26 (0.33–4.79)	0.737
SGLT2 inhibitor (yes)	—	—	1.69 (0.54–5.31)	0.368	1.62 (0.51–5.15)	0.415
Smoking (yes)	—	—	1.54 (0.41–5.87)	0.523	1.47 (0.39–5.61)	0.570
Exercise (yes)	—	—	1.03 (0.35–3.02)	0.960	0.97 (0.33–2.87)	0.950
Alcohol (yes)	—	—	0.13 (0.02–0.75)	0.022	0.11 (0.02–0.67)	0.016
Total energy intake (kcal/kg IBW/day)	—	—	0.91 (0.86–0.97)	<0.001	0.90 (0.85–0.96)	0.002
Protein intake (% Energy)	—	—	0.78 (0.63–0.96)	0.015	0.77 (0.62–0.96)	0.019
Body mass index (kg/m^2)	—	—	—	—	1.06 (0.95–1.19)	0.265
Eating speed						
Fast	1.00 (0.18–5.48)	1.000	1.53 (0.20–11.6)	0.680	1.31 (0.17–10.0)	0.796
Normal	1.29 (0.23–7.23)	0.769	2.17 (0.27–17.5)	0.468	1.95 (0.24–15.6)	0.529
Slow	Reference	—	Reference	—	Reference	—
Age ≥65 years old	**Model 1**		**Model 2**		**Model 3**	
	Odds ratio (95% CI)	***p*–value**	**Odds ratio (95% CI)**	***p*–value**	**Odds ratio (95% CI)**	***p*–value**
Age (year)	—	—	0.95 (0.89–1.01)	0.122	0.95 (0.89–1.01)	0.098
Men	—	—	1.39 (0.70–2.75)	0.337	1.40 (0.71–2.77)	0.328
HbA1c (%)	—	—	1.30 (0.93–1.82)	0.122	1.29 (0.92–1.80)	0.140
Insulin usage (yes)	—	—	1.26 (0.60–2.64)	0.535	1.26 (0.60–2.63)	0.539
SGLT2 inhibitor (yes)	—	—	1.55 (0.60–4.01)	0.369	1.64 (0.62–4.35)	0.318
Smoking (yes)	—	—	0.52 (0.20–1.33)	0.174	0.51 (0.20–1.32)	0.166
Exercise (yes)	—	—	0.96 (0.52–1.77)	0.887	0.95 (0.51–1.76)	0.873
Alcohol (yes)	—	—	0.87 (0.28–2.66)	0.806	0.85 (0.28–2.63)	0.784
Total energy intake (kcal/kg IBW/day)	—	—	0.98 (0.95–1.01)	0.289	0.98 (0.95–1.01)	0.270
Protein intake (% Energy)	—	—	0.97 (0.88–1.06)	0.447	0.97 (0.88–1.06)	0.439
Body mass index (kg/m^2)	—	—	—	—	0.97 (0.89–1.06)	0.555
Eating speed						
Fast	0.44 (0.20–0.97)	0.043	0.40 (0.17–0.93)	0.034	0.42 (0.18–0.98)	0.044
Normal	0.85 (0.38–1.89)	0.694	0.82 (0.35–1.95)	0.656	0.82 (0.36–2.03)	0.715
Slow	Reference	—	Reference	—	Reference	—

Incident muscle mass loss was defined as the rate of skeletal muscle mass index decrease (%) ≥0.5%. SGLT, Sodium–glucose cotransporter, IBW; IBW, ideal body weight.

effects: increasing glucose sensitivity, neogenesis, proliferation, hypertrophy, and transcription of pro–insulin, while reducing apoptosis of beta cells (37–40). Therefore, slow eating speed might have a greater impact on decreasing appetite than on increasing muscle mass. For these reasons, although there were no differences in total energy and protein intakes, muscle mass of patients aged ≥65 years with T2DM decreased in the slow–speed eating group.

In addition, there is a relationship between sarcopenia and swallowing function in elderly people (22), and decreased swallowing function affects the eating speed. Therefore, it is possible that eating speed was slower in patients with reduced muscle mass.

However, this study showed that eating speed did not affect muscle mass loss in patients aged <65 years old. Younger people maintain insulin secretion (41) and anabolic effects (42); therefore, it is possible that muscle mass did not decrease.

The present study also reported that slow–speed eating was related to poor nutrition (17). By contrast, fast eating speed is related to obesity, T2DM, and NAFLD (14–16). The reasons for this may be as follows: slow eating speed leads to lower energy intake (43), but fast eating speed is related to increased energy intake (14, 43, 44). Furthermore, fast speed eating is associated with obesity, reducing energy consumption after meals, phosphorylation of Akt because of postprandial hyperglycemia and hyperinsulinemia (16). However, in this study, there was no significant difference between eating speed, total energy intake, and protein intake, regardless of age. Previous reports have shown that obese patients with T2DM under–report

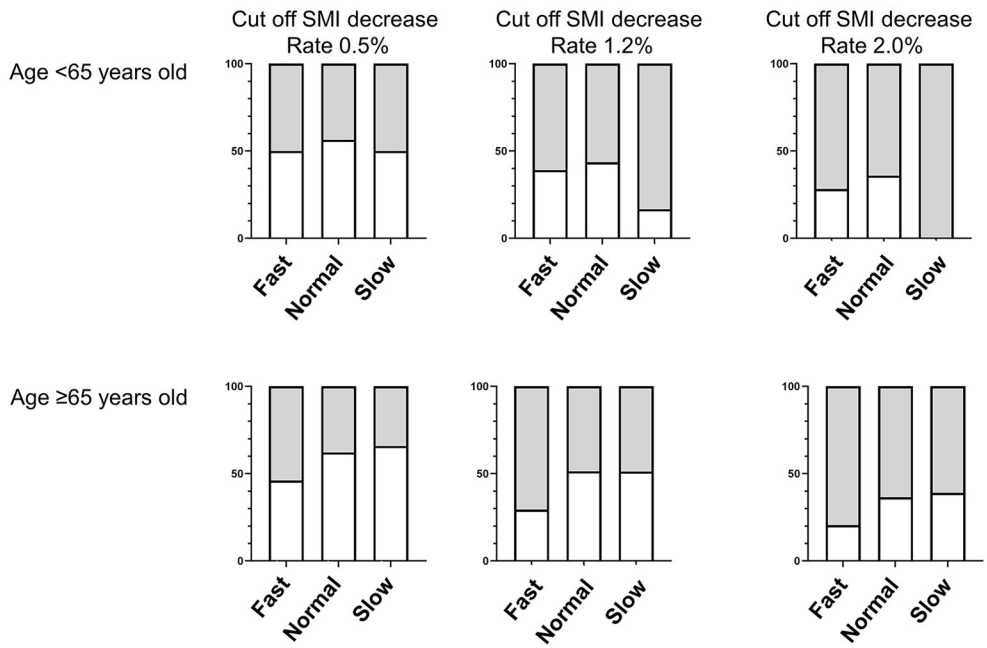

FIGURE 2 | Proportions of muscle mass loss among fast, normal, and slow speed eating by age (Cut–off levels of the rate of SMI decrease Rate 0.5%, 1.2% and 2.0%). Gray square represents the proportion of non–sarcopenia and white square represents the proportion of sarcopenia.

their food intake (45, 46), and this might have had an effect on total energy and protein intakes.

Alcohol consumption causes an increase in cortisol (47) levels and a decrease in mTOR signaling (48, 49), which leads to muscle mass loss (50). By contrast, alcohol intake may be associated with a lower incidence of frailty (51). Modest alcohol consumption leads to higher serum concentrations of dehydroepiandrosterone (DHEA) and testosterone (52, 53). These hormones increase loss of muscle mass (54, 55). In patients aged <65 years, alcohol intake may increase DHEA and testosterone levels and prevent muscle mass loss.

As the number of elderly patients with T2DM increases, the prevalence of sarcopenia also increases. This leads to increased mortality, which is a serious health problem. This study showed that slow–speed eating was associated with muscle mass loss. Therefore, it is important to consider eating speed in elderly patients with T2DM.

This study had some limitations. First, a self–reported questionnaire was used for assessment of eating speed and it was not measured directly. In addition, no clear definition of slow speed eating was showed by using seconds or minutes per specific volume/food type. Thus, there is a possibility that eating speed might have differed from the actual speed of eating. Second, body composition analysis was performed by a BIA for evaluating skeletal muscle mass, although the gold standard method was DEXA. A previous study has reported that BIA underestimated lean mass and overestimates fat mass, compared to DEXA (22). Third, we defined exercises from self–reported questionaries and have not evaluated the actual physical activity by objective criteria such as metabolic equivalents (METs). Fourth, we collected questionnaires only one time at the first time and did not re–evaluate. Lastly, this study included only Japanese patients with T2D; hence, generalization of the results to other groups is still unknown.

CONCLUSION

Slow–speed eating was associated with muscle mass loss in older patients with T2DM; therefore, close attention needed to the eating speed when treating older patients with T2DM.

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 the Ethics Committee of KPUM (No. RBMR–E−466–5). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GK interpreted the data and wrote the manuscript. YH conceived and designed the study, acquired, analyzes, interpreted the data, and contributed discussion. FT acquired and interpreted the data and contributed discussion. AK, MH, and RS conceived and designed the work, acquired the data, and contributed discussion.

TO, NK, HO, NN, SM, TS, EU, MY, and MA acquired the data and contributed discussion. MF conceived and designed the work, acquired and interpreted the data, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.919124/full#supplementary-material

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Conflict of Interest: YH has received honoraria from Kowa Co., Ltd., Sanofi K.K., Takeda Pharmaceutical Co., Ltd., Terumo Corporation, and Chugai Pharmaceutical Co., Ltd., outside the submitted work. HO received grant support from the Japan Society for the Promotion of Science and received personal fees from MSD K.K., Mitsubishi Tanabe Pharma Corporation, Sumitomo Dainippon Pharma Co., Ltd., Novo Nordisk Pharma Ltd., Daiichi Sankyo Co., Ltd., Eli Lilly Japan K.K., Kyowa Hakko Kirin Company Ltd., Kissei Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Kowa Pharmaceutical Co., Ltd., Ono Pharmaceutical Co., Ltd., and Sanofi K.K., outside the submitted work. NN received grant support from Japan Society for the Promotion of Science, the Japan Food Chemical Research Foundation, and received personal fees from Kowa Pharmaceutical Co., Ltd., and Novo Nordisk Pharma Ltd., outside the submitted work. TO has received honoraria from MSD K.K., Sumitomo Dainippon Pharma Co., Ltd., Novo Nordisk Pharma Ltd., DAIICHI SANKYO COMPANY, LIMITED, Eli Lilly Japan K.K., T Takeda Pharmaceutical Co., Ltd., Nippon Boehringer Ingelheim Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Kyowa Kirin Co., Ltd., Kowa Co., Ltd., ONO PHARMACEUTICAL Co., Ltd., TOA EIYO Corporation, and AstraZeneca K.K., grant from Combi Corporation, outside the submitted work. TS has received personal fees from Eli Lilly Japan K.K., Mitsubishi Tanabe Pharma Co., Kowa Pharma Co., Ltd., Astellas Pharma Inc., Takeda Pharma Co., Ltd., Sanofi K.K., Taisho Toyama Pharma Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Kissei Pharma Co., Ltd., MSD K.K., Novo Nordisk Pharma Ltd., Ono Pharma Co., Ltd., outside the submitted work. EU received grant support from the Japanese Study Group for Physiology and Management of Blood Pressure, the Astellas Foundation for Research on Metabolic Disorders, the Japan Society for the Promotion of Science, Mishima Kaiun Memorial Foundation, and received personal fees from Nippon Boehringer Ingelheim Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Daiichi Sankyo Co., Ltd., Takeda Pharmaceutical Co., Ltd., MSD K.K., Kyowa Hakko Kirin Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Kowa Pharmaceutical Co., Ltd., Novo Nordisk Pharma Ltd., Ono Pharmaceutical Co., Ltd., Taisho Pharmaceutical Co., Ltd., AstraZeneca K.K., and Sanofi K.K., outside the submitted work. Donated Fund Laboratory of Diabetes therapeutics is an endowment department, supported with an unrestricted grant from Ono Pharmaceutical Co., Ltd., Taiyo Kagaku Co., Ltd., and Taisho Pharmaceutical Co., Ltd., outside the submitted work. MA received personal fees from Takeda Pharmaceutical Co., Ltd., Abbott Japan Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Kowa Pharmaceutical Co., Ltd., Novo Nordisk Pharma Ltd., Ono Pharmaceutical Co., Ltd., AstraZeneca K.K., and Chugai Pharmaceutical Co., Ltd., outside the submitted work. MH received grants from AstraZeneca K.K., Ono Pharma Co., Ltd., Oishi Kenko inc., Yamada Bee Farm, Nippon Boehringer Ingelheim Co., Ltd., and received personal fees from AstraZeneca K.K., Ono Pharma Co., Ltd., Eli Lilly, Japan, Sumitomo Dainippon Pharma Co., Ltd., Daiichi Sankyo Co., Ltd., Mitsubishi Tanabe Pharma Corp., Sanofi K.K., and Kowa Pharma Co., Ltd., outside the submitted work. MY received personal fees from MSD K.K., Sumitomo Dainippon Pharma Co., Ltd., Kowa Co., Ltd., AstraZeneca PLC, Takeda Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Daiichi Sankyo Co., Ltd., Kowa Pharmaceutical Co., Ltd., and Ono Pharmaceutical Co., Ltd., outside the

submitted work. MF received grants from Ono Pharma Co., Ltd., Oishi Kenko inc., Yamada Bee Farm, Nippon Boehringer Ingelheim Co., Ltd., Kissei Pharma Co., Ltd., Mitsubishi Tanabe Pharma Corp., Daiichi Sankyo Co., Ltd., Sanofi K.K., Takeda Pharma Co., Ltd., Astellas Pharma Inc., MSD K.K., Kyowa Kirin Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Kowa Pharma Co., Ltd., Novo Nordisk Pharma Ltd., Sanwa Kagagu Kenkyusho Co., Ltd., Eli Lilly, Japan, K.K., Taisho Pharma Co., Ltd., Terumo Corp., Tejin Pharma Ltd., Nippon Chemiphar Co., Ltd., Abbott Japan Co., Ltd., and Johnson and Johnson K.K. Medical Co., TERUMO CORPORATION, and received personal fees from Nippon Boehringer Ingelheim Co., Ltd., Kissei Pharma Co., Ltd., Mitsubishi Tanabe Pharma Corp., Daiichi Sankyo Co., Ltd., Sanofi K.K., Takeda Pharma Co., Ltd., Astellas Pharma Inc., MSD K.K., Kyowa Kirin Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Kowa Pharma Co., Ltd., Novo Nordisk Pharma Ltd., Ono Pharma Co., Ltd., Sanwa Kagaku Kenkyusho Co., Ltd., Eli Lilly Japan K.K., Taisho Pharma Co., Ltd., Bayer Yakuhin, Ltd., AstraZeneca K.K., Mochida Pharma Co., Ltd., Abbott Japan Co., Ltd., Teijin Pharma Ltd., Arkray Inc., Medtronic Japan Co., Ltd., and Nipro Corp., TERUMO CORPORATION, outside the submitted work. These sponsors were not involved in the study design, collection, analysis, or interpretation of data, in the writing of this manuscript, or in the decision to submit the article for publication.

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

frontiers | Frontiers in Nutrition

ORIGINAL RESEARCH
published: 23 June 2022
doi: 10.3389/fnut.2022.892719

Linking Mitochondrial Function to Insulin Resistance: Focusing on Comparing the Old and the Young

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Long-term intake of high-energy diet can lead to decreased insulin sensitivity and even insulin resistance, eventually leading to diabetes. Diabetes often occurs in middle-aged and elderly people. However, there is growing evidence that the incidence rate of young body is increasing over the years. This means that insulin resistance can be caused by excessive energy intake in both young and old people. In this study, high-fat diet (HFD) and normal diet were fed to rats of elderly experimental group (EE), elderly control group (EC), young experimental group (YE), and young control group (YC), respectively, for 8 weeks, by which insulin resistance model was obtained. Insulin sensitivity was measured, histopathology changes in liver and skeletal muscle tissues were observed, and mitochondrial fusion and division and cell senescence were detected in four groups of rats. The results showed that both young and elderly rats developed significant insulin resistance, fat deposition, decline of mitochondrial function and mitochondrial biosynthesis in liver and skeletal muscle, and cell aging after HFD feeding. In addition, the degree of mitochondrial dysfunction and aging in young rats was similar to that of aged rats fed a normal diet after HFD. This experiment provides a reference for an in-depth study of the regulatory mechanisms of cellular energy metabolism in this state.

Keywords: insulin resistance, high-fat diet, diabetes, young, elderly, rats

OPEN ACCESS

Edited by:
Ellen E. Blaak,
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Reviewed by:
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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *09 March 2022*
Accepted: *16 May 2022*
Published: *23 June 2022*

Citation:
Wang J, Wu J, Li W, Wang X, Liu R, Liu T and Xiao J (2022) Linking Mitochondrial Function to Insulin Resistance: Focusing on Comparing the Old and the Young. Front. Nutr. 9:892719. doi: 10.3389/fnut.2022.892719

INTRODUCTION

With the improvement of living standards, the incidence of diabetes has also increased, and it has gradually become one of the most important non-communicable diseases threatening global human health. Insulin resistance diseases caused by obesity and overnutrition are increasingly common in humans and animals (1–3). Currently, there are also increasing rates of obesity and insulin resistance in animals around the world. Insulin resistance is the cause of obesity, type 2 diabetes mellitus (T2DM), and other metabolic diseases (4, 5). Insulin plays an important role in glucose homeostasis and is the main regulator of carbohydrate, fat, and protein metabolism (6, 7). *In vivo*, insulin acts in liver, skeletal muscle, and fat by binding to insulin receptors therein (8). When a high-fat diet is fed for a long time, in order to promote the uptake of this glucose by cells, the body compensatory secretes too much insulin, which reduces the sensitivity of liver and skeletal muscle cells to insulin, leading to the occurrence of insulin resistance, which in turn leads to diabetes.

The prevalence of obesity and type 2 diabetes in animals will increase significantly with age, due to which aging will lead to a progressive decline in most endocrine functions and meanwhile result in severe metabolic disorder (9). Previous studies have shown that the prevalence of diabetes in the elderly is high, but at present, many studies show significantly lower insulin sensitivity in obese young adults (10). Young people with T2DM or abnormal glucose tolerance have lower βcell sensitivity than healthy old people, which may be related to the great demand for a role of β cells in regulating blood glucose in young people (11). It is worth noting that the incidence of diabetes in puppies and kittens has been increasing in recent years (12–14). At present, there is no report on young animals.

Growing evidence suggests that insulin resistance caused by high-energy diet can occur in middle-aged and elderly or even young animal individuals, by disturbing a variety of metabolic pathways in the body, which in turn leads to the occurrence of metabolic diseases such as obesity and T2DM. However, several reports have raised the question of whether young and old IR mechanisms are the same (15–17). Therefore, in this experiment, rats will be used as experimental animals to establish a high-energy diet-induced insulin resistance model for youth and old rats, based on which the effects of high-energy diet on the occurrence of insulin resistance, liver, and skeletal muscle as well as mitochondrial function in young and elderly rats will be elucidated, which will have a positive effect on the prevention of energy excess metabolic diseases.

MATERIALS AND METHODS

Animals

The *in vivo* experiment was performed in accordance with The Tab of Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University. Twenty 3-month-old (300 ± 20 g) and twenty 15-month-old (500 ± 40 g) Sprague-Dawley (SD) male rats were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (China) and housed under 12-h light/dark cycle, controlled humidity (40 ± 10%), and constant temperature (24 ± 1°C).

After 1wk of acclimatization, rats of two ages were randomly assigned into four groups ($n = 10$, 5 rats per 549 × 395 × 200 mm cage).

Experimental Design

After a week adaptation period, rats were allocated into four groups ($n = 10$ per group) as follows: (1) elderly experimental group (EE), with high-fat diet; (2) elderly control group (EC), with low-fat diet; (3) young experimental group (YE), with high-fat diet; (4) young control group (YC), with low-fat diet. All animals received water ad libitum, and all rats were maintained for 8 weeks. Glucose tolerance test (IGTT) was performed at the end of week 8, followed by insulin tolerance test (ITT) on day 3, that is, at week 9, rats were euthanized and blood samples were collected by cardiac puncture. Tissues were then harvested. Sera were separated and stored at −20°C for measuring biochemical parameters. Body weight of all rats was measured weekly between 8:00 and 9:00h (**Figure 1**).

Rats were divided into four groups: The 45% high-fat diet (D12451) was purchased from Research Diets Inc (New Brunswick, NJ, USA). The 4% fat rat-chow diet (CS101) was purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). The composition of the diets is shown in **Table 1**.

Establishment of Insulin Resistance Model

Intraperitoneal Glucose Tolerance Test (IGTT)

Intraperitoneal glucose tolerance test was conducted at the end of the 8th week. Rats fasted overnight, and the baseline blood glucose level was measured using a blood glucose monitor (Sinocare, Hunan, China) via tail nick. Animals were injected intraperitoneally with 50% glucose (2 g/kg). Blood glucose levels were then measured at 0, 15, 30, 60, and 120 min after injection. The incremental area under the curve (AUC) of glucose response was calculated using GraphPad Prism 7.0 Software. The blood glucose concentration at 0 min was the fasting blood glucose (FBG, mmol/L) of rats.

Insulin Tolerance Tests (ITT)

After the glucose tolerance experiment, the rats recovered for 3 days. Rats in each group were fasted but not prohibited water for 12 h. Short-acting insulin (0.4 ml/kg body weight; Novo Nordisk, Gentofte) was administered intraperitoneally (i.p.) to rats, and blood samples were taken from the tail vein of the conscious rats before and 15, 30, 60, and 120 min after insulin administration (18–20). Blood glucose levels were immediately measured with a blood glucose monitor (Sinocare, Hunan, China). Total AUC was calculated by the GraphPad Prism 7.0 Software for 120 min after glucose injection.

Homeostatic Model Assessment of Insulin Resistance

The fasting insulin was measured using the ultrasensitive rat insulin enzyme-linked immunosorbent assay kit, and homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as follows:

$$[fasting\ glucose\ (mmol/L) \times fasting\ insulin\ level\ (\mu IU/mL)]/22.5$$

The homeostasis model assessment (HOMA) uses the product of basal insulin and glucose concentrations divided by a constant (21).

Hematology Analysis

Blood Collection

After the ITT, the animals were killed. Blood samples were collected from the animals using cardiac puncture and put into different precooled EDTA containers. The blood samples were centrifuged at 4°C, 2,500 rpm for 15 min to obtain plasma. Each of the plasma was aspirated into plain sample bottles and stored at −80°C freezer until ready for biochemical analysis.

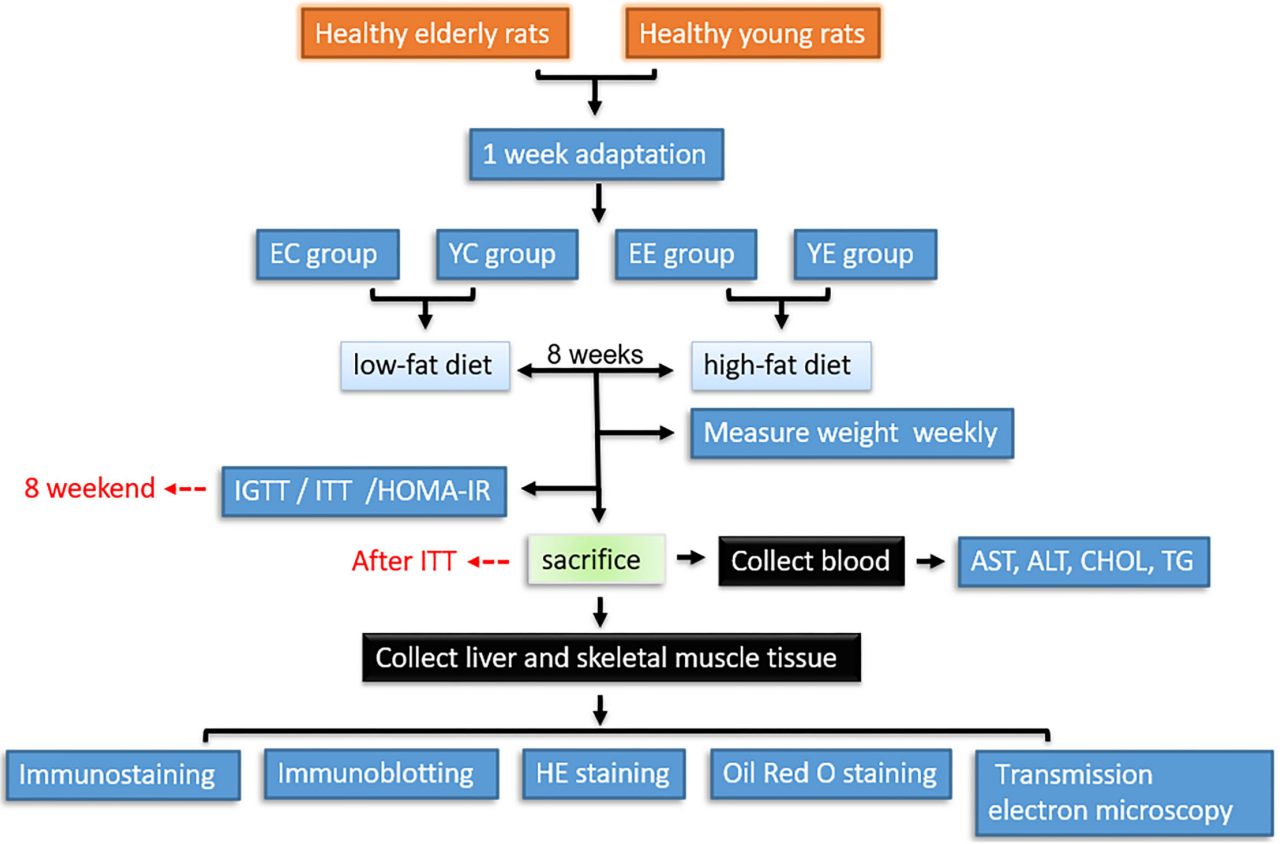

FIGURE 1 | Workflow for hematology, histopathological, and proteomic analysis of tissues from insulin resistance rats. YC, young control group; YE, young experimental group; EC, elderly control group; EE, elderly experimental group.

Biochemical Analysis

Liver enzymes (AST, ALT, CHOL, and TC) in the plasma were analyzed with Mindray BS-280 automatic biochemistry analyzer (BS-280) (Nanjing Baden Medical, Jiangsu, China).

Histopathological Examinations

Tissue Preparation and Histological Analysis

A portion of liver and skeletal muscle tissues were dissected and fixed with 4% paraformaldehyde for 72 h. The specimens were dehydrated in ascending grade of alcohols and paraffin embedding using standard methods. Then, the specimens were then sectioned in sagittal plane with 4-μm thickness and were stained with hematoxylin–eosin (H&E) staining analyzed to elucidate the status of tissue architecture and pathological analysis. Microscopic images were acquired using light microscopy.

The other part was kept frozen in a −80°C freezer for immunoblotting analysis and for making cryosections for Oil Red O staining. Liver and skeletal samples were fixed in 4% buffered formalin. Liver samples were embedded in optimal cutting temperature (OCT) medium and stored at −80°C. OCT embedded, 7 μm sections were stained with ORO for fat content examination. Randomly chosen areas of tissue sections were photographed using a light microscope.

Muscle Triglyceride Analysis

After dissecting any visible adipose tissue, 50 mg of tissue of each animal was weighed and homogenized in a handheld tissue homogenizer, using a volume of distilled water equal to eight times the tissue weight in mg. The protocol for triglyceride extraction was as follows: 5 M NaCl, methanol, and chloroform were added to the homogenate and mixed, and the ternary phase was broken with water and chloroform after 5 min of incubation. The three-phase system was then separated by centrifugation at top speed. The aqueous and protein phases were re-extracted with a 9: 1 chloroform: methanol wash solvent and separated by centrifugation. The organic solvent was dried using a stream of nitrogen gas with an N-EVAP, and the triglyceride was resuspended in isopropanol containing 2% Triton X-100. Glycerol concentration was measured relative to a glycerol standard curve. Standards and samples were loaded on a 96-well-plate, and free glycerol reagent was added to each well. The plate was incubated at 37°C and read on a Kinetic Microplate Reader at 540 nm. Triglyceride reagent was added to each well and

TABLE 1 | Ingredient composition of the diets fed to rats (g/kg).

Ingredient	4% fat g/kg diet	Ingredient	45% fat g/kg diet
Crude ash	80	Casein, 80 Mesh	200
Fiber	50	L-Cystine	3
Mineral mixture	30.215	Corn starch	72.8
Vitamin mixture	318.04	Maltodextrin 10	100
Lysine	8.2	Sucrose	172.8
Methionine + Cystine	5.3	Cellulose, BW200	50
Arginine	9.9	Soybean oil	25
Histidine	4	Lard*	177.5
		Mineral mix S10026	10
		DiCalcium Phosphate	13
		Calcium Carbonate	5.5
		Potassium Citratr, 1 H2O	16.5
		Vitamin Mix V10001	10
		Choline Bitartrate	2
		FD&C Red Dye #40	0.05
Protein kcal (%)	18	Protein kcal (%)	20
Fat kcal (%)	4	Fat kcal (%)	45
		Carbohydrate kcal (%)	35

**Typical analysis of cholesterol in lard = 0.95 mg/gram.*

incubated at 37°C. The plate was read again on the microplate reader at 540 nm, and the background glycerol was subtracted.

Muscle Glycogen Test

Using anthrone method (the kit is provided by Nanjing Jiancheng biology Co., Ltd. Article No.: A043), the test is carried out with method 721 spectrophotometer. Then, take 100 mg fresh muscle, and after treatment, mix the tissue with 300 μL alkali liquor (i.e., sample weight: alkali liquor volume = 1:3) into the test tube, boil with boiling water for 20 min, cool with running water, operate in strict accordance with the instructions of the kit, and substitute it into the formula to calculate the value.

Liver ATP Content Test

All reagents for ATP determination were prepared with redistilled distilled water. The solutions were as follows: (1) adenylate extract (Tris-HCL 20 mmol/L, $MgSO_4$ 2 mmol/l); (2) luciferase buffer: each powder was used to dissolve in 50 mL of redistilled water, containing 50 mmol/L glycylglycine (PH=7.6), 10 mmol/L $MgSO_4$, and 1 mmol/L EDTA buffer.

Standard curve of ATP: ATP was prepared into six tubes of application solution of 1×10^{-10}-5×10^{-5} *mol/L* for determination, and the standard curve was drawn by the *log* value of relative luminescence intensity and ATP concentration.

ATP determination by luciferase-luciferin method: Take 0.1–0.15 g of liver tissue, add it to 1 mL of adenylate extract, homogenize and heat in boiling water for 3 min, then centrifuge at 4,000 rcf/min for 3 min, and take 0.4 mL of supernatant. During detection, add 0.1 mL of liver homogenate to 0.1 mL of double distilled water to dilute, put it into a 2 mL cuvette, put it into the darkroom of FG-200 luminometer, and quickly inject 0.8 mL of luciferase-based buffer into the small hole of the dark chamber cover, and the initial peak of the recorded luminescence curve is the light intensity of the detected sample. The measurement temperature is 25°C, the measurement voltage is 0.5 mV, and the ATP value is detected on the standard curve according to the obtained light intensity (the number of small cells occupied on the measurement curve).

Observation of Mitochondrial Ultrastructure

The ultrastructure of liver mitochondria was observed with transmission electron microscopy (TEM). Pieces of liver tissue (1 mm^2) were picked out within 1 min of death and fixed with 4% glutaraldehyde. Then, the samples were post-fixed in 2% osmium tetroxide, dehydrated in an ascending series of ethanols, and embedded in araldite. Ultrathin sections were cut with an LKB-8800 ultramicrotome (LKB, Sweden) and collected on grids. Sections were stained with uranyl acetate and lead citrate and evaluated with an H-500TEM (Japan) operated at 75 kV.

Western Blot

Samples containing 50 μg proteins were subjected to 10% SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 5% non-fat dry milk to prevent non-specific binding and then incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study were GAPDH (1:750), anti-AMPK (1:3,000), anti-Mfn2 (1:5,000), anti-Opa1 (1:3,000), anti-Drp1 (1:1,500), anti-PGCα (1:1,500), anti-p53 (1:750), anti-p21 (1:1,000), and anti-p16 (1:5,000) antibodies. The membranes were washed in 50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, and 0.1% Tween 20 for 30 min and incubated with appropriate secondary antibody (1:3,000) for 2 h at room temperature. The nitrocellulose membrane was visualized using an ECL luminescent solution, and the film was exposed and visualized and photographed by a fully automated WB chemiluminescence imaging system (Tanon 5200, Shanghai Tanon Technology, China).

Immunostaining

The postfixed specimens of the liver were embedded in paraffin according to standard protocols. Paraffin-embedded sections (4 um) were preheated at 37°C for 10min. followed by incubation in xylene and stepwise rehydration in 100, 95, 70, and 50% ethanol. After slides were washed in TBS, the sections were blocked by the addition of normal goat serum diluted in TBS for 1 h at room temperature. Affinity-purified antibody to Opa1, Mfn2, Drp1, AMPK, PGC-1a, p53, p21, and p16 diluted in blocking solution was added at concentration of 2 ug/ml for 1h at room temperature followed by three 3-min washes with TBS at RT. Antirabbit IgG was added to the sections for 1 h at room temperature followed by three 3-min washes in TBS at room temperature and kept at 4°C until visualized.

Statistical Analysis

The statistics and analysis of all data in this experiment were plotted using GraphPad Prism 8 statistical analysis, and the values were expressed as mean ± standard deviation (Mean

TABLE 2 | Changes in body weight (g) of rats in each group ($n = 10$, ± s).

Group Time (week)	YC	YE	EC	EE
1W	470.8 ± 14.8	472.0 ± 15.0	548.0 ± 43.3	549.8 ± 37.9
2W	496 ± 14.3	506.5 ± 9.6	551.5 ± 46.6	569.5 ± 44.0
3W	516 ± 18.2	533.0 ± 12.0	556.3 ± 55.7	585.8 ± 45.9
4W	534.3 ± 15.2	553.3 ± 9.9	558.8 ± 44.8	593.3 ± 51.1
5W	549.8 ± 11	570.3 ± 14.1	564.3 ± 49.7	606.5 ± 53.2
6W	559.8 ± 10.5	584.0 ± 18.9	566.8 ± 42.0	618.3 ± 50.4
7W	565.8 ± 7.9	597.0 ± 24.5	572.5 ± 33.3	626.5 ± 53.1
8W	571.3 ± 8.2	607.8 ± 19.4	578.8 ± 36.0	634.0 ± 48.5

YC, young control group; YE, young experimental group; EC, elderly control group; EE, elderly experimental group.

± SD). One-way analysis of variance was used to analyze the differences, $P < 0.05$ indicated significant differences, and * indicated differences. $P < 0.01$ indicated extremely significant difference, which was expressed as **. $P > 0.05$ indicates no significant difference, denoted as *ns*.

RESULTS

Basic Characteristics of the Experimental Animals

Appearance and Body Weight

During the feeding process of 8 weeks, the weight of rats in YE and EE groups increased significantly, and the growth rate of YE group was the fastest, with a weight increase of about 32% (**Table 2** and **Figure 3A**). In addition, in terms of appearance, the rats in YE and EE groups fed HFD have rough fur, yellow hair color, depressed spirit, unwilling to exercise, and like to lie down (**Figures 2A–D**).

HFD Feeding Induces Significant Dyslipidemia, Insulin Resistance, and Hepatic Injury

When compared with YE group, the content of AST was significantly increased in EE ($P < 0.01$) (**Figure 3G**), but there was no significant change in ALT ($P > 0.05$) (**Figure 3H**). Serum triglyceride content was not significantly changed between the YE and EE groups ($P > 0.05$) (**Figure 3I**). The content of total cholesterol content (TC) in YE group was significantly higher than that in EE group ($P < 0.05$) (**Figure 3J**).

Intraperitoneal glucose tolerance test (IGTT) showed that compared with the YC and EC groups, the YE and EE groups developed severe glucose intolerance (**Figure 3C**) and the area under the blood glucose concentration curve was significantly increased ($P < 0.05$) (**Figure 3F**). Insulin tolerance test (ITT) results showed that compared with the YC group and the EC group, the YE group and the EE group were less sensitive to exogenous insulin (**Figure 3B**) and the area under the blood glucose concentration curve was significantly different (**Figure 3E**).

Based on these measurements, we calculated the insulin resistance index using the homeostatic model assessment (HOMA) index (**Figure 3D** and **Table 3**). The results showed that compared with the YC and EC groups, the HOMA-IR index of the YE and EE groups increased significantly, and there was no significant difference between the YE and EE groups ($p > 0.05$).

According to these results, it is shown that the 8-week HFD diet successfully induced insulin resistance in the rats of the YE and EE groups.

Liver

The rats in YE (**Figure 2J**) and EE (**Figure 2L**) groups with HFD-fed developed larger livers that were significantly heavier than those of the YC (**Figure 2I**) and EC (**Figure 2K**) group rats. Visually, the livers from the HFD-fed animals were distinguished from those of the control groups rats as yellowing in color, especially YE (**Figure 2J**). Compared with YC (**Figure 2E**) and EC (**Figure 2G**) groups, The presence of more fat around the liver can be observed in rats of the YE group (**Figure 2F**) and EE group (**Figure 2H**). Microscopic examination of tissue slides revealed higher hepatocyte lipid droplet accumulation in the livers from the YE and EE, compared to those from the YC and EC groups (**Figures 5A–D**). *Post-hoc* analysis showed that the degree of liver lipid droplet infiltration in YE group was higher than that in EE group ($P < 0.05$) (**Figure 5A**). TEM showed that there were relatively more liver mitochondria in YE group and EE group than in the control group, and the YE group had more mitochondria in fission state (**Figures 4N,P**).

Skeletal Muscle

The H&E staining results of skeletal muscle in rats are shown in **Figures 4A–D** (Crosscutting) and **Figures 4E–H** (Slitting). In the YC (**Figures 4A,E**) and EC groups (**Figures 4C,G**), the skeletal muscle structure was intact, the muscle fibers were neatly arranged, and there were no fractures, increase in size or migration of nuclei, or inflammatory cell infiltration between muscle fibers. In the YE and EE groups, the muscle fibers were disordered and broken, and displayed inflammatory cell infiltration, non-uniform nuclei, and abnormal locations among the local muscle fibers. YE group was more severe than EE group.

The content of muscle glycogen in YE group was significantly lower than that in YC group ($P < 0.05$) and that in EE group was significantly lower than that in EC group ($P < 0.01$). There was no significant difference between YE group and EE group ($P > 0.05$) (**Figure 3K**). The content of IMTG in the experimental group was higher than that in the corresponding control group. There was a significant difference between YE group and EE group ($P < 0.05$), and the content of IMTG in YE group was the highest (**Figure 3L**).

HFD Diet Promotes Mitochondrial Fission in Liver and Skeletal Muscle of Young Rats

We sought to identify the effect by hypercaloric diet exposure on rats' liver and skeletal muscle mitochondrial dynamics evidenced by changes in the expression of proteins involved in mitochondrial fusion (Mfn 2, Opa 1) and/ or fission (Drp 1).

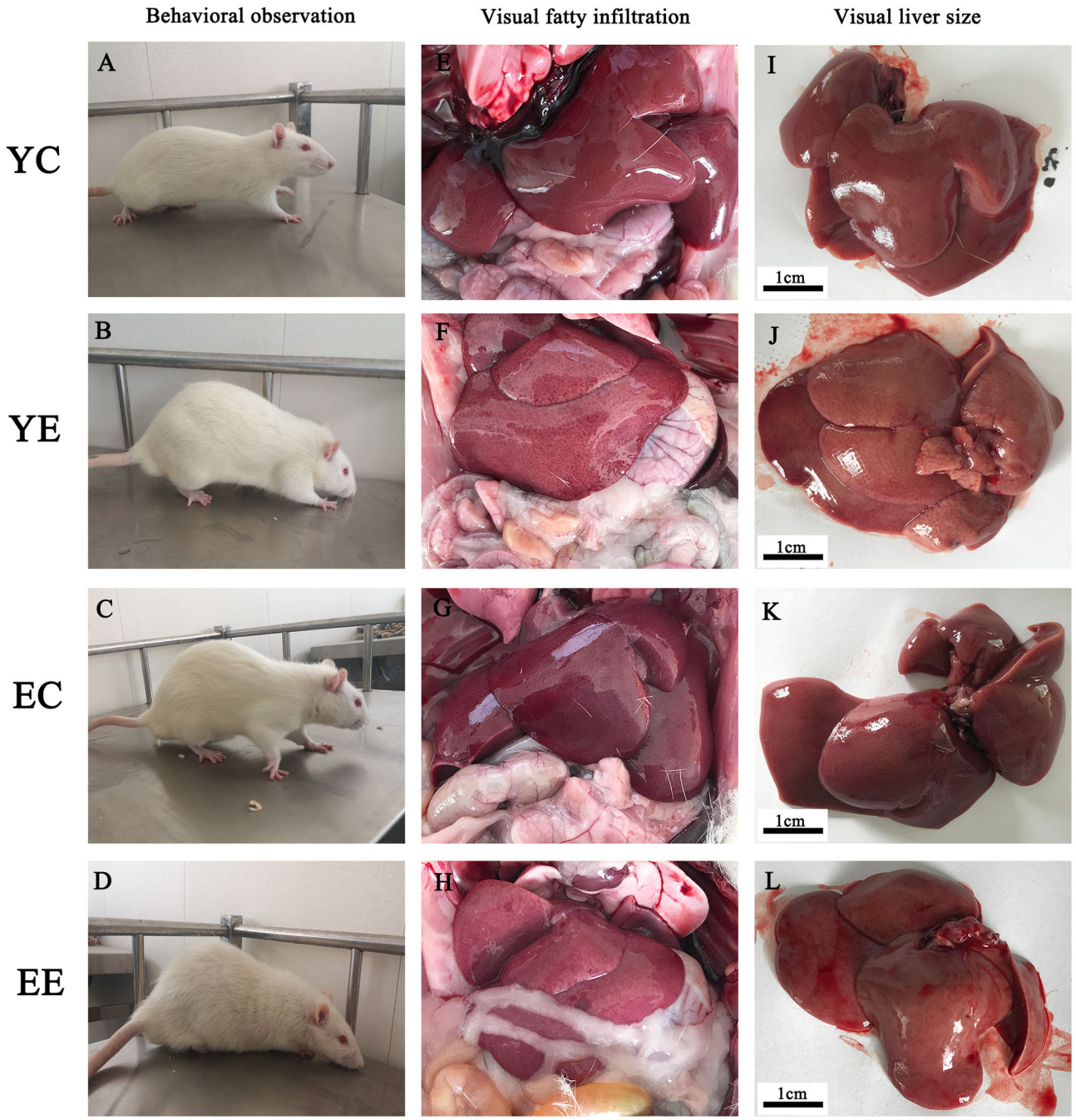

FIGURE 2 | Effect of HFD on behavior and liver of rats. Comparisons among YC, YE, EC, and EE rats in appearance **(A–D)**, liver *in vivo* **(E–H)**, and liver *in vitro* **(I–L)**.

We found that Opa 1 protein in liver of YE group was significantly higher than that in EE group ($P < 0.01$) (**Figure 6A**), and there was no significant difference in skeletal muscle ($P > 0.05$) (**Figure 6B**). Mfn2 protein had no significant difference between YE group and EE group in liver ($P > 0.05$) (**Figure 6C**), but the difference is significant in skeletal muscle ($P < 0.01$) (**Figure 6D**). There was no significant difference in the expression of Drp 1 protein between the YE and EE groups in liver and skeletal muscle ($P > 0.05$) (**Figures 6E,F**).

These data suggest that hypercaloric diet exposure promoted mitochondrial fission in rats, especially young rats. Next, based on the results of protein expression found in the groups of YC, YE, EC, and EE, we quantified their immunofluorescence of Mfn 2, Opa 1, and Drp 1 genes in liver. We identified that the expression of Opa 1 in YE group was higher than that in EE group ($P < 0.01$) (**Figures 5E–H** and 5b), and there was no significant difference in the expression of Mfn2 and Drp1 between YE group and EE group ($P > 0.05$) (**Figures 5C,D**).

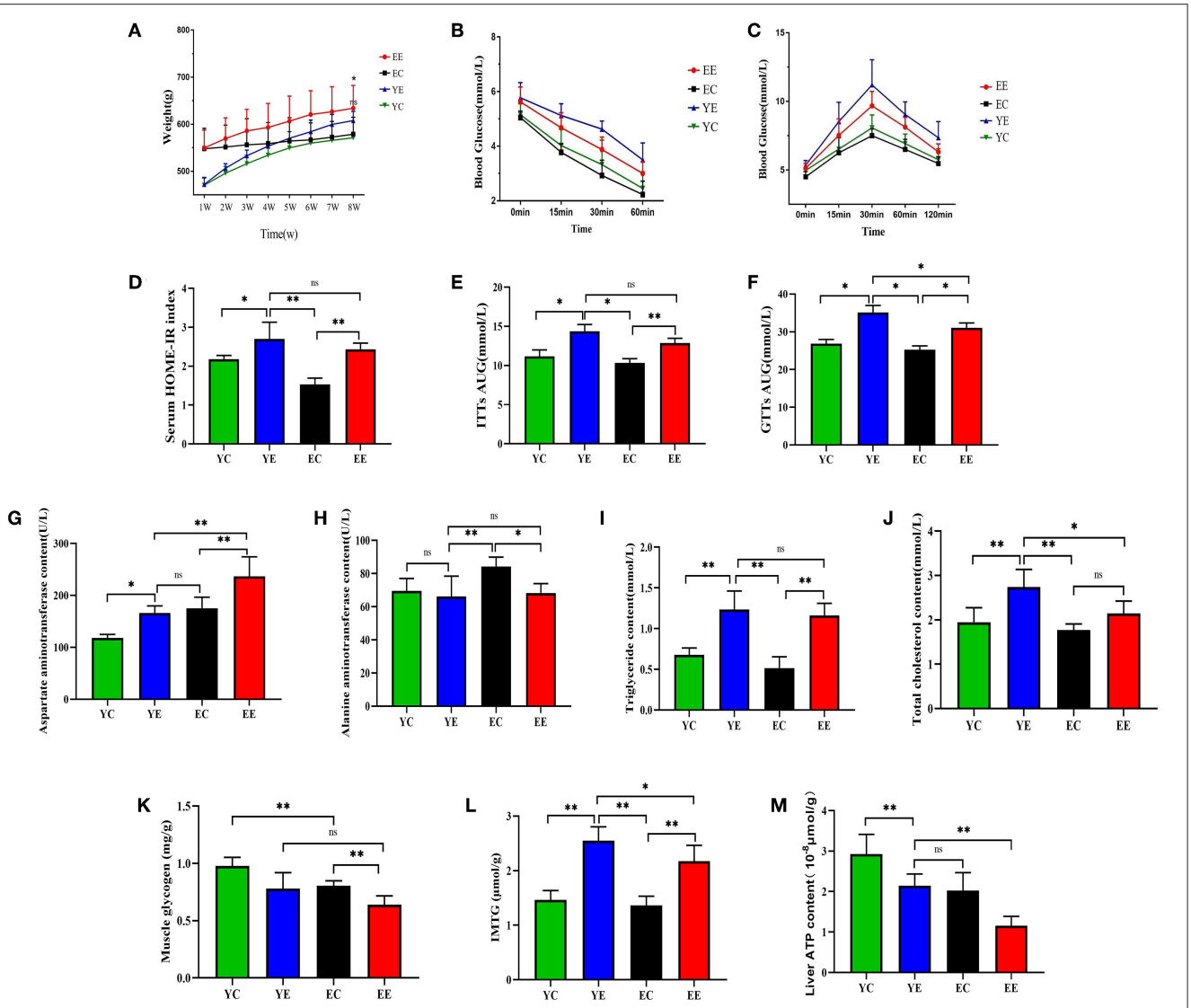

FIGURE 3 | Effect of HFD on weight, insulin sensitivity, and blood biochemistry in EE, EC, YE, and YC rats. **(A)** The change in body weight every week. **(B)** ITTs. **(C)** GTTs. **(D)** HOMA-IR. **(E)** ITTs-AUC. **(F)** GTTs-AUC. **(G)** Effects of HFD by intervention on the plasma AST, **(H)** ALT, **(I)** TC, and **(J)** CHOL in rats. **(K)** Muscle glycogen. **(L)** IMTG. **(M)** Liver ATP content. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{ns}p > 0.05$.

High-Fat Diet Hinders Mitochondrial Biogenesis

We measured two proteins representative of mitochondrial biosynthesis and energy metabolism, AMPK (**Figures 7A–D** and 7a) and PGC-1α (**Figures 7E–H** and 7b), in liver and skeletal muscle, as well as ATP content in liver (**Figure 3M**).

The results show, in liver and skeletal muscle, that the expression of AMPK protein in YE group was significantly higher than that in EE group ($P < 0.01$), but there was no significant change in YE group and EC group ($P > 0.05$) (**Figures 6G,H**); the expression of PGC-1α protein in liver (**Figure 6I**) and skeletal muscle (**Figure 6J**) of YE group was not significantly different from that of EE group ($P > 0.05$). In addition, the results of detecting the ATP content in the liver showed that the YE group was significantly higher than the EE group ($P < 0.01$) (**Figure 3M**).

Senescence in Hepatic and Skeletal Muscle Tissue

Hepatic and skeletal muscle senescence was investigated via Western blot by measuring the protein expression of p53, p21, and p16, which are considered senescence markers.

After 8 weeks of HFD feeding, in the liver, the protein expressions of p53 and p21 in the EE group were significantly higher than those in the YE group ($P < 0.01$) (**Figures 6K,M**), and the p16 protein of EE group was also significantly upregulated compared with YE group ($P < 0.05$) (**Figure 6O**). In the skeletal muscle, the expression of p53 and p21 proteins of

EE was significantly upregulated compared with the YE group ($P < 0.05$ and $P < 0.01$) (**Figures 6L,N**), but there was no significant difference in the expression of p16 protein in two groups ($P > 0.05$) (**Figure 6P**).

Next, we performed immunofluorescence quantitative analysis of p53 (**Figures 7I–L** and 7c), p21 (**Figures 7M–P**), and p16 (**Figures 7Q–T** and 7e) in liver. There was a significant difference in p53 protein expression between YE and EE groups ($P < 0.05$), but no significant difference between YE and EC groups ($P > 0.05$) (**Figure 7C**). There was a significant increase in p21 protein expression in the EE group compared to the YE group ($P < 0.01$) and in the EC group ($P < 0.05$) (**Figure 7D**). There was also a significant difference in p16 protein expression between the YE and EE groups ($P < 0.05$), but no significant difference between the YE and EC groups ($P > 0.05$) (**Figure 7E**).

Overall, the EE group had the most severe aging degree, but the YE group had almost the same degree of aging as the EC group. It is clear from the above results that the high-energy diet induces insulin resistance in rats, which also simultaneously promotes aging of the organism.

TABLE 3 | Descriptive statistics of serum concentrations of FINs and FBG, and HOMA-IR in 40 rats.

Group	FINs (μIU/mL)	FBG (mmol/L)	HOMA-IR
YC	9.865 ± 0.382	4.975 ± 0.206	2.181 ± 0.094
YE	11.736 ± 1.741	5.175 ± 0.171	2.699 ± 0.426
EC	7.678 ± 0.768	4.500 ± 0.392	1.536 ± 0.161
EE	11.241 ± 1.218	4.875 ± 0.250	2.436 ± 0.157

YC, young control group; YE, young experimental group; EC, elderly control group; EE, elderly experimental group; FINs, fasting serum insulin content; FBG, fasting blood glucose concentration.

DISCUSSION

Insulin resistance is the common basis of a variety of metabolic diseases, such as obesity, T2DM, and cardiovascular diseases (22–25). Insulin resistance caused by excess energy mostly occurs in elderly animals. However, more and more evidence shows that the incidence rate of diabetes and insulin resistance is also increasing in young individuals (26, 27). Therefore, insulin resistance in young individuals also needs to be paid attention. *In vivo*, insulin resistance not only disturbs the metabolic process of sugar and fat, but also affects the productivity function of the body, especially the energy biogenesis ability of mitochondria (28, 29). At the same time, insulin resistance will also lead to a large amount of fat deposition in the liver and skeletal muscle and may even aggravate the process of aging (30). Young individuals have stronger cellular metabolic capacity and stronger mitochondrial compensation than older individuals (31). But since insulin resistance and diabetes have occurred, whether similar changes have taken place with older individuals, whether mitochondrial function of young individuals has been severely affected, and whether there has been significant aging in cells has not been reported. Therefore, this study focuses on the changes in mitochondrial function and the degree of cell aging in young insulin-resistant individuals.

To study the effect of HFD-induced insulin resistance on mitochondrial function of liver and skeletal muscle in young and old rats, we used a kind of commercialized high-fat diet to construct the insulin resistance model, which is a representative model of diabetes with related metabolic complications in young and old body (32–34). Clinically, glucose tolerance test, insulin tolerance test, and HOMA-IR are often used to detect insulin resistance (21, 35). Because when insulin resistance occurs, the sensitivity of cells to insulin decreases significantly, which resulted in the elevation of the body's fasting plasma glucose level ultimately (36). This phenomenon was also found in our experiment, even though the blood glucose concentration decreased after prolonged starvation, and the blood glucose in the YE and EC groups was still higher than that in the YC and EC groups. In the glucose tolerance test and insulin tolerance test, it was also found that the blood glucose concentration of the experimental group was higher than that of the control group. In our experiment, the fasting blood glucose concentration and fasting insulin content of rats in each group were incorporated into the HOMA-IR formula to calculate the insulin resistance index. The results showed that the insulin resistance index of the experimental group was significantly higher than that of the control group, indicating that a significant insulin resistance phenomenon occurred after feeding a high-energy diet, which shows that the insulin resistance rat model established in this experiment was successful. In addition, we also found that the YE group had the highest HOMA-IR index, which would normally define the most severe insulin resistance in the YE group, but this is not true, because the HOMA-IR formula is based on multiplying the fasting blood glucose concentration and the serum insulin concentration. However, with the senility of the body tissue in the elderly, the metabolism of pancreatic cells decreases, which causes the gradual aging of the pancreas, which in turn affects the secretion of insulin. Therefore, compared with young rats, the serum insulin concentration of aged rats must be lower, so the HOMA-IR formula cannot fully reflect the degree of insulin resistance in young and old rats. Therefore, this experiment combines mitochondrial dynamics and histopathology to analyze various aspects, and the focus was on differences in liver and skeletal muscle impaired from high-fat diets in young rats compared to older healthy rats.

When carbohydrates and other nutrients are ingested into the body, the body will timely convert them into energy available for cells through various metabolic reactions. When too much energy is continuously ingested, this energy will be transported to liver, skeletal muscle, and other tissues in time for storage (37, 38). In the body, energy storage and utilization will maintain a relative balance and have a certain upper limit of regulation. When the rats in the experimental group ingest too much energy, the balance between energy storage and energy utilization in the body is broken, even exceeding the upper limit of the body. The phenomenon of the high insulin resistance index in the YE and EE groups found in this experiment should also be the result

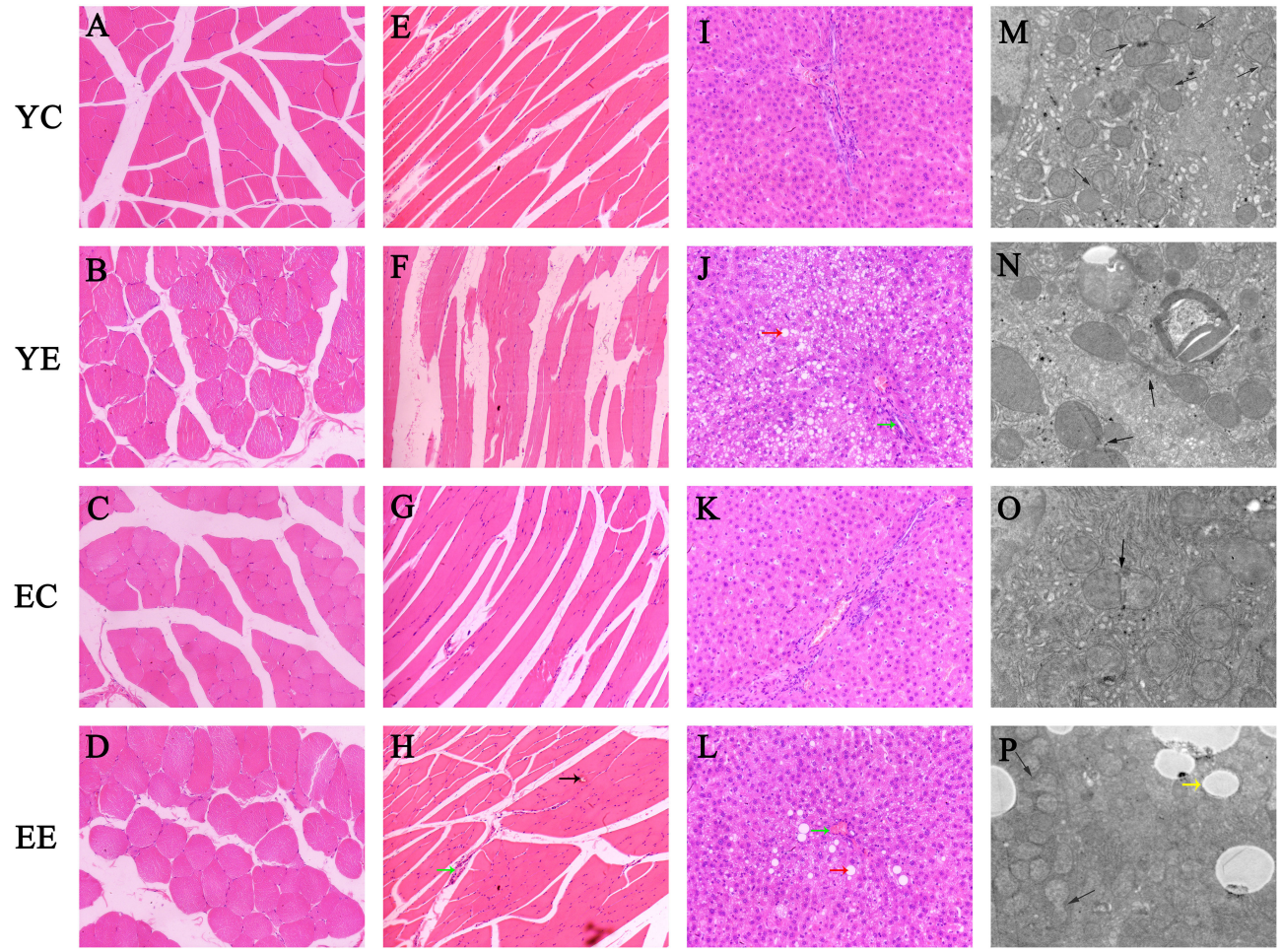

FIGURE 4 | Hematoxylin–eosin staining of transverse **(A–D)** and longitudinal **(E–H)** sections of skeletal muscle. **(I–L)** Hematoxylin–eosin staining of liver tissue. (Green arrow: inflammatory cell infiltration; red arrow: vacuolar degeneration; black arrow: bleeding point). Original magnification: × 200. **(M–P)** Hepatic mitochondrial changes in each group's rats. Bar = 5.0um. (Yellow arrow: white fat droplet). Mitochondrial fission and fusion were evaluated with an inverted fluorescence microscope (Eclipse Ts2, Nikon, China).

that the continuous intake of energy leads to the premature reaching of the upper limit of energy storage and utilization. This result may also be related to the relatively small intake of food in the elderly rats. On the contrary, it also shows that even young individuals will have significant insulin resistance after eating a large amount of high-energy feed, which is not what we usually think of as "young individuals have strong energy processing ability." Young individuals also have an upper limit on energy utilization and storage. If they exceed this upper limit, they will lead to insulin resistance. When animals become obese and insulin-resistant, blood glucose becomes more difficult to be controlled due to the combination of excess hepatic glucagon action and multi-tissue insulin resistance (39, 40).

Studies of liver function in diabetic organisms showed that 28.0 to 36.8% of patients had liver dysfunction (41, 42). Prolonged fatty infiltration of the liver in the diabetic has been shown to lead to hepatic cirrhosis (43). As a result, liver injury is more severe in young rats than in older rats who eat the same high-calorie diet. We thought that this is related to the strong metabolic ability of the rats in the YE group, which can deliver excess energy to the liver for storage timely. In this experiment, compared with other groups of rats, the weight of the rats in the YE group was the heaviest, and the results of Oil Red O staining also indicated that the liver lipid droplet infiltration in the YE group was significantly more extensive than that in the EE group. Meanwhile, it can be seen from the results of ALT and AST that the degree of liver injury in YE group is significantly lower than that in EE group, which explained the strong self-metabolism and repairability of young rats, which can repair or remove damaged hepatocytes in time. In addition, due to the more aging state of EE group, the self-repairability and clearing ability of body tissues are relatively weak.

Mitochondria are always in the process of dynamic regulation including fission and fusion to meet the different energy needs of cells and then maintain mitochondrial and cellular functions (44, 45). In obesity and type 2 diabetes, reduced

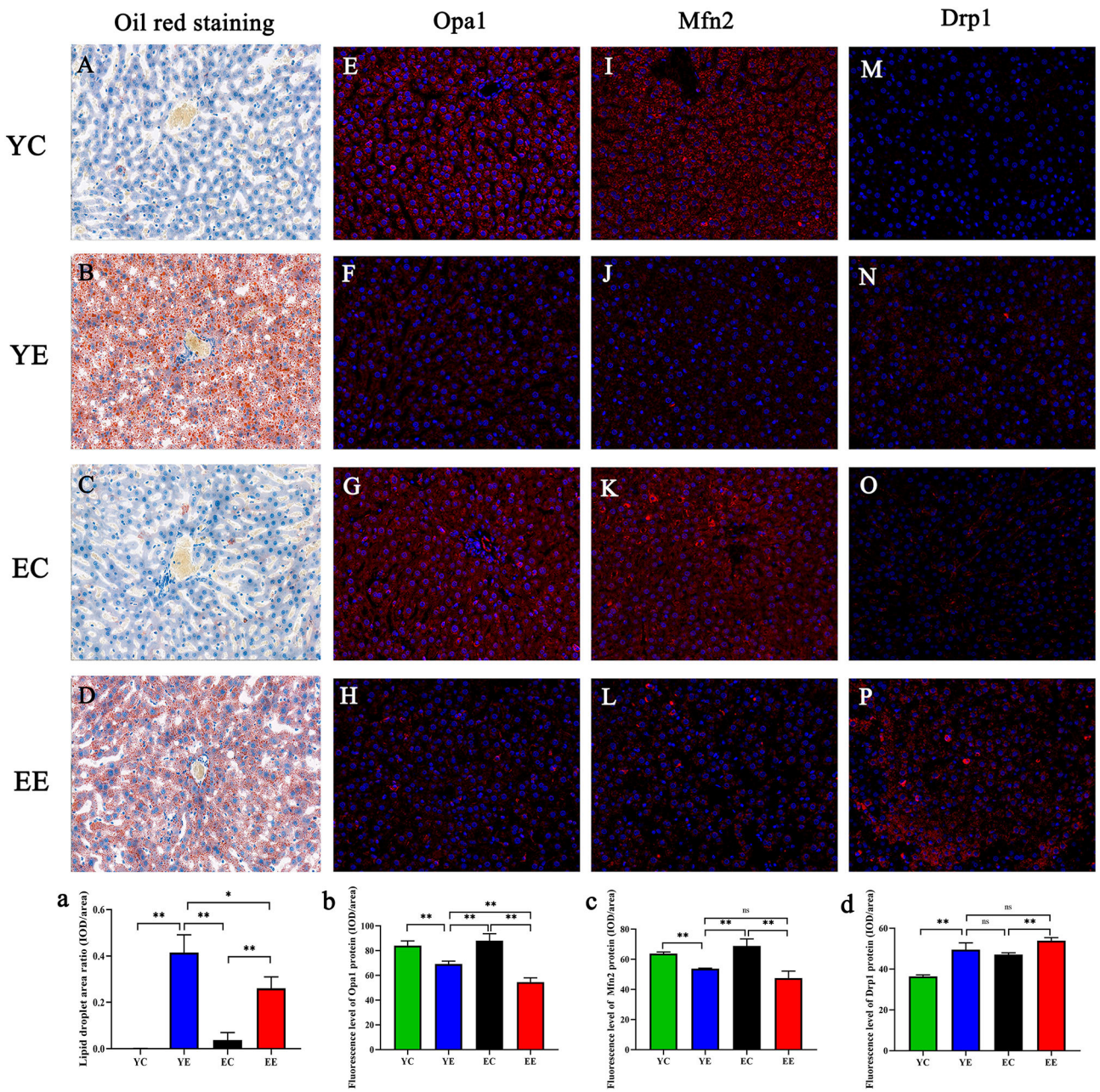

FIGURE 5 | (A–D) Oil Red O staining of the liver. Original magnification: ×200. Opa1 **(E–H)**, Mfn2 **(I–L)**, and Drp1 **(M–P)** in liver mitochondria were labeled with red fluorescence in the cytoplasm and blue fluorescence in the nucleus. Effect of diet on hepatocytes of mice in each group. (a) Data analysis of liver lipid droplet area observed by Oil Red O staining. Opa1(b), Mfn2 (c), and Drp1 (d) protein fluorescence assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{ns}p > 0.05$.

mitochondrial contents have been reported (44, 46). Under a transmission electron microscope, the mitochondria in the livers of experimental and control rats were observed, and the fusion process of liver mitochondria was more pronounced in the control group fed with normal feed, while in the experimental group fed with high-energy feed, it was the division process that was more pronounced. Mitochondrial fusion is promoted by mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (Opa 1), while fission is controlled by proteins such as Drp1 (44, 47). It was found that the expression of two kinds of fusion protein (Mfn 2 and Opa 1) in the control group was significantly higher than that in the experimental group. This is because the rats in the control group were only fed with ordinary feed, which would have a greater demand for energy. Therefore, the mitochondria in the liver of the rats in the control group were fused to generate more energy for cell utilization. There are more fusion phenomena in liver mitochondria of young control group rats, which may be related to the fact

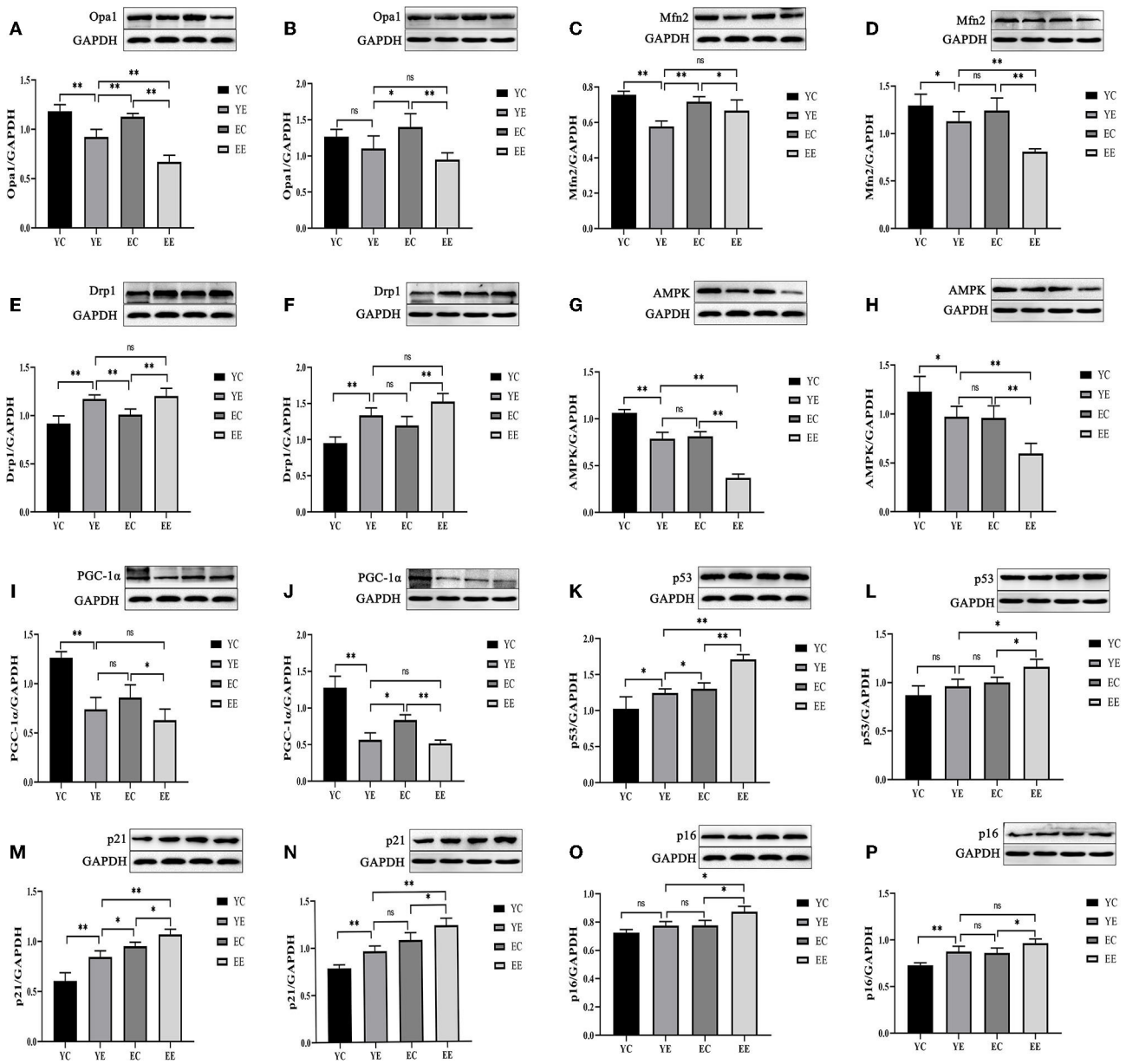

FIGURE 6 | High-fat diet-induced mitochondrial fusion and division, energy stress, and aging in diabetic rats. Protein level of Opa1**(A,B)**, Mfn2 **(C,D)**, Drp1 **(E,F)**, AMPK **(G,H)**, PGC-1α **(I,J)**, p53**(K,L)**, p21 **(M,N),** and p16 **(O,P)**. (Liver: **A,C,E,G,I,K,M,O**; Skeletal Muscle: **B,D,F,H,J,L,N,P**). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{ns}p > 0.05$.

that young control group rats are in the youth stage and their own metabolism and functions are relatively vigorous, so they will produce more ATP through mitochondria for cell utilization. In addition, endoplasmic reticulum stress can inhibit mitochondrial fusion caused by fusion proteins Mfn 1 and Mfn 2 and upregulate mitochondrial division proteins to increase mitochondrial division. Drp 1 is an important protein that regulates mitochondrial fission and clears damaged parts of the cell by regulating autophagy (48). By detecting the expression of Drp 1, it was found that the expression of division protein in the experimental group was also higher than that in the control group. This indicates that continuous high-energy diet will reduce the body's demand for energy. Therefore, mitochondrial division will occur in the liver tissue of rats in the experimental group, which is also the repair of mitochondria after injury. There was no significant difference in the expression of Drp 1 protein between the YE and EE groups, which indicated that the liver and skeletal muscle of the two groups suffered the same degree of injury. However, due to the stronger metabolism and reparability of the young body, the damaged components can be treated faster than the old body. Therefore, according to the results, it can be known that the reparability of the liver and skeletal muscle of the YE group is stronger than that of the EE group.

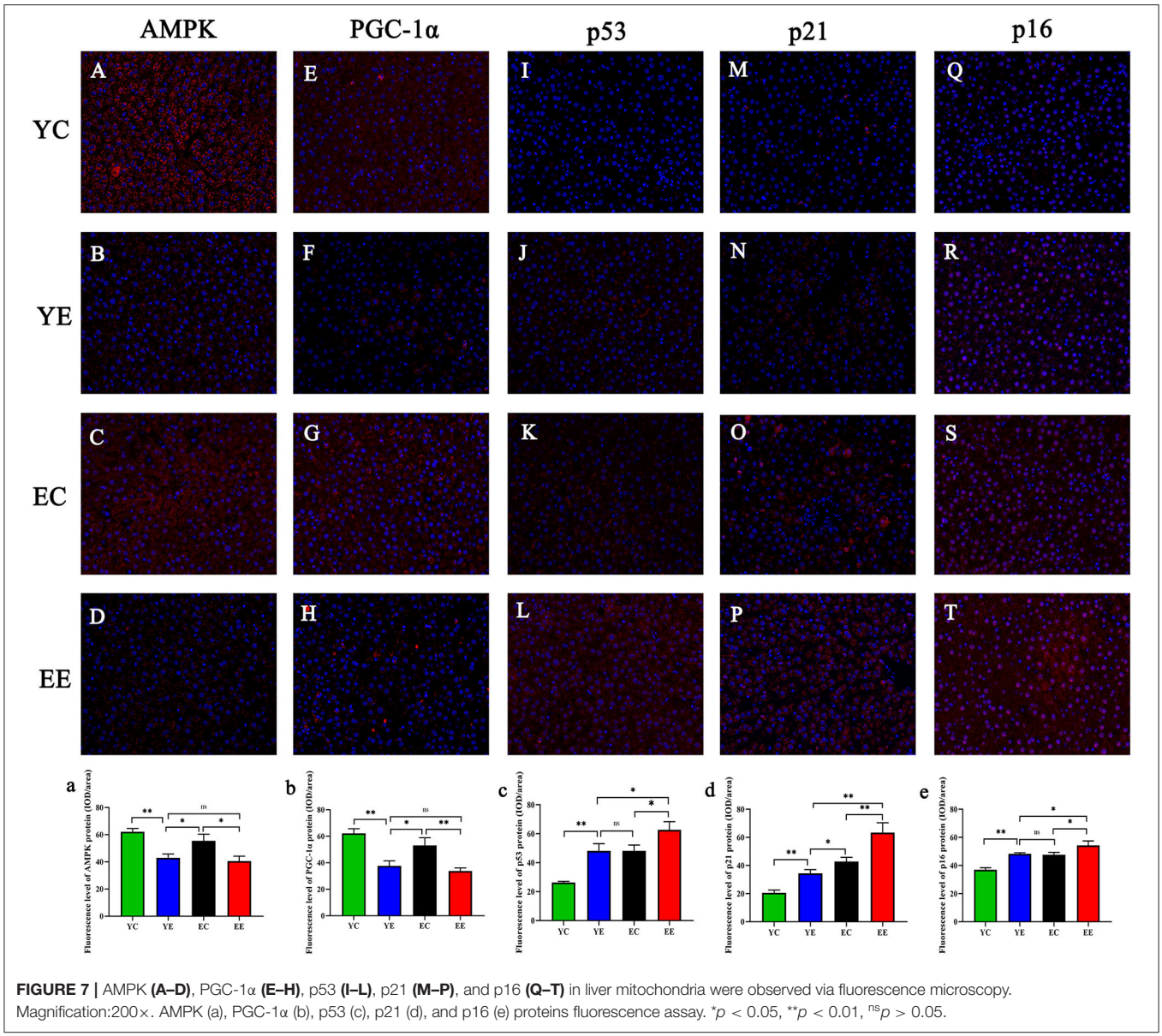

FIGURE 7 | AMPK **(A–D)**, PGC-1α **(E–H)**, p53 **(I–L)**, p21 **(M–P)**, and p16 **(Q–T)** in liver mitochondria were observed via fluorescence microscopy. Magnification:200×. AMPK (a), PGC-1α (b), p53 (c), p21 (d), and p16 (e) proteins fluorescence assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{ns}p > 0.05$.

The study found that skeletal muscle mainly provides energy by oxidizing FFA at rest (49, 50). When high-fat diet and obesity will cause the increase in fatty acids in blood exceeding the oxidation capacity of skeletal muscle, excessive esterified fatty acids are deposited in skeletal muscle, which will affect insulin-mediated signal transduction and lead to insulin resistance (51). The results of this experiment showed that the level of IMTG in the YE group was significantly lower than that in the EE group. This may be because the skeletal muscle of young rats still has a strong oxidative capacity and a relatively high therapeutic level of fatty acid deposition compared to old rats. However, the IMTG levels in the YE group were higher than those in the EC group, suggesting that long-term consumption of a high-fat diet in young people would impair the oxidative capacity of skeletal muscle to fatty acids, even lower than the oxidative level of healthy older bodies, resulting in muscle "premature aging." At the same time, muscle mitochondrial volume was reduced in subjects with insulin resistance or type 2 diabetes (52), and this confirms that the muscle fatty acid oxidation ability of YE rats is stronger than that of EE group. Given that the ability to store glucose in the form of glycogen is a hallmark of insulin sensitivity (53), we measured glycogen content in skeletal muscle. Compared with YC group, the content of muscle glycogen in EC group decreased significantly, indicating that the content of muscle glycogen decreased with the increase in age. This may be because the skeletal muscle in aged rats has an impaired ability to upregulate glycogen synthesis and the insulin function of the elderly body is more defective than that of the young body. But there was no significant difference in muscle glycogen content between YE group and EE group, and it shows that the decrease in insulin sensitivity of skeletal muscle in two groups is similar, which all leads to the decrease in glucose absorption by skeletal

muscle. This maybe because the impairment occurs early in intracellular glucose metabolism concomitantly with an initial, rapid, and disproportionate increase in fat mass, and compared with the old rats, the young rats have stronger glycogen synthesis ability (54).

There is growing evidence support that mitochondrial dysfunction links to diabetes (55), while it has been known for a long time that AMPK and PGC-1α act as two major regulators of mitochondrial function (56). AMPK is activated in response to an increase in the cellular AMP/ATP ratio, indicative of an energy deficit, and acts to switch the cellular metabolic program from ATP consumption to ATP production (57). We therefore measured AMPK and PGC-1α expression in the liver and skeletal muscle of the rats, as well as liver ATP content. The results showed that the protein expression of AMPK and PGC-1α was lower in the YE and EE groups and higher in the YC and EC groups. In addition, the ATP content was lower in the YE and EE groups compared to the YC and EC groups. It indicates that rats in the experimental groups fed high-energy diets have lower energy requirements; at this time, the reserve capacity of ATP is reduced, the expression of AMPK and PGC-1α is downregulated, and the biosynthesis of mitochondria is reduced. Rats in the control group fed a normal diet would not have excess energy, have a higher ATP reserve capacity, and increase mitochondrial biosynthesis by regulating the activation of AMPK and PGC-1a to produce more ATP for cellular use. AMPK protein expression in the YE group was significantly higher than that in the EE group. This suggests that although both the EE and YE groups were fed high-fat diets and both developed insulin resistance, the rats of the YE group may have a greater energy requirement. Therefore, young rats may increase mitochondrial biosynthesis by activating AMPK and PGC-1α. In conclusion, compared with old rats, youth have a high metabolic ability and stronger energy utilization and storage capacity, and when the body requires energy, it immediately mobilizes energy-regulating factors to meet the body's energy needs. This tight regulation of energy also serves as a protection for the body itself against continuous high-energy diet-induced metabolic diseases, such as diabetes. However, due to poor metabolism and self-regulation, aged rats are unable to use or store excessive energy in a timely manner, thus greatly increasing the risk of developing metabolic diseases such as diabetes mellitus.

Numerous studies have shown diabetes affects endothelial cell fate by increasing the expression of p53, p21, and p16 (58). p21 belongs to the cyclin-dependent kinase (CDK) inhibitors that, in concert with various tumor suppressor proteins, such as p53 and p16, induce inhibition of DNA replication and control anti-proliferative programs (59, 60). However, p21 together with the tumor suppressor proteins p53 and p16 is not only an important mediator of quiescence-like growth arrest but also of senescence (61, 62). Remarkably, conditional overexpression of p21 has been reported to be associated with growth arrest and phenotypic features of senescence (63). In this experiment, compared with YC group, the expressions of aging proteins p53, p21, and p16 in liver and skeletal muscle increased in YE group. It shows that the insulin resistance model of rats induced by high-energy feed will increase the aging degree of young rats. Compared with the YE group, the aging phenotype and the expression of aging protein in the EE group were significant increase. During aging, ATM or ATR kinase will activate p53, which inhibits the activity of CDK and blocks the process of cell cycle by upregulating the downstream target gene p21. In addition, p16 can also increase its expression caused by the production of ROS and mitochondrial dysfunction, arrest the cell cycle through p16-pRb pathway, and promote cell aging. Some studies have shown that cell senescence is often beneficial in clinic. When cells accumulate a lot of injury, they will rely on cell cycle points and stress relief mechanism to maintain the stability of cell cycle. However, with the gradual accumulation of injury, the stability of cells will become worse and worse, which will start aging, apoptosis, and other procedures to prevent the malignant development of cells (64). Therefore, the aging of rats in the old experimental group and the young experimental group is more serious. The EE group showed the most severe aging according to the expression levels of p53, p21, and p16 proteins. From the results, the high-fat diet caused the most severe injury to the EE rats. Compared with the YC group, the degree of aging was significantly worse in the YE group, suggesting that a high-fat diet-induced diabetes in young leads to premature aging of the body. Notably, it is possible that the degree of injury was similar in the YE and EE groups, but because the young can clear the broken components of the cells to divide faster and repair the injury better, it resulted in a slightly lower degree of senescence in the YE group compared with the EC.

In summary, our results show that young rats fed a high-fat, high-energy diet have decreased mitochondrial biogenesis, mitochondrial injury, and cellular aging in liver and skeletal muscle close to those of older rats fed an ordinary diet. This suggests that even if young people are stronger than older people, if they suffer from diabetes due to chronic feeding of high-fat, high-energy foods, they will have more severe mitochondrial dysfunction than healthy older people and will cause premature aging of young body cells.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Tab of Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University.

AUTHOR CONTRIBUTIONS

JWa and JWu conceptualized the study. JWa and WL conducted the formal analysis. JWa wrote the original draft. XW, RL, TL, and JX wrote, reviewed, and edited the article. All authors have read and agreed to the published version of the manuscript.

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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 10 August 2022
DOI 10.3389/fnut.2022.935805

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

RECEIVED 04 May 2022
ACCEPTED 11 July 2022
PUBLISHED 10 August 2022

CITATION
Power Guerra N, Leyens K, Müller L, Brauer D, Janowitz D, Schlick S, Pilz K, Grabe HJ, Vollmar B and Kuhla A (2022) The effect of different weight loss strategies to treat non-alcoholic fatty liver disease focusing on fibroblast growth factor 21. *Front. Nutr.* 9:935805. doi: 10.3389/fnut.2022.935805

The effect of different weight loss strategies to treat non-alcoholic fatty liver disease focusing on fibroblast growth factor 21

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Objective: Obesity, often associated with non-alcoholic fatty liver disease (NAFLD), is characterized by an imbalance between energy expenditure and food intake, which is also reflected by desensitization of fibroblast growth factor 21 (FGF21). FGF21 is strongly influenced, among others, by TNFα, which is known to be upregulated in obesity-induced inflammation. Successful long-term treatments of NAFLD might be dietary modification, exercise, or fasting.

Materials and methods: Whether succeeded NAFLD recovery is linked with improved FGF21 sensitivity and finally reverted FGF21 resistance was the focus of the present study. For this purpose, mice received a high-fat diet (HFD) for 6 months to establish obesity. Afterward, the mice were subjected to three different weight loss interventions, namely, dietary change to low-fat diet (LFD), treadmill training, and/or time-restricted feeding for additional 6 months, whereas one group remained on HFD.

Results: In addition to the expected decrease in NAFLD activity with dietary change, this was also observed in the HFD group with additional time-restricted feeding. There was also an associated decrease in hepatic TNFα and FGF21 expression and an increase in ß-klotho expression, demonstrated mainly by using principal component analysis. Pearson correlation analysis shows that independent of any intervention, TNFα expression decreased with improved NAFLD recovery. This was accompanied with higher FGF21 sensitivity, as expressed by an increase in β-klotho and FGFR1c expression and concomitantly decreased FGF21 levels.

Conclusion: In summary, we conclude that successful NAFLD therapy is associated with a reversion of the TNFα-triggered FGF21-resistant state or desensitization.

KEYWORDS

non-alcoholic fatty liver disease, high-fat diet, dietary change, treadmill exercise, time-restricted feeding, FGF21, TNFα, β-klotho

Introduction

Overall, 25% of people worldwide suffer from overweight or obesity, which has reached pandemic proportions. As early as 1989, Kaplan described the "deadly quartet" of abdominal obesity, hypertension, hyperglycemia, and hypertriglyceridemia (1), which is referred to as metabolic syndrome (2, 3). The metabolic syndrome is associated with non-alcoholic fatty liver disease (NAFLD), which is considered as hepatic manifestation of this disease (4–6). One potential reason for the prevalence of NAFLD and many other comorbidities and sequelae of obesity is the persistence of a systemic low-grade inflammation (LGI) (7, 8). In this context, white adipose tissue is capable of expressing both metabolic and immunological mediators (9), which act locally but may also have systemic effects affecting other organs, such as the liver. This is reflected by hepatic and also systemic upregulation of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNFα) (10–12). All these mediators are well coordinated and reciprocally regulated in signaling cascades. An imbalance of these mediators is likely responsible for an LGI-mediated interaction between obesity and NAFLD (13, 14).

In order to better understand the causality of obesity-related inflammatory processes, it is necessary to consider individual hormones, such as leptin, ghrelin, or orexin. They are involved in the regulation of food intake. A dysregulation of these hormones, also triggered by obesity-induced LGI, can further aggravate obesity. In addition, fibroblast growth factor 21 (FGF21), a hormone in addition to fatty acid oxidation, lipolysis, and increased energy dissipation, is also involved in the regulation of food uptake. FGF21 acts *via* its receptor complex of FGF21 receptor (FGFR)1c and β-klotho in an endocrine or paracrine manner (15–17).

Remarkably, on the one hand, FGF21 expression is increased in the liver during fasting states and caloric restriction (18, 19); on the other hand, exceptionally high circulating plasma FGF21 concentrations occur in obese humans and mice (20, 21). Termed the "FGF21 paradox" by Fisher et al. (21), this phenomenon describes, similar to leptin resistance (22), an FGF21-resistant state (21), although more recently, the term FGF21 desensitization has been used in this context (23). Thereby, increased FGF21 concentrations in obesity were associated with a concomitant reduction in the FGF21 receptor complex (21). A link between FGF21 and inflammation was demonstrated by Diaz-Delfin and coworkers in mouse adipocytes, where the application of TNFα inhibited β-klotho expression (24). Furthermore, a long-term study on the adipose tissue of obese mice demonstrated that expression of FGFR1c and β-klotho was markedly decreased and associated with a limited effect of exogenously applied FGF21, implying a decrease in FGF21 sensitivity.

To overcome the vicious cycle of obesity and hence obesity-induced LGI, intervention approaches, such as physical activity, dietary changes, or fasting are appropriate and commonly used methods (25–27). Therefore, the purpose of this study was to investigate to which extent obesity-associated NAFLD and accompanying inflammatory processes are reversible by treadmill training, dietary change, and/or time-restricted feeding, and whether this is associated with enhanced hepatic FGF21 sensitivity. Using principal component and correlation analyses, we tested the hypothesis that recovery from NAFLD is associated with a reversion of FGF21 resistance or desensitization.

Materials and methods

Animals

At the beginning, 90 4-week-old female mice (C57BL/6J) were purchased from Charles River (Sulzfeld, Germany). In compliance with our previous and ongoing investigations, female mice were used for comparability between different studies (28). All animal experimental work was carried out with permission of the local Animal Research Committee [Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) of the state Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-2-001/18, approved on March 1, 2018)] and by following the ARRIVE guidelines. All animals received human care according to the EU Directive 2010/63/EU. The mice were divided in a blinded manner into groups of five mice and were kept in standard cages. The room temperature was controlled (21 ± 3°C), and a 12-/12-h day/night cycle (lights on from 6:00 a.m.

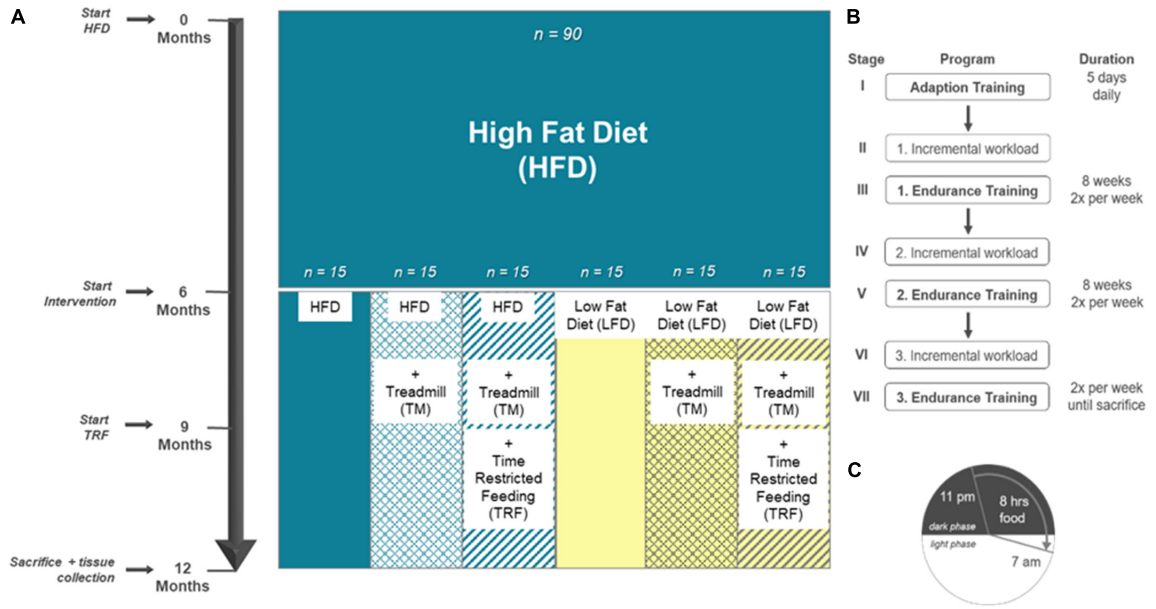

FIGURE 1
Experimental design adapted to Power Guerra et al. (28). **(A)** Female C57BL/6J mice (n = 90) were fed for 6 months with high-fat diet (HFD) to establish the model of diet-induced obesity. Thereafter, mice were divided into six groups. The first group remained on the HFD (n = 90). Groups 2–6 underwent an intervention. Second group: HFD plus treadmill exercise (TM; HFD/HFD + TM, n = 15); third group: HFD plus treadmill exercise and time-restricted feeding (TRF; HFD/HFD + TM + TRF, n = 15); fourth group: dietary change to a low-fat diet (LFD; HFD/LFD, n = 15); fifth group: dietary change plus treadmill exercise (HFD/LFD + TM, n = 15), and sixth group: dietary change, treadmill training, and time-restricted feeding (HFD/LFD + TM + TRF, n = 15). When dietary change was completed, treadmill training started. After 3 months of endurance exercise, time-restricted feeding was introduced. At the end, the mice were killed, and blood and liver tissue were collected. **(B)** Treadmill protocol consists of seven stages with three endurance sections. Prior to endurance training, an incremental workload test was performed to adjust the maximum velocity of the run. **(C)** Mice in the TRF group were restricted food from 7 a.m. to 11 p.m. (16 h). Food supply was provided in the nocturnal active phase for 8 h.

to 6:00 p.m.) was applied. All mice were handled equally for the first 6 months while establishing the model of diet-induced obesity. Therefore, all 90 mice received a high-fat diet (HFD; D12492; Research Diets, New Brunswick, NJ, United States) for 6 months *ad libitum*. According to randomization, the cages were divided into six groups. In the following 6 months, interventions were carried out as previously published by our group (28). The first group (n = 15) remained on an HFD, referred to as "HFD/HFD". In the second group, named "HFD/HFD + TM", treadmill (TM) exercise (TM 303401; TSE Systems Inc., Chesterfield, MO, United States) (n = 15) was added. The third group (n = 15) was trained on treadmills, and additionally, time-restricted food (TRF) intake was introduced after 3 months of intervention, designated as group "HFD/HFD + TM + TRF". The HFD was changed to a low-fat diet (LFD; D12450J; Research Diets, New Brunswick, NJ, United States) for (n = 15), building the fourth group "HFD/LFD". The fifth group (n = 15), called "HFD/LFD + TM", also changed diet to an LFD in addition to treadmill exercise for time of interventions. The last group (n = 15) underwent all given interventions, named "HFD/LFD + TM + TRF". Figure 1A illustrates the experimental design. Body weight was measured weekly and just before killing of the mice.

Interventions

Dietary change to low-fat diet

For the first intervention, 45 mice received an LFD, containing 10% fat, 20% protein, and 70% carbohydrates, matching the HFD in structure of lard and protein composition. Contrary to this, the HFD consists of 60% fat, 20% protein, and 20% carbohydrates.

Treadmill exercise

In addition to dietary change, TM exercise was established for n = 60 mice as the second intervention parameter. The exact TM protocol was previously described by our group (28). In brief, TM exercise was performed twice a week, running through a program, as shown in Figure 1B.

Time-restricted food

To treat obesity and metabolic disorders, time limited restriction of food is described as a beneficial method (29, 30),

which was the third intervention parameter, namely, TRF. After the third phase of TM exercise (**Figure 1C**), TRF was introduced to $n = 30$ mice using the same protocol, as previously described by our group (28). For the last 3 months, TRF was maintained. Food regulation was performed by using an autofeeder (EHEIM, Deizisau, Germany) with an enlarged opening. The food drop was controlled at 11 p.m. *via* a webcam with infrared light. The mice were transferred back to fresh cages at 7 a.m. with water supply and no enrichments.

In vitro experiment

The human hepatoma cell line HepG2 was used for *in vitro* experiments and was cultured as reported by Guy et al. (31). The cells were seeded in six-well plates. After reaching 95% confluence, cells were incubated with 20 μg/mL human TNFα (hTNFα; Sigma Aldrich, Taufkirchen, Germany) or Aqua Dest (B. Braun Melsungen AG, Melsungen, Germany) for 24 h. Afterward, the cells were harvested for analysis of β-klotho protein expression.

Sampling and assays

Under anesthesia (5 vol.% isoflurane; Baxter, Unterschleißheim, Germany), the mice were exsanguinated *via* retrobulbar puncture. Blood was collected and prepared according to Power et al. (31). Thereafter, a laparotomy was performed. The visceral and subcutaneous flanked fat deposits and liver tissue were harvested and weighted. Subsequently, the left lateral liver lobe was fixed in 4% paraformaldehyde (PFA, sc281692 Santa Cruz Biotechnology Inc., Dallas, TX, United States) for 5 days and embedded in paraffin (Carl Roth, Karlsruhe, Germany). The left medial lobe was embedded in Tissue-Tek® (Sakura Finetek Germany GmbH, Umkirch, Germany), snap-frozen in liquid nitrogen with the remaining tissue, and stored at −20°C. For the assessment of liver damage, plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin activities were spectrophotometrically determined (Cobas c111; Roche Diagnostics, Mannheim, Germany) using commercially available reaction kits (Roche Diagnostics, Mannheim, Germany). Measurements of the LDL/VLDL fraction, triglycerides, leptin, insulin, and FGF21 in plasma were performed using the LDL/VLDL cholesterol, leptin, insulin, and FGF21 assay kits according to the manufacturers' instructions (LDL/VLDL: Abcam, Cambridge, United Kingdom; leptin, insulin, FGF21: R&D System, Minneapolis, MN, United States; triglycerides: Cayman Chemical Company, Ann Arbor, MI, United States).

Histology, immunohistochemistry, and image analysis

Hematoxylin and eosin (H&E) staining (Merck, Darmstadt, Germany) was performed using standard protocols on 4 μm thin tissue sections. Images were recorded on a Carl Zeiss Axioskop 40 microscope (Carl Zeiss AG, Oberkochen, Germany) with a Zeiss AxioCamMRc5 camera (Carl Zeiss AG, Oberkochen, Germany) and corresponding Zeiss ZEN2 lite software (Carl Zeiss AG, Oberkochen, Germany).

From the H&E-stained specimen, the NAFLD Activity Score (NAS) was assessed in a blinded manner to characterize diet-induced liver damage. Following the description by Kleiner et al. (32) and our previous work (33), the parameters steatosis (scores 0–3), hepatocellular ballooning (scores 0–2), and lobular inflammation (scores 0–3) were used to calculate the NAS (total scores 0–8). Steatosis was assessed at 50× magnification and ballooning at 100× magnification. Inflammation was assessed by counting inflammatory foci from 10 representative low-power fields (LPF) (200× magnification), characterized as a grouping of at least five inflammatory cells in the tissue that are not arranged in a row (34). For Oil Red O staining of lipids, the frozen liver tissue was cut in 8 μm thick sections, air-dried, and fixed in paraformaldehyde. The staining was performed using Oil Red O (Sigma-Aldrich Corp., St. Louis, MO, United States) and counterstained with hematoxylin. In total, 10 images at 400× magnification were taken per sample. Quantitative analysis of the red-stained area was conducted using ImageJ (v 1.52, Wayne Rasband, National Institutes of Health, United States) (protocol provided in the supplements as ImageJ Code S1) analyzing the percentage of red pixels per image.

To substantiate inflammatory processes in the liver, naphthol-AS-C-chloracetate esterase (CAE) staining (Sigma Aldrich Corp., St. Louis, MO, United States) was used for characterizing granulocytes. After fixing in paraformaldehyde and embedding, sections were stained with CAE and counterstained with hematoxylin. The ratio of CAE-positive cells (CAE+) and the total number of hepatocytes in 10 consecutive high power fields (HPF) at 400× magnification were used to quantify granulocytes in a blinded manner. Macrophages were immunohistochemically stained for the indication of cellular hepatic LGI. Therefore, overnight incubation (4°C) with a rat anti-mouse-F4/80 (MCA497; Bio-Rad, Hercules, CA, United States) was followed by 1 h incubation at room temperature with the second antibody (goat anti-rat; abcam 97054; Abcam, Cambridge, United Kingdom). Afterward, the cells were stained with the chromogen Permanent Red (Ref. K0640, DAKO GmbH, Jena, Germany) and counterstained with hematoxylin. For quantification, the total number of F4/80-positive cells (F4/80+)

and the total number of hepatocytes were also counted in a blinded manner in 10 consecutive HPF at 400× magnification.

Western blot analysis

The harvested liver tissue and HepG2 cells incubated with TNFα were further processed for protein isolation. For this purpose, the liver tissue and cells were homogenized in lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X100, 0.02% NaN3, and 0.2 mM PMSF, protease inhibitor cocktail), incubated for 30 min on ice, and centrifuged for 10 min at 4°C and 10,000 × *g*. Protein contents were assayed by using the bicinchoninic acid method (Pierce Biotechnology Inc., Thermo Fisher Scientific, Waltham, MA, United States), with 2.5% BSA (Pierce Biotechnology Inc., Thermo Fisher Scientific, Waltham, MA, United States) as the standard. On an 8% SDS gel (FGFR1c and pFGFR1c) and a 10% Mini-PROTEAN® TGX Stain-FreeTM (Bio-Rad Laboratories, Munich, Germany) gel (β-klotho), 20 (liver tissue) or 10 (HepG2 cells) µg protein was separated. Mini-PROTEAN TGX gel was captured using the ChemiDoc XRS System (Bio-Rad Laboratories, Munich, Germany) before being transferred to a polyvinyldifluoride membrane (Immobilon-P; Millipore, Eschborn, Germany). After blockade with 2.5% BSA (Santa Cruz Biotechnology, Santa Cruz, CA, United States), membranes were incubated overnight at 4°C with a rabbit polyclonal anti-β-klotho (1:1.000, LSBioScience, Seattle, WA, United States), a rabbit polyclonal anti-pFGFR1c (Tyr653/654; 1:1.000, Cell Signaling Technology, Cambridge, United Kingdom), or a rabbit monoclonal anti-FGF21 [EPR8314(2), only HepG2 cells, 1:1.000, abcam, Cambridge, United Kingdom] antibody, respectively. Afterward, a secondary peroxidase-linked anti-rabbit antibody (β-klotho and pFGFR1c, 1:10.000; Cell Signaling Technology, Cambridge, United Kingdom) or only HepG2 cells (FGF21, 1:3.000) was applied. Protein expression was visualized by means of luminol-enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech, Freiburg, Germany) and digitalized using the ChemiDoc™ XRS System (Bio-Rad Laboratories, Feldkirchen, Germany). Signals were densitometrically assessed (Quantity One; Bio-Rad Laboratories, Munich, Germany) and normalized either to the GAPDH signals (β-klotho and FGF21, HepG2 cells, and mouse monoclonal anti-β-GAPDH antibody; 1:20.000; Millipore, Eschborn, Germany, followed by secondary anti-mouse antibody, 1:40.000, Sigma Aldrich Corp., St. Louis, MO, United States) or to whole protein (β-klotho, liver tissue). To analyze the phosphorylation status of FGFR1c, signals of pFGFR1c were normalized to rabbit polyclonal anti-FGFR1c (clone D8E4, 1:1.000, Cell Signaling Technology, Cambridge, United Kingdom).

Quantitative real-time polymerase chain reaction

Ribonucleic acid (RNA) isolation and transcription into cDNA were performed as already published (28). mRNA expression analyses were performed *via* quantitative real-time polymerase chain reaction (PCR) in a BioRad iQ5 Multicolor Real Time PCR Detection System (Conquer Scientific, San Diego, CA, United States) with an iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Munich, Germany). Primer sequences are shown in **Table 1**. Measurement results are corrected against the housekeeping gene 40S ribosomal protein S18 (RPS18), and relative quantification was carried out *via* the $2^{-\Delta\Delta CT}$ method.

Statistics

Statistical analysis was performed using GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA, United States) as previously described by our group (28). Data from animals that died during the experimental period or showed abnormalities during organ removal were excluded from all analysis. For expressional analyses of liver FGF21, IL-1β, IL-6, IL-10, and TNFα, each $n = 7$ samples and for HDL, LDL, and cholesterol assays, each $n = 5$ samples were measured. The ROUT method based on the false discovery rate ($Q = 0.01$) was applied to remove outliers. All results are presented as mean ± standard deviation (SD), and statistical significance was set at $p < 0.05$. For further details, see figure legends.

To represent correlation between all observational parameters, Pearson correlation was performed by measuring linear dependence of two parameters. Clustering between the six groups is represented as a dot plot of principal component analysis (PCA). For the parameters TNFα, FGF21, and β-klotho expressions in the liver, all data from $n = 84$ mice are included. Correlation between all 26 parameters (for AST, ALT, insulin, and leptin, see **Supplementary Figure 1**) is shown in a heatmap, indicating a strong positive correlation by red color (1.00–0.70), a strong negative correlation by blue color (−0.70 to −1.00),

TABLE 1 Primers used for quantitative real-time polymerase chain reaction (PCR).

Transcript	Forward primer (5′–3′)	Reverse primer (5′–3′)
fgf21	GCTGTCTTCCTGCTGGGG	CCTGGTTTGGGGAGTCCTTC
tnfα	ACATTCGAGGCTCCAGT GAATTCGG	GGCAGGTCTACTTTGGAGT CATTGC
il-1β	CCCAAGCAATACCCAAAGAA	TTGTGAGGTGCTGATGTACCA
il-6	TCTGACCACAGTGAGGA ATGTCCAC	TGGAGTCACAGAAGGAGT GGCTAAG
il-10	GCCTTGCAGAAAAGAGAGCT	AAAGAAAGTCTTCACCTGGC
rps18	AGGATGTGAAGGATGGGAAG	TTGGATACACCCACAGTTCG

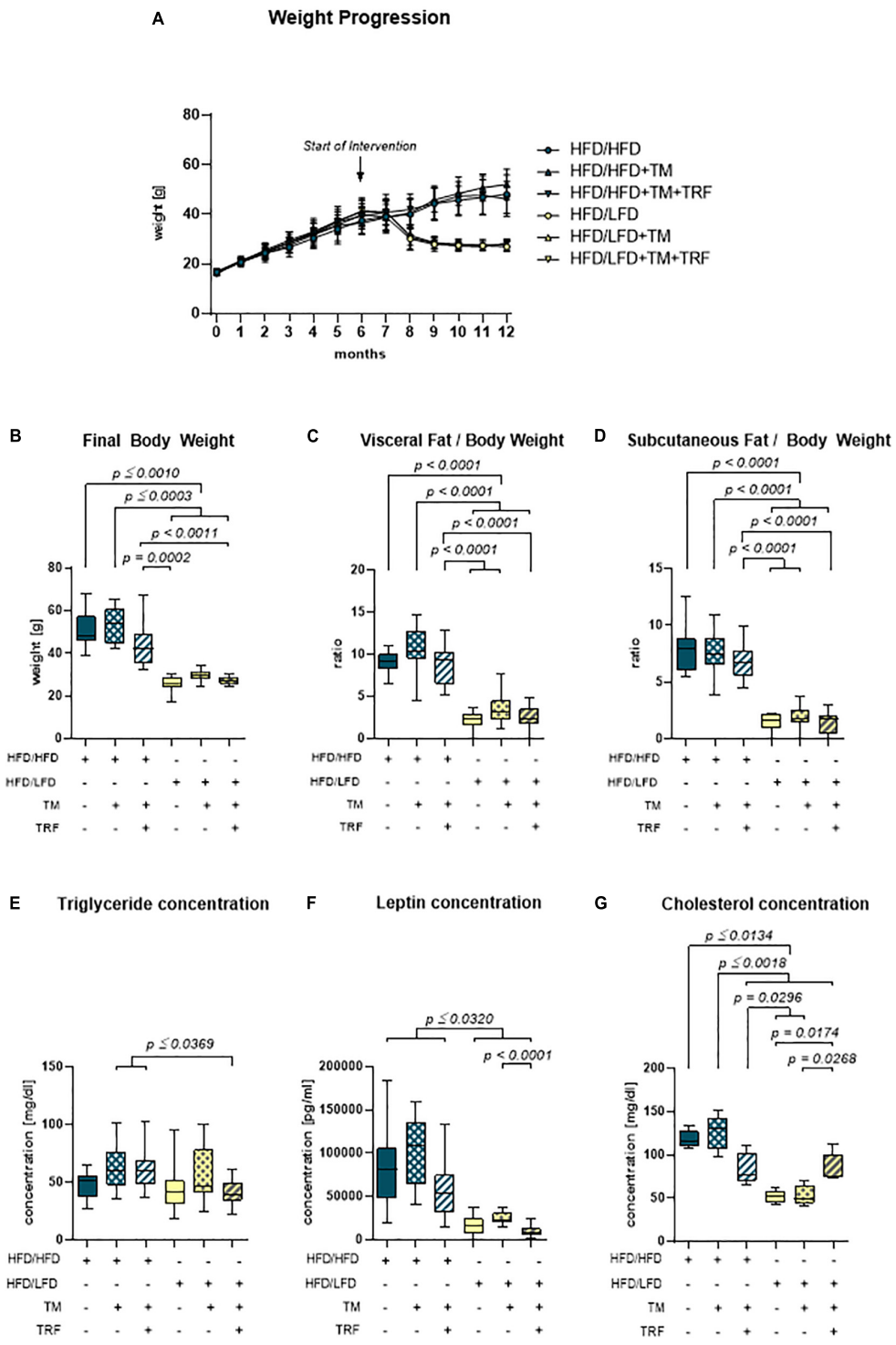

FIGURE 2
Body and fat composition. **(A)** Monthly weight progression with n = 90 mice at the beginning and n = 84 mice at the final time point. **(B)** Final body weights [g] before euthanasia (HFD/HFD: n = 13, HFD/HFD + TM: n = 13, HFD/HFD + TM + TRF: n = 15, HFD/LFD: n = 14, HFD/LFD + TM: n = 15, HFD/LFD + TM + TRF: n = 13; total n = 83). **(C)** Ratio of visceral body fat deposits to body weight. **(D)** Ratio of subcutaneous flanked fat deposits to body weight (**C,D** HFD/HFD: n = 13, HFD/HFD + TM: n = 13, HFD/HFD + TM + TRF: n = 15, HFD/LFD: n = 14, HFD/LFD + TM: n = 15, HFD/LFD + TM + TRF: n = 14; total n = 84). All HFD/LFD groups showed a significant fat loss with $p < 0.0001$ when compared to all three HFD/HFD groups, respectively [**A–D** from Power Guerra et al. (28)]. **(E)** Plasma triglyceride [mg/dL] (HFD/HFD: n = 13, HFD/HFD + TM: n = 13, HFD/HFD + TM + TRF: n = 15, HFD/LFD: n = 14, HFD/LFD + TM: n = 15, HFD/LFD + TM + TRF: n = 14; total n = 84). **(F)** Plasma leptin [pg/mL] (HFD/HFD: n = 13, HFD/HFD + TM: n = 13, HFD/HFD + TM + TRF: n = 14, HFD/LFD: n = 14, HFD/LFD + TM: n = 15, HFD/LFD + TM + TRF: n = 13; total n = 82) and **(G)** plasma cholesterol [mg/dL] (HFD/HFD: n = 5, HFD/HFD + TM: n = 5, HFD/HFD + TM + TRF: n = 5, HFD/LFD: n = 5,

(Continued)

FIGURE 2
HFD/LFD + TM: n = 5, HFD/LFD + TM + TRF: n = 5; total n = 30). Blue dots and box plots indicate HFD groups, and yellow dots and box plots indicate dietary change to LFD. Table displays the individual groups, respectively. Table is read from top to bottom; "+" denotes implementation of a given diet or intervention and "−" its absence. Significance of differences between groups was tested with either the Kruskal–Wallis test, followed by Dunn's *post hoc* test for multiple comparisons **(B)**, the Brown–Forsythe test, and Welch's ANOVA with the Tamhane T2 *post hoc* test for multiple comparisons [**C:** F value (F) = 51.82, degree of freedom (DF) = 5; **D:** F = 66.19; DF = 5. **F:** F = 22.68, DF = 5], or by ordinary one-way ANOVA with Tukey's *post hoc* test for multiple comparisons **(E)**. Data are presented as mean ± SD, and statistical significance was set at $p < 0.05$. HFD, high-fat diet; LFD, low-fat diet; TM, treadmill; TRF, time-restricted feeding.

and a moderate correlation by light colors (>0.40 or <-0.40) (35). The analysis was carried out in R (version 4.0.2, R studio, Boston, United States) *via* the prcomp method. Missing values were imputed ahead of analysis using the mean of the respective experimental group.

Results

Diet-induced obesity is attenuated by intervention strategies

Continuous administration of the HFD caused a large increase in body weight within the first 6 months (**Figure 2A**). After the introduction of the intervention approaches, such as LFD, TM training, and TRF, only the dietary change to LFD resulted in weight loss within a few weeks (**Figure 2A**, yellow vs. blue). Also, final body weight, and visceral and subcutaneous fat-to-body weight ratios were about 50% lower than those in all HFD/HFD groups (blue) (**Figures 2B–D**, yellow vs. blue). While in the HFD/LFD groups triglyceride concentrations were only decreased in tendency (**Figure 2E**), the concentrations of leptin and cholesterol were significantly reduced (yellow; **Figures 2F,G**).

High-fat diet-induced non-alcoholic fatty liver disease is treatable by intervention strategies leading to reduced pro-inflammatory tumor necrosis factor alpha expression

Accordingly, liver fat content, evaluated by Oil Red O staining, was found to be significantly diminished after dietary change (**Figures 3A,B**), which was also reflected by a significantly lower steatosis score (**Figures 3C,D**). Notably, livers of the HFD mice receiving TM and TRF displayed almost the same histological changes as observed in all LFD groups. In particular, Oil Red O staining showed a significant reduction in liver lipid in the HFD/HFD + TM + TRF group compared to the HFD/HFD group (**Figure 3B**). Although the parameters of hepatocellular ballooning and lobular inflammation were largely unchanged upon interventions, the NAFLD score was significantly decreased in all groups with dietary change, and in particular upon TM and TRF in the HFD group (**Figure 3E**). Despite the strong decrease in the lipid content after dietary change, there was no reduction in the number of resident macrophages and granulocytes in the liver, as indicated by no significant differences in the number of F4/80 (**Figures 4A,B**) and CAE (**Figures 4C,D**)-positive cells between the individual experimental groups. Consistent with this, mRNA expressions of the pro-inflammatory cytokines IL-1β and IL-6 were also nearly unchanged (**Figures 5A,B**), but the anti-inflammatory cytokine IL-10 showed tendencies to be increased mainly upon dietary change (**Figure 5C**). Noteworthy, the mRNA expression of TNFα was significantly decreased in all LFD vs. HFD groups (**Figure 5D**).

Reduced tumor necrosis factor alpha expression is accompanied by restoration of an fibroblast growth factor 21 sensitive state

Parallel to the reduction of TNFα, a significant decrease in the FGF21 concentration in plasma (**Figure 5F**) was observed. This was in line with the significant reduction of hepatic FGF21 mRNA expression (**Figure 5E**), whereby HFD mice receiving additional TM and TRF showed similar values, indicating that not only dietary change but also physical activity and intermittent fasting may trigger FGF21 levels. Of particular interest was the significant increase in hepatic β-klotho expression in all dietary change groups (**Figure 5G**), which was also associated with increased phosphorylation of FGFR1c, although this was only seen in the LFD groups with additional TM and TRF (**Figure 5H**). These results now suggest that especially with the dietary change, FGF21 sensitivity could be restored, that is, reduced FGF21 expression and increased expression of its receptors. The statistical results were confirmed by PCA (**Figure 6**). PCA showed for hepatic TNFα (A), FGF21 (B), and β-klotho (C) that the LFD groups clustered mainly on one side and the HFD groups on the other. In addition to this, the trained HFD group with additional intermittent fasting clustered more with the LFD groups. A further consideration of the correlation analysis (**Figure 6D**) showed that TNFα correlated with parameters, such as body weight, percentage of adipose tissue, and liver fat content (on average with r = 0.6;

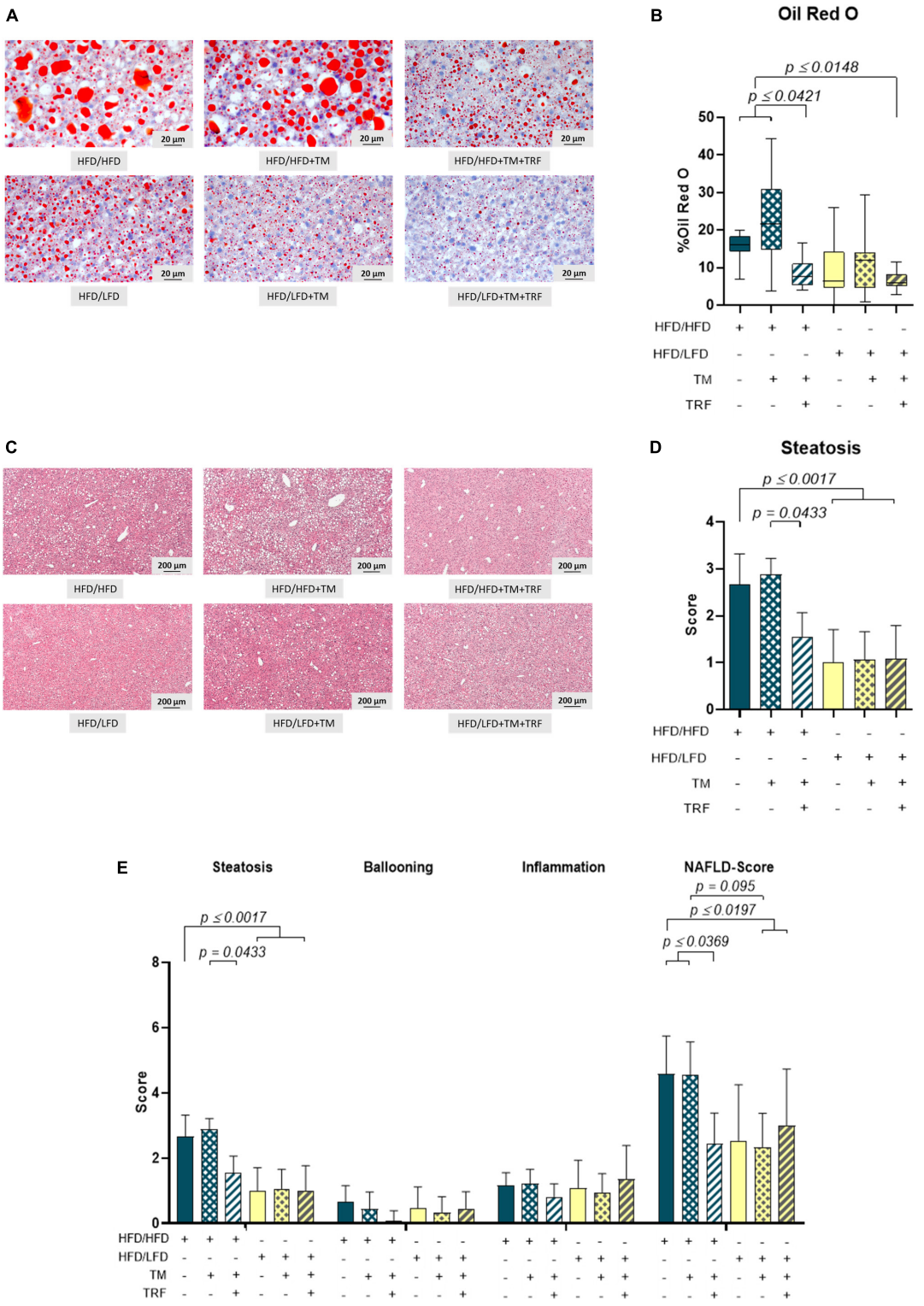

FIGURE 3
Representative images of Oil Red O **(A)** (400× magnification, scale bar represents 200 µm) and HE-stained liver specimen. **(C)** (50× magnification, scale bar represents 200 µm) and quantitative analysis of the Oil Red O-stained area in percentage **(B)** (HFD/HFD: *n* = 11, HFD/HFD + TM: *n* = 11, HFD/HFD + TM + TRF: *n* = 12, HFD/LFD: *n* = 13, HFD/LFD + TM: *n* = 15, HFD/LFD + TM + TRF: *n* = 10; total *n* = 72) and steatosis score **(D)** (HFD/HFD: *n* = 12, HFD/HFD + TM: *n* = 9, HFD/HFD + TM + TRF: *n* = 11, HFD/LFD: *n* = 13, HFD/LFD + TM: *n* = 15, HFD/LFD + TM + TRF: *n* = 11; total *n* = 71). Assessments of scores for steatosis, ballooning, and inflammation, as well as calculation of NAS for
(Continued)

FIGURE 3
the groups, are represented in panel **E**. Blue dots and box plots indicate HFD groups, and yellow dots and box plots indicate dietary change to LFD. Table displays the individual groups, respectively. Table is read from top to bottom; "+" denotes implementation of a given diet or intervention and "−" its absence. Significance of differences between groups was tested with either the Kruskal–Wallis test, followed by Dunn's *post hoc* test for multiple comparisons **(B)**, the Brown–Forsythe test, and Welch's ANOVA with the Tamhane T2 *post hoc* test for multiple comparisons [**D:** *F* value (*F*) = 8.297, degree of freedom (DF) = 5, or by ordinary one-way ANOVA with Tukey's *post hoc* test for multiple comparisons (**E:** *F* = 9.765, DF = 5)]. Data are presented as mean ± SD, and statistical significance was set at $p < 0.05$. HFD, high-fat diet; LFD, low-fat diet; TM, treadmill; TRF, time-restricted feeding.

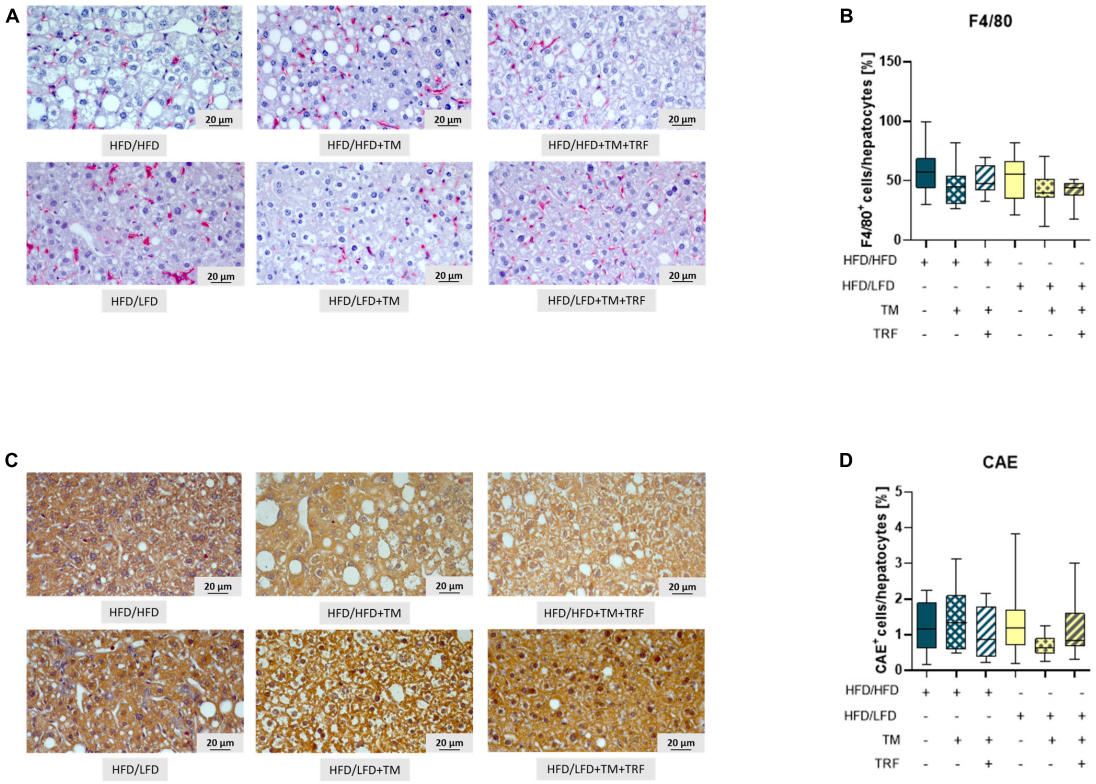

FIGURE 4
Representative images (both at 400× magnification, scale bar representing 20 µm) of F4/80 **(A)** as well as CAE-stained livers **(C)** and quantitative analysis of F4/80+ (**B:** HFD/HFD: *n* = 12, HFD/HFD + TM: *n* = 10, HFD/HFD + TM + TRF: *n* = 12, HFD/LFD: *n* = 13, HFD/LFD + TM: *n* = 15 or HFD/LFD + TM + TRF: *n* = 11; total *n* = 73) as well as CAE+ (**D:** HFD/HFD: *n* = 12, HFD/HFD + TM: *n* = 9, HFD/HFD + TM + TRF: *n* = 12, HFD/LFD: *n* = 13, HFD/LFD + TM: *n* = 13, HFD/LFD + TM + TRF: *n* = 11; total *n* = 70). Blue dots and box plots indicate HFD groups, and yellow dots and box plots indicate dietary change to LFD. Table displays the individual groups, respectively. Table is read from top to bottom; "+" denotes implementation of a given diet or intervention and "−" its absence. Significance of differences between groups was tested with either the Kruskal–Wallis test, followed by Dunn's *post hoc* test for multiple comparisons **(B)** or the Brown–Forsythe test, and Welch's ANOVA with the Tamhane T2 *post hoc* test for multiple comparisons **(D)** [*F* value (*F*) = 1.313, degree of freedom (DF) = 5]. Data are presented as mean ± SD, and statistical significance was set at $p < 0.05$. HFD, high-fat diet; LFD, low-fat diet; TM, treadmill; TRF, time-restricted feeding.

all $p \leq 0.05$). Of particular interest is that the NAFLD score positively correlated with hepatic TNFα ($r = 0.38$; $p \leq 0.05$), and particularly with systemic FGF21 ($r = 0.41$; $p \leq 0.05$).

Furthermore, the degree of dependency between TNFα and FGF21 sensitivity was investigated by correlation analysis (Figure 6D). We found that hepatic β-klotho correlated negatively with hepatic TNFα ($r = -0.38$; $p \leq 0.05$) and with hepatic FGF21 ($r = -0.32$; $p \leq 0.05$), while hepatic FGF21 correlated strongly positively with TNFα ($r = 0.82$; $p \leq 0.05$). This finding was partly supported by the *in vitro* analysis in HepG2 cells, showing that TNFα is indeed able to significantly reduce β-klotho expression (Figure 7A), whereas the phosphorylation of FGFR1c and protein expression of FGF21 was almost unchanged (Figures 7B,C).

Discussion and conclusion

The main finding of the study was that reversion of NAFLD is achieved not only by dietary change but also by continued

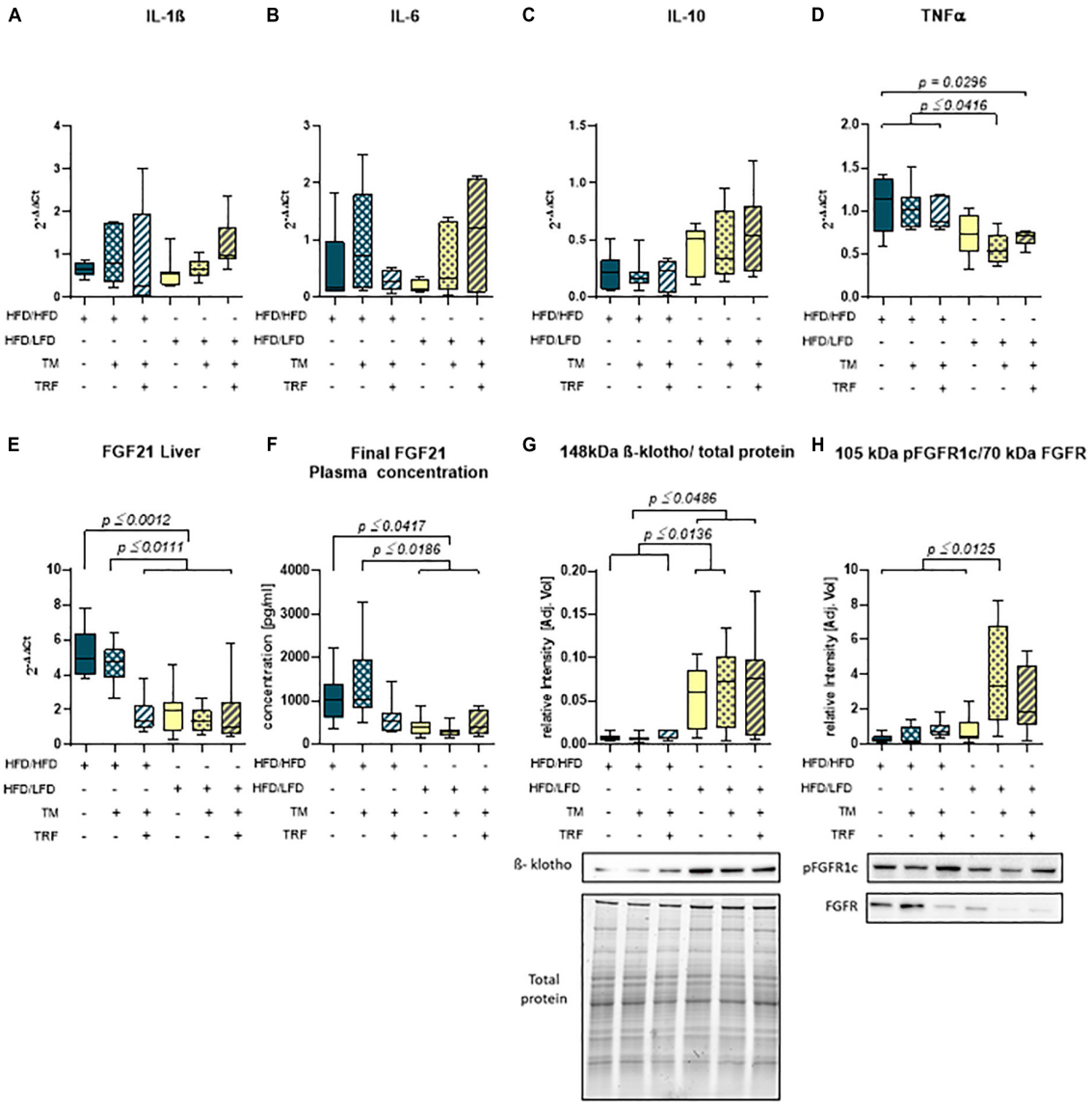

FIGURE 5
Hepatic mRNA expression of IL-1β **(A)** (HFD/HFD: *n* = 6, HFD/HFD + TM: *n* = 7, HFD/HFD + TM + TRF: *n* = 7, HFD/LFD: *n* = 7, HFD/LFD + TM: *n* = 7, HFD/LFD + TM + TRF: *n* = 7; total *n* = 41), IL-6 **(B)** (HFD/HFD: *n* = 6, HFD/HFD + TM: *n* = 7, HFD/HFD + TM + TRF: *n* = 7, HFD/LFD: *n* = 6, HFD/LFD + TM: *n* = 7, HFD/LFD + TM + TRF: *n* = 7; total *n* = 40), IL-10 **(C)** (HFD/HFD: *n* = 7, HFD/HFD + TM: *n* = 7, HFD/HFD + TM + TRF: *n* = 6, HFD/LFD: *n* = 7, HFD/LFD + TM: *n* = 7, HFD/LFD + TM + TRF: *n* = 7; total *n* = 41), TNFα **(D)** (HFD/HFD: *n* = 6, HFD/HFD + TM: *n* = 7, HFD/HFD + TM + TRF: *n* = 7, HFD/LFD: *n* = 7, HFD/LFD + TM: *n* = 7, HFD/LFD + TM + TRF: *n* = 7; total *n* = 41), plasma concentration **(E)** (HFD/HFD: *n* = 13, HFD/HFD + TM: *n* = 13, HFD/HFD + TM + TRF: *n* = 15, HFD/LFD: *n* = 12, HFD/LFD + TM: *n* = 14, HFD/LFD + TM + TRF: *n* = 11; total *n* = 78), and hepatic mRNA expression **(F)** (HFD/HFD: *n* = 6, HFD/HFD + TM: *n* = 6, HFD/HFD + TM + TRF: *n* = 7, HFD/LFD: *n* = 7, HFD/LFD + TM: *n* = 7, HFD/LFD + TM + TRF: *n* = 7; total *n* = 40) of FGF21. Data presented as $2^{-\Delta\Delta Ct}$ values determined by quantitative real-time PCR. Quantitative analysis of hepatic protein expression of β-klotho **(G)** (HFD/HFD: *n* = 12, HFD/HFD + TM: *n* = 9, HFD/HFD + TM + TRF: *n* = 10, HFD/LFD: *n* = 12, HFD/LFD + TM: *n* = 15, HFD/LFD + TM + TRF: *n* = 11; total *n* = 69) and phosphorylated form of the FGF receptor **(H)** (HFD/HFD: *n* = 12, HFD/HFD + TM: *n* = 10, HFD/HFD + TM + TRF: *n* = 10, HFD/LFD: *n* = 9, HFD/LFD + TM: *n* = 10, HFD/LFD + TM + TRF: *n* = 8; total *n* = 59) both with representative Western blots. Signals were normalized either to total protein (β-klotho) or to the non-phosphorylated FGF receptor. Blue dots and box plots indicate HFD groups, and yellow dots and box plots indicate dietary change to LFD. Table displays the individual groups, respectively. Table is read from top to bottom, and "+" denotes implementation of a given diet or intervention and "—" its absence. Significance of differences between groups was tested with either the Kruskal–Wallis test, followed by Dunn's *post hoc* test for multiple comparisons **(B)**, Brown–Forsythe, and Welch's ANOVA with the Tamhane T2 *post hoc* test for multiple comparisons [**A:** *F* value (*F*) = 1.165, degree of freedom (DF) = 5; **D:** *F* = 5.742, DF = 5. **E:** *F* = 11.65, DF = 5, **F:** *F* = 9.902, DF = 5 **G:** *F* = 10.33; DF = 5, **H:** *F* = 9.214; DF = 5], or by ordinary one-way ANOVA with Tukey's *post hoc* test for multiple comparisons **(C)**. Data are presented as mean ± SD, and statistical significance was set at $p < 0.05$. HFD, high-fat diet; LFD, low-fat diet; TM, treadmill; TRF, time-restricted feeding.

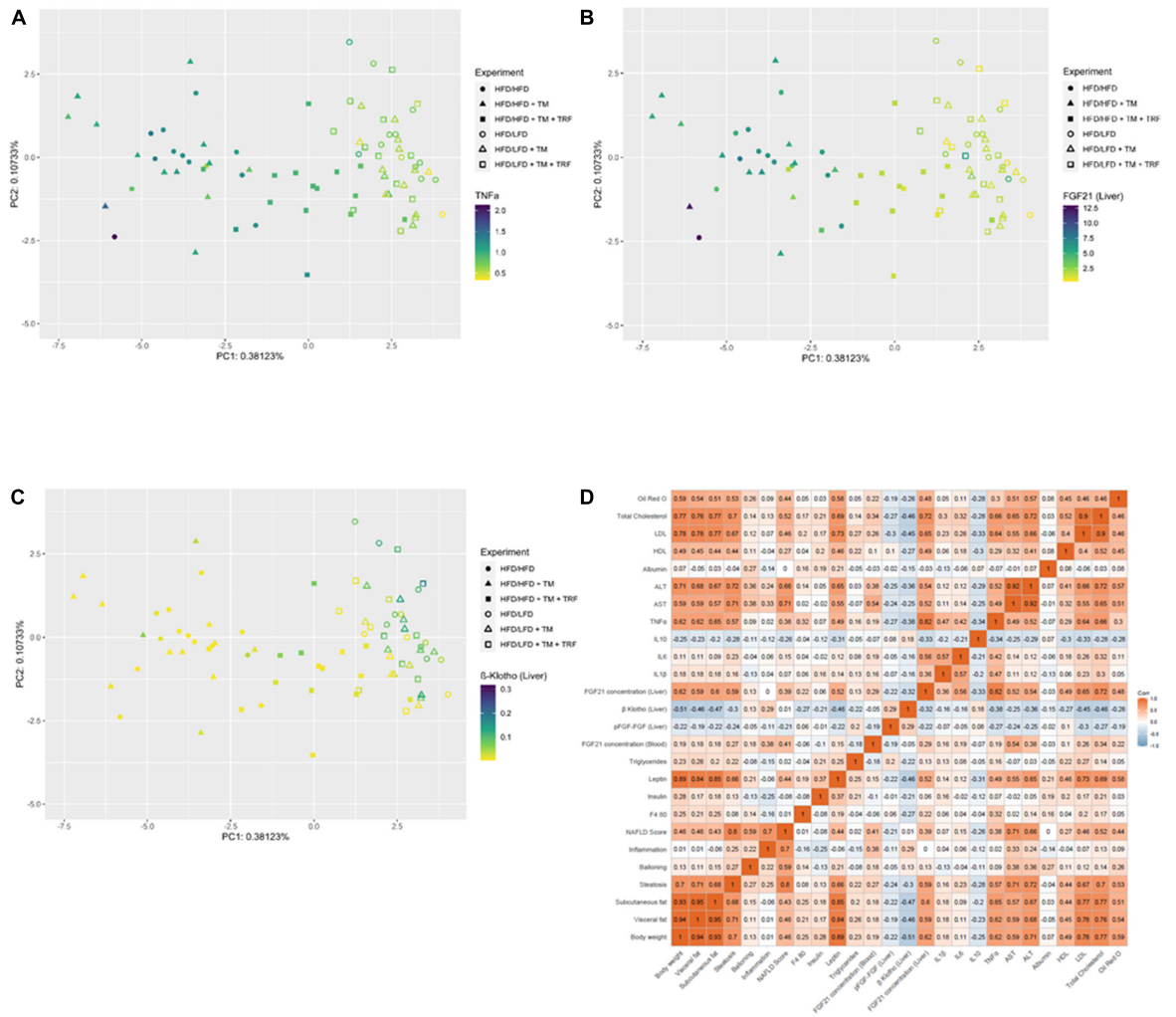

FIGURE 6
Dot plot of principal component analysis (PCA) for TNFα **(A)**, FGF21 in the liver **(B)**, and β-klotho in the liver **(C)** and correlation (Pearson, two-sided test, statistical significance was set at $p < 0.05$) represented as a multidimensional heatmap **(D)**. All parameters from previously acquired experiments were further used for PCA construction. TNFα (**A:** HFD/HFD $n = 13$, HFD/HFD + TM $n = 13$, HFD/HFD + TM + TRF $n = 15$, HFD/LFD $n = 14$, HFD/LFD + TM $n = 15$, HFD/LFD + TM + TRF $n = 14$; total $n = 84$), FGF21 liver (**B:** HFD/HFD $n = 13$, HFD/HFD + TM $n = 13$, HFD/HFD + TM + TRF $n = 15$, HFD/LFD $n = 14$, HFD/LFD + TM $n = 15$, HFD/LFD + TM + TRF $n = 14$; total $n = 84$), and β-klotho liver (**C:** HFD/HFD $n = 13$, HFD/HFD + TM $n = 13$, HFD/HFD + TM + TRF $n = 15$, HFD/LFD $n = 14$, HFD/LFD + TM $n = 15$, HFD/LFD + TM + TRF $n = 14$; total $n = 84$). In panels **A–C**, filled circles, triangles, and squares represent groups with HFD, and non-filled indicate dietary change to LFD. In panel **D**, red color indicates positive correlation, blue color indicates negative correlation, while white indicates no correlation. HFD, high-fat diet; LFD, low-fat diet; TM, treadmill; TRF, time-restricted feeding.

HFD combined with exercise and intermittent fasting. In addition, by observing decreased TNFα and FGF21 expression with concomitant increased β-klotho expression and a strong negative correlation between TNFα and β-klotho expression, we conclude that NAFLD recovery is associated with reversal of a TNFα-triggered FGF21-resistant state or desensitization.

For the treatment of NAFLD, next to bariatric surgery and pharmacological approaches, a less invasive method, namely, dietary change, is also feasible in some obese cases (36, 37). Thus, reduced calorie intake by lowering the fat content is recommended for sustainable weight loss (36) and thus for NAFLD recovery. As proof of principal, this was confirmed in the present study as indicated by the lowered liver fat content and steatosis score. Supporting this, our correlation analyses showed that a decrease in body weight and subcutaneous, as well as visceral fat, is associated with a reduced NAFLD score. In addition to dietary modification, physical activity is another modality for weight reduction in NAFLD therapy (38). However, in the present study, independent of LFD or HFD, no additional benefit was reached with treadmill exercise, as also shown by Ringseis et al. (39). This may be due to the frequency of training as daily training is recommended

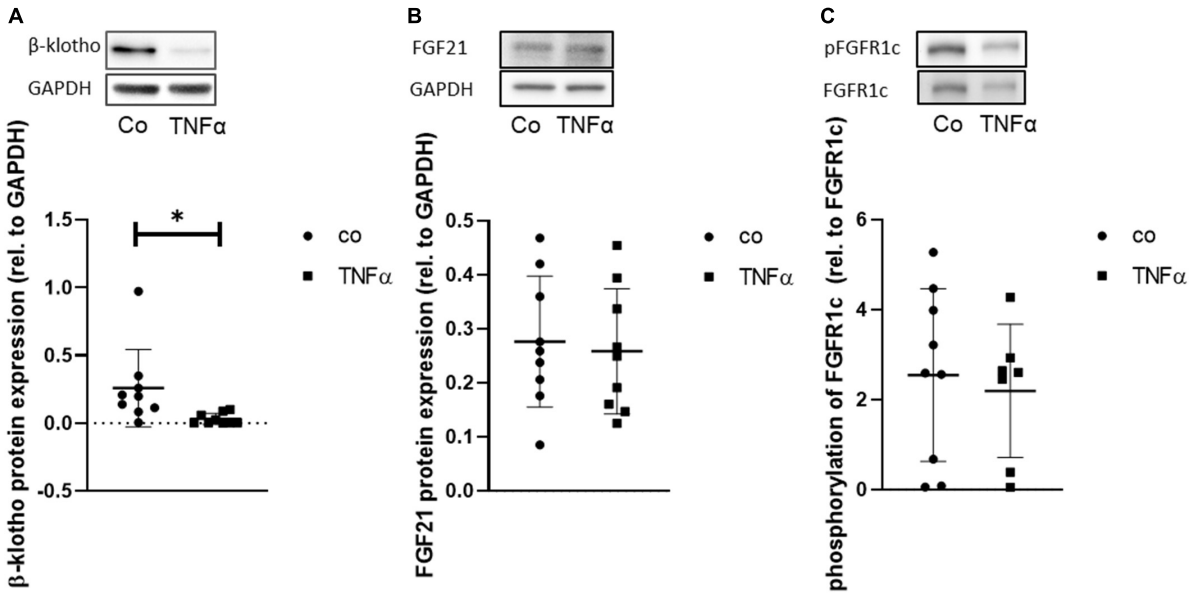

FIGURE 7
Representative Western blot and quantitative analysis of protein expression of β-klotho **(A)** and of FGF21, **(B)** and phosphorylation of FGFR1c **(C)** in HepG2 cells upon incubation of TNF for 24 h. Signals were normalized to GAPDH or FGFR1c, respectively. Data are presented as mean ± SD; $n = 9$ independent experiments; unpaired Student's t-test, $^*p < 0.05$ vs. control (Co).

for a reduction in body weight and thus for NAFLD therapy (37). Furthermore, the currently widespread intermittent fasting may have beneficial effects on NAFLD, especially since is knowing that this contributes to weight loss in obesity (40). Contrary to expectation, only the HFD mice receiving a time-restricted feeding additional to exercise revealed a reduced NAFLD score. As exercise alone did not benefit in NAFLD recovery, we conclude that in particular intermittent fasting in continued HFD is responsible for the observed effect. Similarly, cholesterol concentration was reduced only in the HFD group that received intermittent fasting in addition to exercise, which contradicts the study by Swift et al. (41), who showed that exercise alone reduced blood lipid levels. However, the extent to which the two interventions—treadmill training and intermittent feeding—influence each other in the context of a continued HFD and possibly overshadow any protective effect of training by intermittent fasting or vice versa can only be speculated. Nevertheless, the beneficial effect of time-restricted feeding is in line with the study of Chaix et al. (40) showing that body weight and body fat composition were markedly reduced in the HFD mice, which may be causative for recovery of NAFLD, as shown in the present study.

Obesity *per se* is often associated with increasing inflammatory mediators, such as TNFα (8). In turn, the reversion of NAFLD correlated positively with the reduction of TNFα expression, which persisted not only in dietary change but also in the HFD group receiving treadmill training and time-restricted feeding. TNFα has the ability to decrease the expression of β-klotho but not the phosphorylation of FGFR1c, as shown by Diaz-Delfin et al. (24) in adipocytes. This finding was underlined by the current study in hepatocytes, further highlighting the stronger role of β-klotho in FGF21 signaling. This statement is further supported when considering the groups with NAFLD recovery, where there was a stronger negative correlation of TNFα with β-klotho than with pFGFR1c, confirming the assumption that TNFα may alter FGF21 responsiveness, mainly *via* β-klotho. Although no increase in FGF21 expression could be detected after TNFα application *in vitro*, hepatic FGF21 expression, especially the circulating FGF21 plasma concentration, was decreased in mice that received dietary change in general. Interestingly, the exercising HFD group receiving additional time-restricted feeding also showed reduced FGF21 levels, suggesting that not only dietary change alone but also intermittent fasting may recover from obesity-induced FGF21 desensitization (20). This is also confirmed by the distribution of TNFα, FGF21, and β-klotho data points in the PCA plots, as indicated by clustering not only within the LFD groups but also within the treadmill training HFD group, which additionally underwent intermittent fasting. Accordingly, intermittent fasting has also been described to protect against consequences of obesity (27, 40), including the FGF21-resistant state (21). Since FGF21 has a circadian rhythm that is disrupted by a high-fat diet, intermittent fasting is thought to rebalance the oscillation of FGF21 by coupling food intake in a time-of-day-dependent manner (42, 43) and

thus counteracts obesity. Moreover, Geng et al. (26) showed that physical activity can decrease FGF21 expression and restore FGF21 sensitivity in obese mice and rebalance the metabolic interaction between the adipose tissue, liver, and skeletal muscle. However, this was not observed in the current study because exercise alone in the LFD as well as in the HFD group did not provide any additional benefit—neither in ameliorating NAFLD nor in reversing FGF21 sensitivity. Nevertheless, correlation analysis revealed, independent of any interventions, that with enhanced NAFLD recovery, TNFα expression was decreased, leading to increased FGF21 sensitivity expressed as an increase in β-klotho and FGFR1c expression with concomitantly reduced FGF21 levels. Thus, we conclude that hepatic FGF21 resistance or desensitization is most likely TNFα-dependent. Moreover, it was also observed that mainly circulating FGF21 correlates with the NAFLD score, suggesting a potential dependency between treated NAFLD and FGF21. This finding could provide a basis for considering non-invasive determination of plasma FGF21 as a possible marker to monitor NAFLD activity. This approach might have a high translational potential in treatment of NAFLD in obese patients.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Ethics statement

This animal study was reviewed and approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) of the state Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-2-001/18, approved on March 1, 2018).

Author contributions

AK: conceptualization, funding acquisition, and project administration. NPG: data curation, investigation, and validation. KL and LM: formal analysis. NPG, KL, LM, and DB: methodology. BV: resources. AK and BV: supervision. KL: visualization. AK, NPG, KL, and LM: roles and writing—original draft. DJ, SS, KP, BV, and HG: writing—review and editing. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn, Germany (KU 3280/1-2 and JA 2872/1-2).

Acknowledgments

We cordially thank the technicians of the Institute for Experimental Surgery and of the Central Animal Care Facility, Rostock University Medical Center, for their valuable assistance.

Conflict of interest

HG has received travel grants and speakers honoraria from Fresenius Medical Care, Neuraxpharm, Servier, and Janssen Cilag, as well as research funding from Fresenius Medical Care.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.935805/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 19 August 2022
DOI 10.3389/fnut.2022.933118

OPEN ACCESS

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

RECEIVED 30 April 2022
ACCEPTED 21 July 2022
PUBLISHED 19 August 2022

CITATION
Thomsen MN, Skytte MJ, Samkani A, Astrup A, Fenger M, Frystyk J, Hartmann B, Holst JJ, Larsen TM, Madsbad S, Magkos F, Rehfeld JF, Haugaard SB and Krarup T (2022) Weight loss improves β-cell function independently of dietary carbohydrate restriction in people with type 2 diabetes: A 6-week randomized controlled trial. *Front. Nutr.* 9:933118. doi: 10.3389/fnut.2022.933118

Weight loss improves β-cell function independently of dietary carbohydrate restriction in people with type 2 diabetes: A 6-week randomized controlled trial

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Background: Carbohydrate restriction may benefit β-cell function and glucose metabolism in type 2 diabetes (T2D) but also leads to weight loss which in itself is beneficial.

Methods: In order to determine the additional effect of carbohydrate restriction in addition to a fixed body weight loss, we randomly assigned 72 adults with T2D and obesity (mean ± SD HbA_{1c} 7.4 ± 0.7%, BMI 33 ± 5 kg/m^2) to a carbohydrate-reduced high-protein diet (CRHP; energy percent from carbohydrate/protein/fat: 30/30/40) or an isocaloric conventional diabetes diet (CD; 50/17/33) for 6 weeks. All foods were provided free of charge and total energy intake was tailored individually, so both groups lost 6% of baseline body weight.

Results: Despite significantly greater reductions in HbA_{1c} (mean [95% CI] −1.9 [−3.5, −0.3] mmol/mol) after 6 weeks, the CRHP diet neither improved glucose tolerance, β-cell response to glucose, insulin sensitivity, during a 4-h oral glucose tolerance test, nor basal proinsulin secretion when compared to the CD diet, but increased C-peptide concentration and insulin secretion rate (area under the curve [AUC] and peak) significantly more (∼10%, $P \leq 0.03$ for all). Furthermore, compared with the CD diet, the CRHP diet borderline increased basal glucagon concentration (16 [−0.1, 34]%, $P = 0.05$), but decreased glucagon net AUC (−2.0 [−3.4, −0.6] mmol/L × 240 min,

$P < 0.01$), decreased basal triglyceride and total AUC (~20%, $P < 0.01$ for both), and increased gastric inhibitory polypeptide total AUC (14%, $P = 0.01$).

Conclusion: A moderately carbohydrate-restricted diet for 6 weeks decreased HbA_{1c} but did not improve β-cell function or glucose tolerance beyond the effects of weight loss when compared with a conventional diabetes diet in people with T2D.

Clinical trials registration: www.Clinicaltrials.gov, Identifier: NCT02472951.

KEYWORDS

β-cell function, carbohydrate restriction, insulin sensitivity, low-grade inflammation, type 2 diabetes, weight loss

Introduction

Type 2 diabetes (T2D) is characterized by hyperglycemia, which occurs when insulin resistance is not adequately compensated by hyperinsulinemia. In fact, despite ongoing insulin resistance, T2D does not manifest until pancreatic β-cells fail to produce sufficient amounts of insulin to maintain normal glucose tolerance (1). At this point, patients have lost over 80% of their β-cell function and the overworked β-cells are believed to be "exhausted" although the exact mechanisms are yet to be identified (2). "Lipotoxicity," i.e., chronically elevated levels of circulating non-esterified fatty acids (NEFAs), may impair normal insulin secretion (3), but the weight of evidence suggests that "glucotoxicity" is primarily responsible for β-cell function impairment (4). Indeed, short-term amelioration of hyperglycemia by intensive insulin therapy may to some extent improve β-cell responsiveness to glucose and incretin hormones (5, 6). Furthermore, decreasing the β-cell workload, without changing plasma glucose concentrations, by overnight exogenous infusion of somatostatin, enhances first-phase insulin secretion and decreases proinsulin/insulin ratio (7), which is suggestive of more appropriate intracellular insulin processing and less β-cell stress (8). Accordingly, increased plasma proinsulin relative to insulin or C-peptide has been suggested to be a sensitive index of β-cell failure, often found in individuals with T2D in the fasted state but, particularly, when requirements for insulin secretion increase (9).

Lifestyle modification is pivotal in T2D management, with weight loss being the cornerstone (10). In fact, remission of T2D can be achieved after a >15% weight loss in most patients with short-term diabetes (11), partly associated with the recovery of first-phase insulin secretion, traditionally believed to be irreversibly lost in long-lasting T2D (12). Even without significant changes in body weight, however, several meta-analyses have suggested that additional metabolic benefits, in terms of lower concentrations of glycated hemoglobin (HbA_{1c}), triglyceride, and high-density lipoprotein cholesterol, are achievable following dietary carbohydrate restriction, at least in the short term (13–15). Accordingly, carbohydrate restriction has been suggested as the first approach in diabetes management (16), and the American Diabetes Association (ADA) recently recognized carbohydrate restriction as a viable dietary strategy to improve glycemic control (17).

Previously, we demonstrated that a carbohydrate-reduced high-protein (CRHP) diet improved glycemic control and lipid metabolism when compared with a conventional diabetes (CD) diet (18), which noteworthy also improved β-cell responsiveness to glucose and proinsulin processing although the participants had a mean T2D duration of 7 years (19). Thus, the loss of β-cell function may be at least partially restored by restricting carbohydrate intake independently of weight loss, even in patients with a longer duration of T2D. Unfortunately, carbohydrate-restricted eucaloric diets readily reduce body weight despite considerable efforts to prevent this from happening (18, 20); hence, the results of carbohydrate restriction beyond weight loss are often difficult to interpret. Accordingly, we aimed to evaluate the effects of matched 6% weight loss, induced by 6 weeks of a hypocaloric CRHP or CD diet, on β-cell function and insulin sensitivity, and pancreatic and gut hormone secretion in people with T2D and overweight or obesity. The current study represents a secondary analysis of a trial for which the primary and secondary outcomes (glycemic control, basal triglyceride, and ectopic fat) have been reported elsewhere (21).

Materials and methods

Study design and eligibility criteria

This open-labeled, parallel, randomized clinical trial was conducted at Copenhagen University Hospital Bispebjerg from January 2018 to July 2019 and included people with T2D from the Capital Region of Denmark. A full list of eligibility criteria has been provided previously (21). In brief, individuals with an HbA_{1c} of 6.5–11.0% (48–97

mmol/mol), body mass index (BMI) >25 kg/m^2, and glucose-lowering therapy restricted to metformin and/or dipeptidyl peptidase 4 (DPP-4) inhibitors, and without critical illness, renal dysfunction (estimated glomerular filtration rate <30 mL/min/1.73 m^2 or urine albumin/creatinine ratio >300 mg/g), and without treatment with systemic corticosteroids, sulfonylureas, sodium–glucose co-transporter 2 inhibitors or injectable hypoglycemic medications, were eligible for enrolment. The patients consented in writing, after appropriate oral and written information, to participate in the study, which was approved by the Health Ethics Committee of Copenhagen and the Danish Data Protection Agency. The study was registered with clinicaltrials.gov (NCT02472951) and was conducted in accordance with the Declaration of Helsinki.

Diet intervention and weight loss management

Participants were randomly assigned in a 1:1 ratio to 6 weeks of a fully provided hypocaloric CD or CRHP diet consisting of 50 and 30% of total energy (E%) from carbohydrates, 17 and 30 E% from proteins, and 33 and 40 E% from fats, respectively. Other dietary components varied with the foods included, for instance, the contents of monounsaturated fat (higher in the CRHP diet) and fiber (higher in the CD diet; Supplementary Table 1). Diets were provided free of charge two times weekly and included three main meals with or without two snacks and all daily calories. Alcohol, soft drinks, and any other calorie-containing foods or beverages not provided by the investigational team were not allowed during the study. Participants were instructed to consume all meals, and dietary adherence was evaluated at each diet provision by the use of food records. Meals were prepared in the metabolic kitchen at the Department of Nutrition, Exercise and Sports (NEXS), University of Copenhagen, as seven different daily menus.

The weight loss regimen aimed for a 6% reduction in baseline body weight over the first 5 weeks and stabilization at this new lower body weight during the last week, so that the post-intervention testing was performed in a state of relative energy balance without being confounded by possible acute effects of energy restriction. A fixed weight loss algorithm was applied to each participant, which has been detailed elsewhere (21). To ensure the targeted weight loss, body weight was evaluated two times weekly and, if necessary, adjustments in dietary energy were made by adding or subtracting CRHP or CD food items. Participants were instructed not to change their habitual physical activity during the study, and the International Physical Activity Questionnaire (IPAQ) long form was used at baseline and week 5 to assess adherence. All pharmacotherapy affecting glucose, lipids, or blood pressure was kept constant in the 2 months prior to study commencement and throughout the intervention.

Oral glucose tolerance test and analyses

An oral glucose tolerance test (OGTT) was performed on weeks 0 and 6 to assess β-cell function, insulin sensitivity, and hormonal responses involved in glucose homeostasis and satiety. Participants were instructed not to participate in any strenuous activities for 48 h prior to testing. In the morning of the testing days, after an overnight 10-h fast, a cannula was inserted in an antecubital vein and two fasting blood samples were drawn (at time points −10 and 0 min). Then, a standardized OGTT solution (75 g glucose dissolved in 300 ml of water) was ingested over 5 min and additional blood samples were drawn at time points 10, 20, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min. Participants remained sedentary in a reclined position throughout the OGTT. Blood was processed accordingly for the separation of plasma or serum and stored at −80°C until analysis.

Plasma was analyzed for glucose, cholecystokinin (CCK), and gastrin at all available time points and glucagon, glucagon-like peptide-1 (GLP-1), and gastric inhibitory polypeptide (GIP) at times 0, 30, 60, 90, 120, 150, 180, and 240 min; whereas serum was used for the measurement of insulin, C-peptide, triglyceride, and NEFAs at every time point (21). Concentrations of total glucagon, GLP-1, and GIP were determined (following extraction from plasma with 70% ethanol) by radioimmunoassay (RIA) using C-terminally directed antisera code nos. 4305, 89390, and 867, respectively (22). Likewise, CCK concentrations were measured by RIA using an antiserum directed at the C-terminal sequence (Ab. no. 92128), which specifically binds all the bioactive forms of CCK without cross-reactivity with any of the homologous gastrin (23). The gastrin concentrations were also measured by RIA, but using an antiserum (Ab. no. 2604), which specifically binds all the bioactive forms of gastrin without cross-reactivity with any of the homologous CCK peptides (24).

Fasting samples (at time point −10 min) were used for measuring C-reactive protein (CRP), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 as markers of inflammation, and intact proinsulin (IP) and 32,33 split proinsulin (SP) as indicators of proinsulin processing, as these comprise the major portion of circulating proinsulin-like molecules (25). Serum CRP was measured by enzyme-linked immunosorbent assay (VICTOR Nivo; PerkinElmer, MA, USA), and serum TNF-α, IL-6, and IL-8 by multi-spot immunoassay (V-PLEX; Meso Scale Discovery, MD, USA); all were measured in duplicate where an intra-assay coefficient of variation (CV) of >20% excluded data from analysis. Serum IP and SP were determined in duplicate with a fluorometric immunoassay

(Auto-DELFIA; PerkinElmer, MA, USA) with a lower detection limit of 1.25 pmol/L (26). The results were summed to give total proinsulin. Considerable cross-reactivity between these two assays exists which has been accounted for previously (19). The intra-assay CV for duplicate measurements of CRP, TNF-α, IL-6, and IL-8 was 6, 3, 5, and 3%, respectively.

Outcomes and calculations

Basal concentrations were obtained from samples taken at time point 0 min or as the mean of time points 0 and −10 min when both were available. Responses to the OGTT were evaluated as the area under the curve (AUC), calculated by the trapezoidal method including net AUC (i.e., total AUC minus the area below baseline) where variables suppressed by the OGTT, i.e., triglyceride, NEFAs, and glucagon, took negative values. In addition, we determined the peak/nadir values, defined as the greatest increment/decrement above/below the baseline, as well as the time point when these values were reached.

Prehepatic insulin secretion rates (ISR) were estimated by the deconvolution of C-peptide concentrations using the ISEC software which integrates postprandial peripheral concentrations of C-peptide and individual subject characteristics in a two-compartment model (27). ISR and its relation to plasma glucose were used to evaluate the β-cell responsiveness or sensitivity to glucose in the early phase from 0 to 30 min and expressed as the insulinogenic index (IGI_{30} = [ISR $AUC_{0-30min}$]/[glucose $AUC_{0-30min}$]). IGI_{240} = [ISR $AUC_{0-240min}$]/[glucose $AUC_{0-240min}$] was calculated as an index of the full insulin response during the OGTT. B_{total} was calculated as an index of changes in insulin secretion relative to changes in glucose and incretin hormones, represented as the slope of individual regression lines achieved by the cross-correlation of corresponding values of ISR and plasma glucose during the total 240 min of the OGTT.

As a marker of whole-body insulin sensitivity, the composite index (ISI_{comp}) was calculated as follows: $10{,}000/\sqrt{}$[basal glucose x basal insulin x $\bar{G}$ x $\bar{I}$; where $\bar{G}$ is mean glucose and $\bar{I}$ is mean insulin during the 4-h OGTT (28). Included values of glucose and insulin were in units of mg/dL and μU/ml [conversion factor: 1 μU/ml = 6.0 pmol/L (29)], respectively. Given the hyperbolic relationship between insulin sensitivity and β-cell responsiveness to glucose, which has been validated in another study (30), the disposition index, calculated as their product (D_i = ISI_{comp} x B_{total}), was used to assess β-cell function (31). The metabolic clearance rate of insulin was estimated as the ratio between newly secreted insulin and total serum concentration of insulin throughout the OGTT after adjustment for body weight by using the following formula: MCRi = [ISR $AUC_{0-240min}$]/[insulin $AUC_{0-240min}$] × body weight (30).

Sample size, statistics, and randomization

Sample size calculations, together with primary and secondary outcomes, have been reported elsewhere (21). In brief, the study evaluated changes in HbA_{1c} and liver fat as its primary and leading secondary outcomes, respectively, and adequate power was expected with 80 participants enrolled, allowing for 20% attrition. β-cell function, insulin sensitivity, and hormonal responses following the OGTT, presented here, were pre-specified exploratory outcomes and interpreted as such. Accordingly, no adjustments were made for multiplicity in data analysis, and the two-sided statistical tests were considered significant when $P < 0.05$.

Summary statistics included only completing participants. All data—except for net AUCs and time to peak/nadir—were log-transformed because of distribution skewness. Therefore, unless otherwise stated, baseline data are shown as geometric means (95% CI), and changes from baseline are shown as a percentage. Statistical inference was accomplished using all available data in a constrained linear mixed model with inherent baseline adjustment, and the treatment effect was evaluated as the marginal mean (95% CI) of CRHP vs. CD diet. The model used an unstructured pattern of covariance to account for repeated measurements. Missing data were assumed to be missing at random and implicitly handled by maximum likelihood estimation. Model assumptions were assessed from residual diagnostics, and skewed variables were handled by log-transformation prior to analysis, in which case differences between diets are given in percentage. To account for possible confounders, a secondary model was adjusted for the covariates sex, age, duration of T2D, BMI, and therapy with metformin and DPP-4 inhibitors.

An unrelated study nurse was responsible for the randomization which upon the allocation of the participants was unblinded for the investigators; whereas participants were kept blind until the first meal provision. Randomization was performed in blocks of random size through a generated randomization list which was conducted in R (Version 3.6.0, R, Boston, MA, USA) together with all statistical analyses and graphics.

Results

Participants and baseline characteristics

From a total of 338 telephone pre-screenings and 102 screening visits, 72 participants were enrolled in the present study of whom 67 completed all visits; three withdrew consent during the intervention, and two before the first visit but after randomization. Reasons for withdrawal have been described elsewhere (21) but were not related to trial outcomes or any study-related adverse events. Because of

10.3389/fnut.2022.933118

TABLE 1 Baseline characteristics of participants with T2D.

Characteristic	CD diet	CRHP diet
Participants/white ethnicity, *n*	33/33	34/34
Male/female sex, *n*	15/18	20/14
Age, years	67.0 (±8.8)	66.4 (±6.9)
Duration of T2D, years	7.7 (2.8, 10.1)	8.5 (3.5, 11.9)
HbA_{1c}, %	7.40 (±0.70)	7.42 (±0.77)
HbA_{1c}, mmol/mol	57.4 (±7.7)	57.6 (±8.4)
Body mass index, kg/m^2	33.2 (±5.1)	33.6 (±4.6)
Body weight, kg	97.5 (±25.4)	98.0 (±14.2)
Estimated daily TEE, kcal	2,600 (±632)	2,652 (±364)
Medication use, *n* (%)		
No glucose-lowering therapy	12 (36)	8 (24)
Glucose-lowering therapy	21 (64)	26 (76)
1 hypo-glycemic agent	18 (54)	16 (47)
2 hypo-glycemic agent	3 (9)	10 (29)
Metformin	21 (64)	25 (74)
DPP-4 inhibitors	3 (9)	11 (32)
Lipid-lowering therapy	23 (70)	26 (76)
Anti-hypertensive therapy	26 (79)	29 (85)

Data are presented as means (±SD), medians (25th, 75th percentiles), or frequencies. CD, conventional diabetes; CRHP, carbohydrate-reduced high-protein; DPP-4, dipeptidyl peptidase 4; T2D, type 2 diabetes; TEE, total energy expenditure.

the COVID-19 pandemic lockdown, fewer than the expected 80 participants were enrolled but power was sufficient due to lower than anticipated attrition rates (CD 8.3%, CRHP 5.6%). Randomization was successful and, apart from uneven distribution of sex and use of DPP-4 inhibitors, the baseline characteristics were well balanced between groups (Table 1). Importantly, the results were robust when adjusting for baseline differences in sex, age, duration of T2D, BMI, and use of metformin and DPP-4 inhibitors.

Weight loss

Weight loss in the two diet groups was well-matched (CHRP vs. CD: 0.1 [−0.6, 0.7] kg, $P = 0.83$) with a 5.8 kg mean decrease in both groups. No differences in dietary energy restriction (4 [−149, 158] kcal/day, $P = 0.95$) or physical activity level (0 [−27, 37]%, $P = 0.99$) were found. These outcomes have been published in detail elsewhere (21).

Glucose and lipid metabolism

Overall, glycemic control was improved by the CRHP diet after 6 weeks compared with the CD diet as HbA_{1c} decreased by 1.9 [−3.5, −0.3] mmol/mol and diurnal mean glucose by −0.8 (−1.2, −0.4) mmol/L; both reported elsewhere (21).

Basal concentrations of glucose, insulin, C-peptide, and ISR did not differ between diets and were all reduced after the 6-week intervention (Table 2). When compared with baseline, weight loss with both diets increased total AUC, net AUC, peak, and time to peak of insulin, C-peptide, and ISR; and decreased total AUC, net AUC, and peak of glucose (Table 2; Figure 1). After 6 weeks, the CRHP diet increased total AUC (9 [2, 18]%) and net AUC (203 [11, 394] pmol/L × 240 min) of C-peptide and ISR response (10 [1, 19]%) ($P < 0.05$ for all) to a significantly greater extent compared with the CD diet, but no differences in glucose response were found (Table 2; Figure 1). Peak values of C-peptide and ISR also increased to a significantly greater extent by the CRHP than the CD diet (Supplementary Table 2).

Triglyceride and NEFA total AUCs and nadirs were reduced with both diets when compared with baseline. The CRHP diet reduced basal triglyceride (−19 [−30, −6]%) and triglyceride total AUC and nadir (−21 [−31, −9]% and −25 [−35, −13]%, respectively) ($P < 0.01$ for all) and tended to also suppress NEFA nadir (−20 [−36, −0.1]%, $P = 0.05$) to a greater extent than the CD diet.

Insulin sensitivity and β-cell function

Weight loss induced by either diet significantly increased ISI_{comp} by approximately 42% and indices of β-cell responsiveness (B_{total}, IGI_{30}, and IGI_{240}) and function by 24–68% and 138%, respectively ($P < 0.001$ for all). Improvements were seen in most participants: 88% for ISI_{comp}, 93% for B_{total}, and 99% for D_i (Figure 2). The CRHP diet did not improve measures of insulin sensitivity, β-cell responsiveness to glucose, or β-cell function when compared with the CD diet (Supplementary Table 3). No difference was found for MCRi between diets (Figure 2), and only small changes were observed when compared with baseline.

Glucagon and gut hormones

Changes in basal concentrations of GLP-1, GIP, CCK, and gastrin after 6 weeks were similar between diets, and all hormones except for gastrin decreased significantly with weight loss (Table 3). Basal glucagon tended to decrease to a lesser extent following the CRHP diet when compared with the CD diet (16 [−0.1, 34]%, $P = 0.05$), whereas glucagon net AUC was reduced significantly more after the CRHP diet (−2.0 [−3.4, −0.6] mmol/L × 240 min, $P < 0.01$, Figure 3). Moreover, GIP total AUC, but not net AUC, was higher on the CRHP than on the CD diet (14 [3, 27]%, $P = 0.01$), and a tendency for higher peak was found (Supplementary Table 2). No differences between diets were found in the OGTT response of GLP-1, CCK, and gastrin (Figures 3, 4). Nonetheless, peaks and total AUCs

TABLE 2 Basal concentrations and responses to an oral glucose tolerance test of glucose, insulin, C-peptide, triglyceride, NEFAs, and insulin secretion at baseline and after matched ~6% weight loss by a CD or a CRHP diet in individuals with T2D and overweight or obesity.

	CD diet, $n = 33$		CRHP diet, $n = 34$		Between diets	
	Baseline	Change[a]	Baseline	Change[a]	Difference[b]	*P* value
Plasma glucose						
Basal glucose, mmol/L	8.8 (8.2, 9.5)	−21 (−26, −16)‡	8.7 (8.1, 9.3)	−22 (−26, −17)‡	−2 (−8, 5)	0.63
AUC, mmol/L x 240 min	13.9 (12.8, 15.1)	−19 (−24, −15)‡	14.1 (13.3, 15.0)	−18 (−23, −14)‡	1 (−5, 9)	0.70
Serum insulin						
Basal insulin, pmol/L	125 (106, 148)	−26 (−36, −16)‡	117 (101, 135)	−27 (−35, −18)‡	−0.4 (−16, 18)	0.96
AUC, pmol/L x 240 min	281 (223, 355)	2 (−7, 11)	266 (215, 328)	13 (4, 23)†	11 (−0.5, 24)	0.06
Serum C-peptide						
Basal C-peptide, pmol/L	1,252 (1,125, 1,394)	−15 (−22, −8)‡	1,249 (1,126, 1,386)	−12 (−18, −6)‡	3 (−6, 14)	0.50
AUC, pmol/L x 240 min	2,501 (2,183, 2,865)	8 (1, 14)†	2,464 (2,171, 2,797)	18 (11, 25)‡	9 (2, 18)	0.02
Insulin secretion rate						
Basal ISR, pmol x kg^{-1} x min^{-1}	3.3 (3.0, 3.6)	−11 (−17, −4)†	3.2 (3.0, 3.5)	−8 (−13, −2)*	3 (−6, 13)	0.50
AUC, pmol x kg^{-1} x 240	7.2 (6.2, 8.2)	14 (7, 22)‡	7.0 (6.2, 7.8)	26 (18, 34)‡	10 (1, 19)	0.03
Serum triglyceride						
Basal triglyceride, mmol/L	1.7 (1.5, 1.9)	−13 (−24, −0.7)*	1.6 (1.3, 1.9)	−27 (−37, −15)‡	−19 (−30, −6)	<0.01
AUC, mmol/L x 240 min	1.5 (1.3, 1.8)	−15 (−25, −5)†	1.5 (1.2, 1.8)	−31 (−40, −21)‡	−21 (−31, −9)	<0.01
Serum NEFA						
Basal NEFA, mmol/L	0.71 (0.64, 0.78)	−11 (−17, −4)	0.61 (0.55, 0.68)	−5 (−14, 6)	−1 (−11, 10)	0.85
AUC, mmol/L x 240 min	0.32 (0.29, 0.36)	−14 (−18, −9)†	0.29 (0.26, 0.33)	−13 (−23, −1)‡	−4 (−14, 7)	0.46

Data are presented as mean (95% CI) following log-transformation. Between-diet differences are estimated marginal means (CRHP vs. CD) derived from constrained linear mixed models with inherent baseline adjustment using all available data.
[a]Relative change (%) from baseline.
[b]Relative difference (%) between diets.
*$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$ vs. baseline.
CD, conventional diabetes; CRHP, carbohydrate-reduced high-protein; ISR, insulin secretion rate; NEFAs, non-esterified fatty acids.

for these hormones except for gastrin decreased with weight loss which only minutely affected net AUCs and time to peak/nadir.

Proinsulin-like molecules

When compared with the CD diet, the CRHP diet did not reduce absolute concentrations of intact, 32,33 split, or total proinsulin; or total proinsulin in relation to insulin or C-peptide (Table 4). However, all variables were reduced significantly and similarly after weight loss (by 28–51%, $P < 0.001$ for all). Participants benefitted robustly as all but one experienced a reduction in the total proinsulin/C-peptide ratio.

Low-grade inflammation

None of the included markers of inflammation differed between groups after the diet interventions, and few changed with weight loss, except for CRP and TNF-α which were reduced and increased, respectively, on the CD diet (Table 4). Notably, the data included much variation and outliers for TNF-α and IL-6 (Supplementary Figure 1), but the results remained robust after excluding these data points (not shown).

Adverse events

No serious adverse events occurred during the study. Reported adverse events were generally mild and not statistically different between groups. Nevertheless, one participant (CRHP) experienced episodes of transient excessive sweating and increased plasma creatinine concentration but no underlying medical cause was identified. Also, more time of the day was spent with plasma glucose below 3.9 mmol/L (from 7-day continuous glucose monitoring) while on the CRHP diet (21) which, however, did not correspond to symptoms of hypoglycemia. Most adverse events experienced by the participants were gastrointestinal with symptoms of constipation being the most predominant (CD 5, CRHP 8); all but one was easily remedied by sufficient fluid intake and laxatives. Few episodes of diarrhea (CD 2, CRHP 2), dizziness (CD 1, CRHP 2), and feelings of increased tiredness or lack of energy (CD 0, CRHP 2) occurred.

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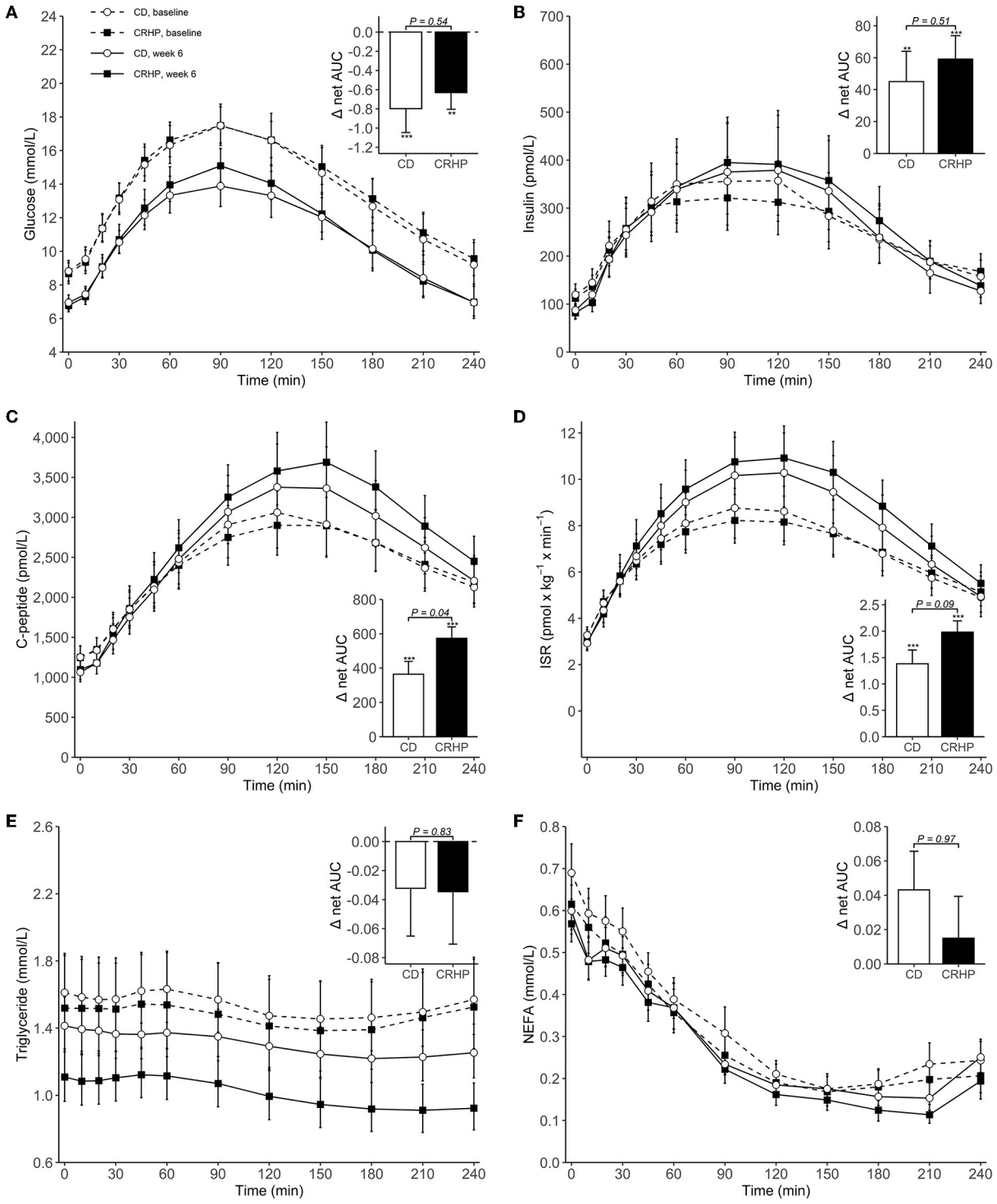

FIGURE 1
Concentrations of plasma glucose **(A)**, serum insulin **(B)**, serum C-peptide **(C)**, insulin secretion rate (ISR) **(D)**, serum triglyceride **(E)**, and serum non-esterified fatty acids (NEFAs) **(F)** during an OGTT at baseline and after 6 weeks of a CD or CRHP diet. Inserted plots represent net AUC in the units stated x 240 min. Data are presented as mean (±SEM) following log-transformation, except for net AUCs; $^{**}P < 0.01$; $^{***}P < 0.001$.

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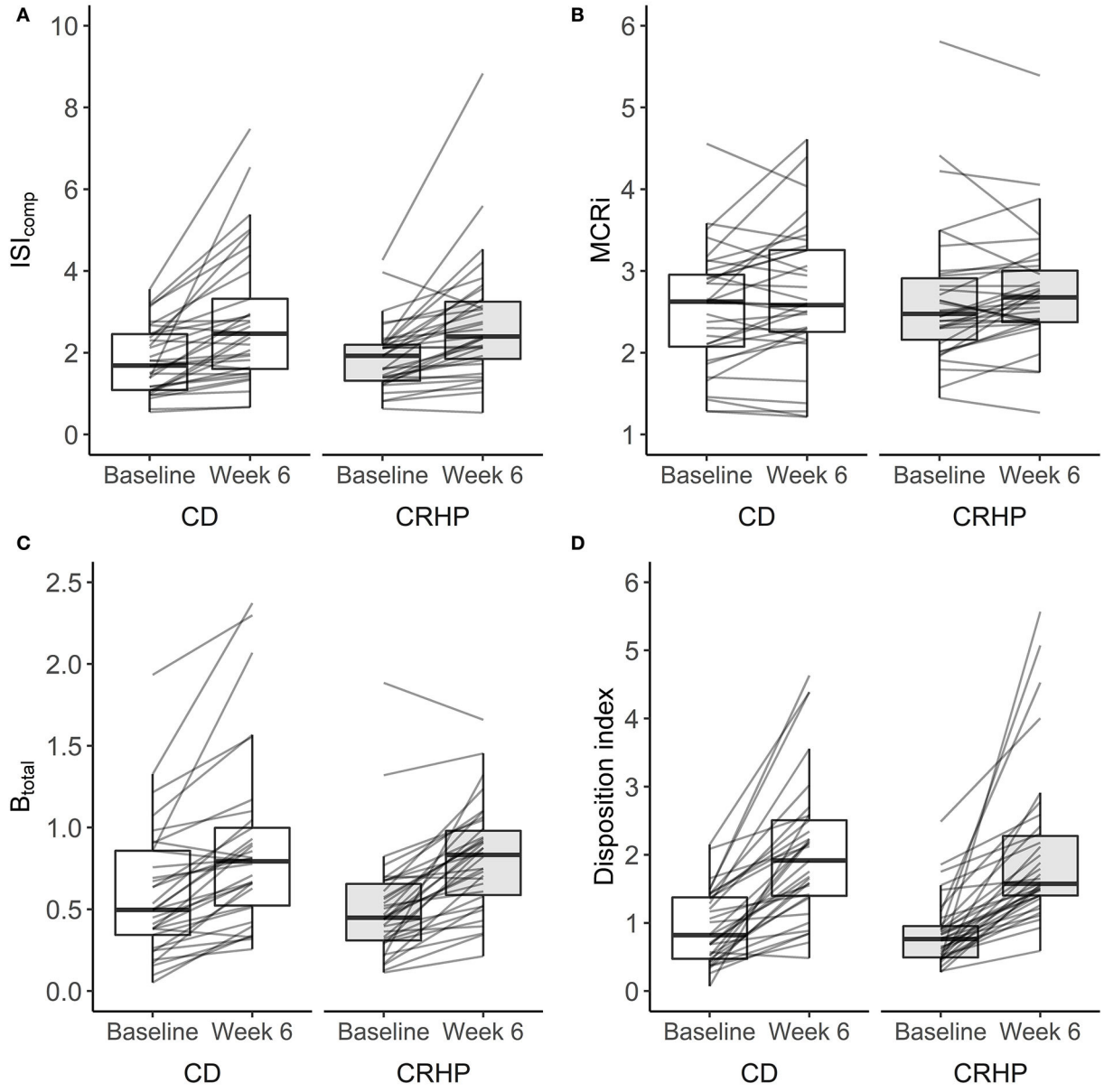

FIGURE 2
Changes in the composite index (ISI_{comp}) **(A)**, metabolic clearance rate of insulin (MCRi) **(B)**, β-cell responsiveness to glucose (B_{total}) **(C)**, and disposition index (D_i) **(D)** derived from an OGTT at baseline and after 6 weeks of a CD or CRHP diet. Data are presented as median (25th, 75th) with individual changes. No differences between diets were evident from linear mixed model analysis: $P = 0.47$, $P = 0.77$, $P = 0.65$, and $P = 0.99$ for **(A–D)**, respectively. Estimates are in units: $L^2 \times mg^{-1} \times \mu U^{-1} \times 10^{-4}$ (ISI_{comp}), $L \times min^{-1}$ (MCRi), $L \times kg^{-1} \times min^{-1} \times 10^{-9}$ (B_{total}), and $L^3 \times g^{-2} \times min^{-1} \times \mu U^{-1} \times 10^{-1}$ (D_i).

Discussion

We hypothesized that a clinically relevant weight loss induced by a CRHP diet would ameliorate β-cell dysfunction, impaired glucose tolerance, and stress on the β-cells to a greater extent than a matched weight loss induced by a CD diet in individuals with T2D and overweight or obesity. Instead, we found no improvements in these measures when consuming a CRHP diet relative to a CD diet, but rather a small increase in the secretion of insulin and C-peptide during an oral glucose challenge, despite similar decrements in glucose. Nevertheless, weight loss, as expected, was highly effective in improving β-cell function, insulin sensitivity, and proinsulin processing independently of diet composition.

Weight loss has consistently been shown to be a key component of effective treatment in T2D; indeed, a 15 kg

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TABLE 3 Basal concentrations and responses to an oral glucose tolerance test of glucagon, GLP-1, GIP, CCK, and gastrin at baseline and after matched ∼6% weight loss by a CD or a CRHP diet in individuals with T2D and overweight or obesity.

	CD diet, $n = 33$		CRHP diet, $n = 34$		Between diets	
	Baseline	Change[a]	Baseline	Change[a]	Difference[b]	*P* value
Plasma glucagon						
Basal glucagon, pmol/L	17.6 (15.7, 19.7)	−30 (−37, −22)‡	17.8 (16.1, 19.6)	−19 (−28, −9)‡	16 (−0.1, 34)	0.05
AUC, pmol/L x 240 min	14.2 (12.7, 15.8)	−30 (−39, −20)‡	13.7 (12.6, 14.9)	−27 (−33, −20)‡	3 (−10, 19)	0.65
Plasma GLP−1						
Basal GLP−1, pmol/L	6.6 (5.1, 8.7)	−65 (−75, −51)‡	5.7 (4.2, 7.7)	−42 (−62, −14)‡	48 (−4, 128)	0.08
AUC, pmol/L x 240 min	13.3 (11.2, 15.8)	−30 (−43, −14)‡	10.8 (9.4, 12.5)	−20 (−32, −7)†	3 (−18, 30)	0.79
Plasma GIP						
Basal GIP, pmol/L	9.9 (8.0, 12.4)	−59 (−69, −45)‡	7.5 (6.0, 9.5)	−43 (−58, −23)‡	22 (−17, 78)	0.31
AUC, pmol/L x 240 min	28.7 (25.7, 32.0)	−17 (−25, −9)‡	23.4 (21.2, 25.9)	−4 (−10, 1)	14 (3, 27)	0.01
Plasma CCK[c]						
Basal CCK, pmol/L	1.1 (0.8, 1.7)	−40 (−55, −20)‡	1.1 (0.7, 1.6)	−30 (−49, −4)*	14 (−24, 72)	0.52
AUC, pmol/L x 240 min	1.4 (1.0, 1.8)	−22 (−30, −12)‡	1.3 (0.9, 1.8)	−12 (−21, −2)*	12 (−4, 31)	0.14
Plasma gastrin[c]						
Basal gastrin, pmol/L	13.2 (10.0, 17.4)	−11 (−22, 2)	10.3 (8.1, 13.2)	−11 (−21, 0.3)*	−4 (−19, 13)	0.61
AUC, pmol/L x 240 min	14.3 (11.5, 17.8)	−8 (−15, −2)*	11.3 (9.4, 13.6)	−2 (−8, 4)	3 (−5, 12)	0.43

Data are presented as mean (95% CI) following log-transformation. Between-diet differences are estimated marginal means (CRHP vs. CD) derived from constrained linear mixed models with inherent baseline adjustment using all available data.

[a]Relative change (%) from baseline.

[b]Relative difference (%) between diets.

[c]Total analyzed $n = 66$ (CD 32 and CRHP 34). Missing data due to insufficient plasma required for analysis.

*$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$ vs. baseline.

CCK, cholecystokinin; CD, conventional diabetes; CRHP, carbohydrate-reduced high-protein; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1.

reduction has been shown to almost eliminate hyperglycemia and result in the remission of T2D (11). In fact, this amount of weight loss and/or severe energy restriction may lead to the normalization of β-cell function and hepatic insulin sensitivity to levels typical of individuals without T2D (33, 34). Accordingly, in the present study, we found that the modest 6% weight loss significantly improved β-cell function and responsiveness to glucose and whole-body insulin sensitivity in response to an OGTT, independent of diet composition. We have previously demonstrated—using diets of similar composition and for the same duration—that a moderate substitution of carbohydrate with protein and fat improves β-cell responsiveness to glucose in response to CHRP vs. CD meals (19), but this could have been because of the acute changes specific to the different composition of the CRHP and CD meals (22). This is likely, given that we did not confirm these observations in this study, in response to the same oral challenge (OGTT). It is also likely that the effects of concurrent weight loss in the present study far outweighed and masked any independent effects of macronutrient composition. However, the use of an OGTT may be limited when evaluating carbohydrate restriction as the response in healthy individuals was found to depend on the carbohydrate content of the preceding dinner meal (32).

The β-cell sensitivity to glucose and to incretin hormones seems to improve from short-term amelioration of hyperglycemia by intensive insulin therapy (5, 6), which possibly relieves β-cells from the toxic byproducts of increased insulin production in response to hyperglycemia and the deteriorating effect of glucotoxicity (35). In T2D, stressed β-cells increase the proportion of secreted proinsulin and proinsulin conversion intermediates that remain inactive precursors of insulin, altogether suggestive of impaired intracellular processing of proinsulin to insulin (9, 36). Accordingly, β-cell rest induced by overnight administration of somatostatin reduces proinsulin secretion and improves β-cell function (7). We recently demonstrated that the release of proinsulin-like molecules was decreased after 6 weeks of a weight-maintaining CRHP diet when compared with a CD diet, which we suggested was the result of decreased demand on the β-cells (19), caused by reductions in postprandial hyperglycemia occurring after the CRHP meals (18, 22). We could not replicate this finding in the present study, as there was no difference in basal proinsulin secretion between diets despite the improved glycemic control including a considerably larger reduction in mean diurnal glucose, by 0.8 mmol/L (∼50%), after the CRHP diet compared with the CD diet [discussed in Thomsen et al. (21)]. In fact, in response to the OGTT, we found minute increments in ISR and C-peptide total AUCs and peak values after the CRHP diet compared with the CD diet (by ∼10%, $P < 0.05$ for both); this was despite identical decreases in glucose responses to the

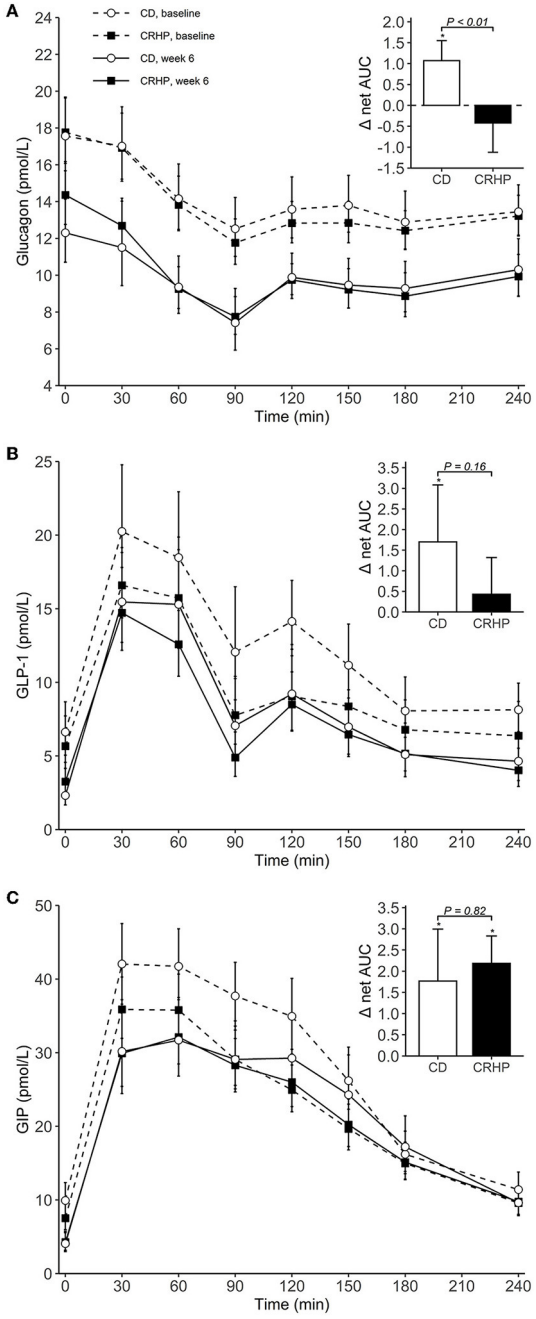

FIGURE 3
Plasma concentrations of glucagon **(A)**, GLP-1 **(B)**, and GIP **(C)** during an OGTT at baseline and after 6 weeks of a CD or CRHP diet. Inserted plots represent net AUC in the units stated x 240 min. Data are presented as mean (±SEM) following log-transformation, except for net AUCs; *$P < 0.05$.

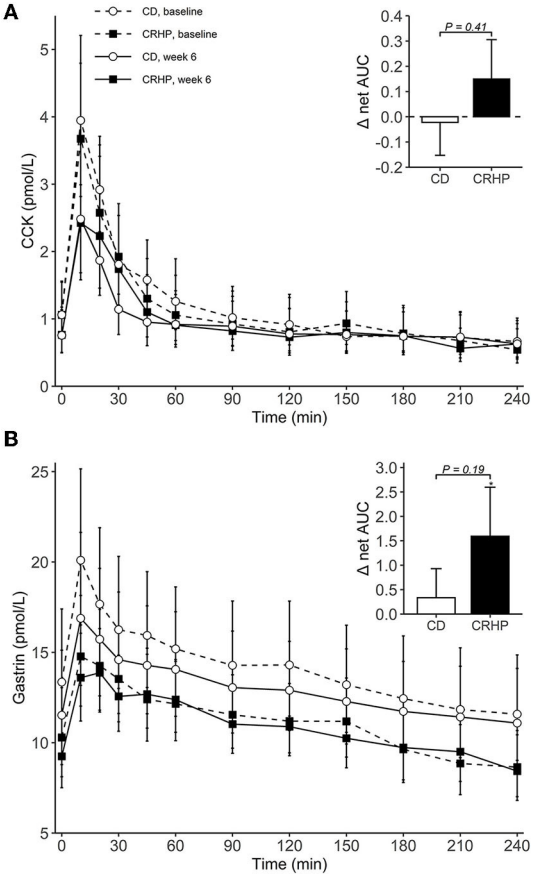

FIGURE 4
Plasma concentrations of CCK **(A)** and gastrin **(B)** during an OGTT at baseline and after 6 weeks of a CD or CRHP diet. Inserted plots represent net AUC in the units stated x 240 min. Data are presented as mean (±SEM) following log-transformation, except for net AUCs; *$P < 0.05$.

OGTT. The clinical relevance of this finding remains uncertain, but could potentially translate into increased demand on the β-cells when transitioning from a carbohydrate-restricted diet to a carbohydrate-rich diet. Nevertheless, weight loss in the present study, induced by either diet, ameliorated inappropriate proinsulin secretion efficiently, consistent with what others have found (37).

GLP-1 and GIP have well-known insulinotropic properties during glucose stimulation, which account for most of the incretin effect (38), but other secretagogues including glucagon through intra-islet crosstalk (39) and circulating CCK and gastrin are also recognized for contributing to insulin secretion (40). In the present study, only GIP total AUC increased significantly after the CRHP diet compared with the CD diet, which may explain the increase in insulin secretion although the action of GIP in T2D is usually impaired (41). Basal glucagon concentration tended to be reduced to a smaller extent by the CRHP diet than the CD diet, but was suppressed similarly during the OGTT, resulting in a significant reduction in net AUC. Several studies have found that fasting hyperglucagonemia is associated with fasting hyperglycemia through increased hepatic glucose production (39, 42), but the higher glucagon

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TABLE 4 Basal concentrations of proinsulin-like molecules and markers of inflammation at baseline and after matched ~6% weight loss by a CD or a CRHP diet in individuals with T2D and overweight or obesity.

	CD diet, $n = 33$		CRHP diet, $n = 34$		Between diets	
	Baseline	Change[a]	Baseline	Change[a]	Difference[b]	*P* value
Proinsulin[c]						
Intact proinsulin, pmol/L	9.5 (7.6, 12.0)	−45 (−54, −35)‡	9.4 (7.8, 11.3)	−43 (−50, −36)‡	1 (−16, 22)	0.88
Split 32,33 proinsulin, pmol/L	14.5 (11.3, 18.6)	−49 (−58, −38)‡	12.8 (10.2, 16.2)	−51 (−58, −43)‡	−9 (−27, 15)	0.43
Total proinsulin[d], pmol/L	24.2 (19.1, 30.7)	−47 (−56, −37)‡	23.0 (19.1, 27.6)	−48 (−55, −40)‡	−4 (−22, 17)	0.66
Total proinsulin / insulin	0.19 (0.17, 0.23)	−28 (−36, −19)‡	0.20 (0.17, 0.23)	−29 (−39, −19)‡	−3 (−17, 13)	0.69
Total proinsulin / C-peptide	0.019 (0.016, 0.023)	−38 (−45, −29)‡	0.018 (0.016, 0.021)	−41 (−48, −34)‡	−9 (−21, 4)	0.18
Inflammation markers[d]						
CRP, ng/mL	1.4 (0.9, 2.0)	−33 (−50, −10)†	1.5 (0.9, 2.4)	−14 (−45, 33)	43 (−10, 127)	0.14
TNF-α, pg/mL	2.0 (1.8, 2.2)	8 (0.8, 17)†	2.0 (1.8, 2.2)	1 (−3, 6)	−7 (−14, 1)	0.08
IL-6, pg/mL	1.1 (1.0, 1.3)	0.1 (−10, 12)	1.2 (1.0, 1.4)	11 (−8, 35)	12 (−9, 38)	0.29
IL-8, pg/mL	7.4 (6.6, 8.4)	−1 (−13, 11)	6.8 (6.1, 7.5)	−5 (−11, 2)	−6 (−17, 6)	0.28

Data are presented as mean (95% CI) following log-transformation. Between-diet differences are estimated marginal means (CRHP vs. CD) derived from constrained linear mixed models with inherent baseline adjustment using all available data.

[a]Relative change (%) from baseline.

[b]Relative difference (%) between diets.

[c]Total analyzed $n = 64$ (CD 30 and CRHP 34). Missing data due to hemolyzed ($n = 1$) or insufficient serum required for analysis ($n = 2$).

[d]Total analyzed $n = 52$ (CD 25 and CRHP 27) for CRP and $n = 66$ (CD 33 and CRHP 33) for IL-6. Missing data due to measurements under ($n = 2$) and above ($n = 3$) the detection range, and intra-assay CV >20% exclusion ($n = 11$).

† $P < 0.01$ and ‡ $P < 0.001$ vs. baseline.

Total proinsulin, sum of intact and split 32,33 proinsulin; CD, conventional diabetes; CRHP, carbohydrate-reduced high-protein; CRP, C-reactive protein; TNF-α, tumor necrosis factor α; IL, interleukin.

concentration in the CRHP group was not accompanied by a concomitant increase in basal glucose concentration in the present study. We have previously demonstrated that a CRHP meal elicits an increased postprandial glucagon response (because of its higher protein content) even after 4 h (19, 22) and, thus, the observed difference may simply reflect a higher postprandial glucagon secretion from the preceding CRHP dinner, maintained overnight, although some studies suggest that this effect dissipates after an overnight fast (43, 44).

In the present study, the triglyceride total AUC was reduced more by the CRHP diet than the CD diet. This was likely because of the greater reduction in basal triglyceride concentration, maintained during the OGTT. We have previously speculated that decreased *de novo* lipogenesis following each CRHP meal accounted for the observed intrahepatic fat mobilization and improved dyslipidemia (21). Lipotoxicity may affect the β-cells (45), and several studies have found intrapancreatic fat accumulation to be inversely associated with β-cell function in T2D (46). Accordingly, the normalization of β-cell function by short-term severe energy restriction and weight loss was found to depend on the reduction in pancreatic fat in one study (33), which was partly corroborated by the present study as participants in both groups experienced a decrease in intrapancreatic fat [previously published in Thomsen et al. (21)] and an increase in β-cell function. Nonetheless, the role of macronutrient composition is still unclear (18, 21).

Chronic low-grade inflammation is involved in β-cell dysfunction in T2D (47), presumably due to increased proinflammatory adipokine release from enlarged and dysfunctional adipose tissue depots (48). Accordingly, diet-induced weight loss attenuates markers of systemic inflammation in individuals with obesity and T2D (49) and in individuals with obesity irrespectively of dietary carbohydrate content (50). In contrast, individuals with T2D may benefit more from weight loss induced by a severely carbohydrate-restricted diet (20E% vs. 55–60E% from carbohydrates) (51). At odds with these observations, the systemic inflammation (CRP, IL-6, IL-8, and TNF-α) was not ameliorated by the moderate carbohydrate restriction in the present study or by weight loss itself, which suggests that the resolution of inflammation is not an important factor in the metabolic improvement after modest weight loss. These findings corroborated those of a weight-maintaining CRHP diet in a similar study design reported by us previously (52).

The primary strength of this study was the highly controlled setting with full provision of the experimental diets, which we believe is necessary to minimize poor dietary adherence in assessing different eating patterns. Adherence to the present diets was confirmed by measuring 24-h urea excretion in urine [i.e., a surrogate marker of protein intake, published in Thomsen et al. (21)]. Moreover, conclusions on dietary regimens are often confounded by competing lifestyle interventions, e.g., exercise or weight loss unequally distributed between groups or with

significant interindividual variation, which we minimized by inducing a matched weight loss between groups and reinforcing and confirming the maintenance of habitual physical activity throughout the study period (21). Medication use was also unchanged. Nevertheless, our study has several limitations, namely, unblinded study design, lack of objective assessment of physical activity, imbalance in sex and DPP-4 inhibitor use between groups, and issues of multiple comparisons that require our results to be interpreted with caution. In addition, we did not properly assess gastric emptying which may increase following a CRHP meal (19) and thereby could influence the metabolic response to the OGTT. However, as the time of peak plasma glucose did not differ between diets, differences between groups in gastric emptying of ingested glucose were likely negligible. When substituting carbohydrates with protein and fat, we allowed other dietary components to vary naturally, reflecting the real foods used. As such, the CRHP diet had more monounsaturated fat and less dietary fiber than the CD diet, and these nutrients may have affected our outcomes independent of carbohydrate restriction. Finally, the duration of our intervention was 6 weeks which may not be sufficient to allow for all metabolic changes to manifest and thus, may not accurately reflect what could be expected in people with T2D undergoing dietary modification for a longer period of time. This is particularly true for the primary outcome of this study, HbA_{1c}, which may not have reached a new steady state within 6 weeks, as discussed previously (21).

In conclusion, a modest ∼6% weight loss induced after 6 weeks on a diet moderately restricted in carbohydrates and enriched in protein and fat did not improve markers of β-cell function, insulin sensitivity, or proinsulin processing to a greater extent than the same amount of weight loss induced after 6 weeks on a conventional carbohydrate-rich diet despite improved glycemic control. Weight reduction in itself was very efficient in ameliorating metabolic dysfunction, independently of dietary macronutrient composition. These results reinforce the key role of weight loss in the management of T2D.

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 Health Ethics Committee of Copenhagen. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MT was involved in study planning and execution, including the collection and analysis of data, and the writing of the manuscript. MS and AS assisted with study planning and data interpretation. AA, JH, SM, and JF contributed to the study conception and design as well as the data production and interpretation. TL and FM contributed to the study design, supervised the food preparation and distribution, and assisted in data interpretation and manuscript writing. MF, BH, and JR contributed to the production and interpretation of data. SH and TK were responsible for conceptualizing the study, obtaining funding, supervising the study, and assisting with data interpretation. As guarantors of this paper, MT, SH, and TK take responsibility for the data integrity and accuracy of the data analysis. All authors contributed to the critical revision of the manuscript and gave their approval to the final version for publication.

Funding

This study was funded by grants from Arla Foods amba, The Danish Dairy Research Foundation, and Copenhagen University Hospital Bispebjerg Frederiksberg. These study sponsors were not involved in the study's design, data collection, analysis, interpretation, or report writing, and they did not place any limits on the publication of the report.

Acknowledgments

We are grateful to the study participants for their tireless efforts in making this study possible. Special thank also go to the kitchen personnel at the Department of Nutrition, Exercise and Sports, University of Copenhagen, as well as the research staff at the Copenhagen University Hospital Bispebjerg, particularly Martin H. Carl and Philip Weber. We offer gratitude to the research team at the Core Biochemical Assay Laboratory, Cambridge University Hospitals National Health Service Foundation Trust, Cambridge, United Kingdom, especially Keith Burling, for analyzing proinsulin. The copyright holder has been granted permission to report previously published data (21).

Conflict of interest

Author AA is a member of the advisory board/consultant for Gelesis (USA), Groupe Éthique et Santé (France), and Weight Watchers (USA) as well as co-owner of the University

of Copenhagen spin-off Flax-Slim ApS and co-inventor on a pending provisional patent application for the use of biomarkers to predict responses to weight loss diets and other related patents and patent applications that are all owned by the University of Copenhagen in accordance with Danish law. He has also co-authored several diets and cookery books, including books on personalized diets. He is not a proponent of any particular diet (e.g., veganism, Atkins diet, gluten-free diet, high animal protein diet, or dietary supplements). Author JH is a member of advisory boards for Novo Nordisk. Author TL is an advisor for the "Sense" diet program.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.933118/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 15 September 2022
DOI 10.3389/fnut.2022.947697

OPEN ACCESS

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

RECEIVED 19 May 2022
ACCEPTED 30 August 2022
PUBLISHED 15 September 2022

CITATION
Bu Q, Zhang J, Guo X, Feng Y, Yan H, Cheng W, Feng Z and Cao M (2022) The antidepressant effects and serum metabonomics of bifid triple viable capsule in a rat model of chronic unpredictable mild stress. *Front. Nutr.* 9:947697. doi: 10.3389/fnut.2022.947697

The antidepressant effects and serum metabonomics of bifid triple viable capsule in a rat model of chronic unpredictable mild stress

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Background: Probiotics have shown potential antidepressant effects. This study evaluated the effect and probable mechanisms of bifid triple viable capsules (BTVCs) on a rat model of chronic unpredictable mild stress (CUMS).

Materials and methods: Rats were randomly divided into Normal, CUMS model, fluoxetine hydrochloride (FLX), BTVCs, and FLX+BTVCs groups. Depressive-like behaviours, pathological changes in the hippocampus, changes in serum metabolites and potential biomarkers, and metabolic pathways were detected *via* behavioural tests, haematoxylin-eosin staining, nissl staining, non-targetted metabolomics, and ingenuity pathway analysis (IPA).

Results: The rats displayed depressive-like behaviours after CUMS exposure, but BTVCs ameliorated the depressive-like behaviours. In addition, the pathological results showed that the hippocampal tissue was damaged in rats after CUMS exposure and that the damage was effectively alleviated by treatment with BTVCs. A total of 20 potential biomarkers were identified. Treatment with BTVCs regulated D-phenylalanine, methoxyeugenol, (±)-myristoylcarnitine, 18:3 (6Z, 9Z, 12Z) /P-18:1 (11Z), propionyl-L-carnitine, and arachidonic acid (AA) concentrations, all compounds that are involved with biosynthesis of unsaturated fatty acids, glycerophospholipid metabolism, linoleic acid metabolism and AA metabolism. The IPA demonstrated that endothelin-1 signalling and cyclic adenosine monophosphate response element binding protein (CREB) signalling in neurons may be involved in the development of depression.

Conclusion: Our findings suggest that BTVCs can alleviate depressive-like behaviours, restore damage to the hippocampus in CUMS rats and regulate serum metabolism, which may be related to endothelin-1 signalling or CREB signalling in neurons.

KEYWORDS

depression, bifid triple viable capsule, hippocampal damage, serum metabolomics, chronic unpredictable mild stress

Introduction

Depression is a frequently recurrent psychiatric disorder characterised by feelings of pessimism, despair, anhedonia, and even suicidal ideation (1). The 12-month prevalence of major depressive disorder varies considerably across countries but is approximately 6% worldwide. The lifetime risk of depression is 15- -18%, and one in ten patients, on average, presents with depressive symptoms (2). According to the World Health Organization, unipolar depressive disorders ranked as the third leading cause of the global disease burden in 2004 and predicted as the leading cause by 2030 (3). Selective serotonin reuptake inhibitors (SSRIs) are still the mainstay of medical management, but side effects and the risk of adverse drug reactions have drawn concerns (4).

Probiotics can alleviate depressive-like behaviours (5, 6) and avoid side effects and addictions, in contrast to current treatments (7). Probiotics have been proven to attenuate depressive-like behaviour by modulating imbalances in gut microbiota (8). In recent years, preclinical studies have shown that probiotics can effectively treat neurological diseases. Wallace et al. demonstrated that probiotics could alleviate symptoms of depression (9). The efficacies of probiotics and prebiotics on depression have been validated in various preclinical studies and clinical trials (10). Bifidobacterium, a probiotic, plays a fundamental role in maintaining the gut microbiota ecosystem in humans and animals, as it alleviates various diseases by changing the composition of intestinal microflora (11). Zhu et al. reported that Bifidobacterium has beneficial effects on cognition *via* the concentration increase of brain-derived neurotrophic factor (BDNF) and modulation of the gut microbiome (12). A placebo-controlled trial showed that probiotic *Bifidobacterium longum* NCC3001 reduced depression scores and responses to negative emotional stimuli in multiple brain areas, including the amygdala and fronto-limbic regions. However, to date, little evidence has been found associating the therapeutic effect of Bifidobacterium on depression at the level of serum small-molecule metabolites.

Metabolomics, a branch of omics science that systematically analyses the concentration profiles of small-molecule endogenous metabolites generated by living systems, is a promising approach for identifying new biomarkers and novel metabolic pathways in several diseases (13, 14). In the past decade, the rapid development of liquid chromatography–mass spectrometry (LC–MS) has facilitated non-targetted metabolomics. By mining metabolomes more deeply, researchers are now primed to uncover key metabolites and their associations with diseases (15, 16). Metabolomics now has unique and established advantages for developing biomarkers for several diseases, and investigating the association between phenotype and metabolomics changes (17, 18). Serum metabolomics has been widely used in diagnostic and treatment studies on depression (19). Metabolomics may be a valuable tool for predicting antidepressant outcomes (20). However, few studies have investigated the association between Bifidobacterium and serum metabolites in depression.

Bifid triple viable capsule (BTVC) is a kind of probiotics composed of bifidobacterium, Lactobacillus and enterococcus. In this study, LC–MS serum metabolomics was used to explore the therapeutic effect of BTVC on chronic unpredictable mild stress (CUMS) rats, and to reveal the potential mechanism of BTVCs in the treatment of depression from the metabolite level.

Materials and methods

Animals

Adult male Sprague–Dawley (SD) rats (200∼300 g) were purchased from the Experimental Animal Center of China Three Gorges University. Licences no. SCXK (E) 2017-0061. The experimental protocol was approved by the Ethical Committee in Research Medical College of China Three Gorges University of Medical Sciences (NO.202012B0A).

Chemicals and reagents

A concentration of 0.2 mg/ml fluoxetine hydrochloride (FLX, Eli Lilly Pharmaceutical Co., Ltd, Suzhou) in deionised

water was (21). Bifid triple viable capsules (BTVCs, Shangyao Xinyi Pharmaceutical Co., Ltd, Shanghai) were prepared in a 2 mg/ml suspension in deionised water. Mass spectrometry grade methanol (Fisher Scientific, USA), mass spectrometry acetonitrile (Fisher Scientific, USA), and formic acid (Sigma, USA) were used.

Equipment

A Triple TOF® 6600 high-resolution mass spectrometry system (AB SCIEX, USA) equipped with an ACQUITY UPLC I-Class ultrahigh-performance liquid system (Waters, USA) and Analyst® TF data acquisition software (AB SCIEX, USA) was used. We used a 5417R centrifuge (Eppendorf, Germany); Vortex Mixer T1 vortex oscillator (Titan SCIENTIFIC LAB); TIMI-10K micromini centrifuge (Titan SCIENTIFIC LAB); and a Labconco centrifugal concentrator (Labconco, USA). Ultrapure water (18.2 MΩ•cm) was prepared using a Milli-Q purified water system (Merck Millipore, USA).

Drug treatment and groups

The FLX solution was given to the animals by oral gavage. Drugs were prepared as specified for rat weight. Rats were randomly assigned to one of the following groups: Normal group, CUMS model group, FLX group (2 mg/kg/d, FLX), BTVCs group (20 mg/kg/d, BTVC), FLX+BTVCs group (2 mg/kg/d FLX + 20 mg/kg/d BTVCs). Each group contained 5 rats. All rats were given agents by gavage at a dose-dependent on body weight once daily for 20 days. Rats in the control and model groups were fed deionised water. Rats were kept under standardised temperature (23 ± 1°C) and a 12 h light/dark cycle (lights on 07:00 –19:00) and had free access to food. The rats lived in abnormally hygienic "specific pathogen free" (SPF) facilities. The experimental timeline is shown in **Figure 1**.

Chronic unpredictable mild stress procedure

Except for Normal rats, all groups underwent daily exposures in random order to one of the following chronic unpredictable mild stress for 34 days: 24-h fasting, 24-h water-deprivation, 5-min ice water swimming at 4°C, 2-min tail clamping (1 cm from the tail root), 24 h of a reversed light/dark cycle, 24 h of the cage being tilted, 24-h strange smell (glacial acetic acid sprayed on the litter for 24 h) and 24-h damp bedding. CUMS was randomly performed as summarised in **Figure 1** described the experimental schedule (22–24).

Behavioural tests

The rats were weighed weekly. After 46 days of CUMS exposure, the neurological behaviours of rats were evaluated in the open field test (OFT) and elevated plus-maze (EPM) test. The OFT and EPM tests were recorded, results analysed with Top Scanlife.

The OFT was used to measure exploratory behaviour and general behaviours. Three days before the start of the experiment, the rats were placed in the experimental environment for 5 min every day. The apparatus was a 40 cm × 40 cm × 47 cm box with normal lighting and temperature. A video recording system was stationed above the apparatus to capture the movement of rats within the box. Subsequently, each rat was placed in the centre of the open field, and its movements were video-recorded for 5 min. The apparatus was cleaned between subjects to remove the odour of the previously tested rat. During the test, the observers stayed away from the apparatus. Upon-the OFT finished, the video was analysed using Top Scanlife, and the distance travelled in the centre area, total distance travelled and latency to move were assessed.

The EPM was elevated 50 cm above the ground, constructed from wood, and consisted of two open arms (50 cm × 15 cm) and two enclosed arms (50 cm × 15 cm × 40 cm) with an open top. Rats were placed in the centre of the apparatus, facing one of the open arms, and allowed 5 min of free exploration. Their activity was observed and recorded for 5 min. The percentage of time spent in the open arms was calculated as the percentage of the total time the rat spent on the maze [time in the open arms/ (time in open arms+time in the closed arms) × 100], and the percentage of open arm entries were calculated as a percentage of the total number of rat entries into the maze [number of open arm entries/ (number of open arm entries+number of closed arm entries) × 100] (25).

Sample collection

After the last behavioural assessment, the blood was collected by the abdominal aortic method. Serum was separated by centrifugation (3,500 rpm, 10 min), and the supernatant was stored at −80°C until analysis. Each brain was dissected on ice; the left hemisphere was removed and fixed with 4% paraformaldehyde solution until tissue sections were obtained. Right hippocampal tissue was isolated and stored at −80°C.

Paraffin sectioning

The left hemispheres of the brains of the experimental animals were placed in 4% paraformaldehyde, dehydrated with a gradient of alcohol and xylene, and then embedded in paraffin.

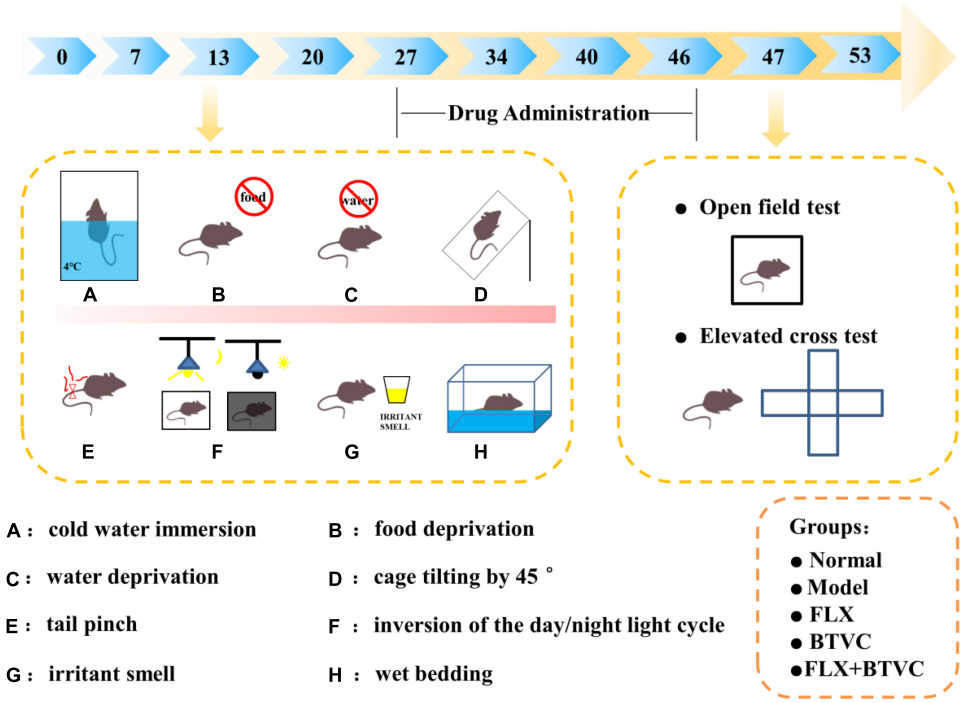

FIGURE 1
The experimental timeline. **(A–H)** The CUMS procedure.

After the wax solidified, the wax block was placed on a paraffin microtome to slice; slices were baked in a 60°C oven.

Hematoxylin and eosin staining

The slices were washed 2 times with xylene (20 min per wash) and anhydrous ethanol (10 min per wash), and then the slices were immersed in 95% alcohol for 5 min, 90% alcohol for 5 min, 80% alcohol for 5 min, 70% alcohol for 5 min, and distilled water. The slices were stained in Harris haematoxylin for 3–8 min, washed with tap water, washed 1% hydrochloric acid alcohol for a few seconds, washed with tap water, stained blue with 0.6% ammonia water, and washed again with tap water. Then, the slices were stained in eosin staining solution for 1–3 min, placed in 95% alcohol (5 min per time), anhydrous ethanol (5 min per time), and twice in xylene (5 min per time) for dehydration and to render transparent, then taken out of the xylene and naturally dried. The slices were sealed with neutral resin.

Nissl stain

The slices were washed in xylene (20 min per wash) and washed twice in anhydrous ethanol (5 min per wash), placed in 75% alcohol for 5 min, and washed with running water. The tissue slices were put into toluidine blue for 5 min, washed with water, and differentiated with 1% glacial acetic acid. The reaction was terminated by washing with tap water. After washing with tap water, the slices were dried in an oven. The slices were immersed in xylene for 5 min and sealed with neutral resin. Elemental composition was calculated from the high-resolution spectra using CasaXPS with measurements.

Metabolomics

Metabolites were extracted from serum samples. Briefly, cold methanol containing internal standards: deuterated carnitine, FFA, CDCA, CA, Trp, Phe, LPC 19:0, SM 12:0, and choline was added to 50 μL serum. The samples were centrifuged at 14,000 × *g* for 15 min at 4°C, and then the supernatant was subjected to LC-MS. For non-targetted metabolomics assays, an ultra-performance liquid chromatography (UPLC, Waters, USA) system coupled with a Q-TOF high-resolution mass spectrometer (Triple TOF 6600, AB SCIEX, USA) was employed. A more detailed description can be found in the **Supplementary material**. For quality control, an equal volume of all the serum samples was mixed and inserted per 10 samples in the analytical process to evaluate the reliability of the whole procedure including sample preparation and UPLC-HRMS analysis.

Ingenuity pathway analysis

The canonical pathways enriched by the differential metabolites were detected with the Ingenuity Pathway Analysis (IPA) suite (version 1.0, QIAGEN, USA). IPA is a web-based software application[1]that identifies biological pathways and functions relevant to biomolecules of interest. The PubChem CID, *p*-value and fold change (FC) of each metabolite were uploaded to the database to construct a core analysis, and then a list of differential metabolites was uploaded to the IPA along with their Human Metabolome Database (HMDB) identification, FDR (false discovery rate) and logarithmic fold change. Enriched pathways of differential metabolites was generated based on the Ingenuity Pathway Knowledge Database, elucidating the potential targets and pathways of BTVCs in the treatment of depression.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (Chicago, IL, USA). Data were expressed as the mean ± standard deviation ($\bar{x} \pm SD$). If the data were normally distributed, comparisons were performed between two groups using the independent-sample *t*-test and among three or more groups using the one-way analyses of variance (ANOVA) test. Pairwise comparisons were made using the least significant difference (LSD) *post-hoc* test after one-way ANOVA. If the data were not normally distributed, the non-parametric Kruskal-Wallis test was used to analyse data. The statistical significance was set at $P < 0.05$.

Results

Bifid triple viable capsule improved the behavioural changes of chronic unpredictable mild stress rats

As shown in **Figure 2A**, compared with the Normal group, rats in the Model group travelled significantly less distance in the open field. Compared with the Model group, all the drug intervention groups showed significant increases in total distance travelled. As shown in **Figure 2B**, compared with the Normal group, travel distances were significantly lower in the Model group. Compared with the Model group, the total distance travelled in the FLX group and FLX+BTVCs group were significantly increased ($P < 0.05$). The BTVCs group displayed a significantly increased total distance travelled ($P < 0.05$). As shown in **Figure 2C**, compared with the Normal group, the Model group displayed a significant trend toward decreased movement in the central area, but this difference was not statistical significance ($P > 0.05$). The distance travelled in the central area was significantly increased in the FLX group and FLX+BTVCs group than in the Model group ($P < 0.05$). The BTVCs group displayed a trend toward increased movement in the central area, but this difference was not statistical significance ($P > 0.05$). As shown in **Figure 2D**, the immobility time in the CUMS group was significantly higher than in the Normal group ($P < 0.05$). Compared with the Model group, the Normal group and drug intervention groups had significantly shorter latencies; among them, the latency of the FLX group and FLX+BTVCs group showed significant difference ($P < 0.05$), shorter than that of the BTVCs group.

The traces in the EPM test are shown in **Figure 2E**. Compared with the Normal group, the Model group entered significantly fewer open arms and travelled significantly lower distances in the open arms. Compared with the Model group, the drug intervention groups exhibited a higher number of entries into the open arms and travelled further in the open arms. Compared with the FLX group, the BTVCs group exhibited a denser trace (**Figure 2E**). The percentage of open arm entries significantly decreased in the model group than in the Normal group ($P < 0.05$); While the percentage was reversed dramatically by the FLX treatment, and the percentage in the BTVCs group of entries of the open arms further increased significantly ($P < 0.05$, **Figure 2F**). Compared with the Normal group, the Model group decreased the percentage of time spent in the open arms, but the behaviour of the drug intervention groups was improved by treatment. The drug intervention groups spent more time in the open arms. Compared with the Model group, the BTVCs groups spent more time exploring the open arms ($P < 0.05$, **Figure 2G**), but there were no significant differences among the drug intervention groups ($P > 0.05$, **Figure 2G**).

Bifid triple viable capsules protects the hippocampus of chronic unpredictable mild stress rats

The results showed that the cell nucleus was full and clear, and the cells were arranged closely and neatly in the Normal group. Cells from the Model group lacked nuclei, were disordered, shrunken, and irregularly arranged, and displayed a tendency to spread to the outer layer (black arrows). We found that treatment improved the arrangement of cells compared to that of the Model group. Hippocampal cells had a normal structure, clear nucleus, regular and tighter cell arrangement, and normal cytoarchitecture, and damage to the hippocampal structure was decreased in the drug intervention groups (black arrows), shown in **Figures 3A,B**.

1 http://www.ingenuity.com

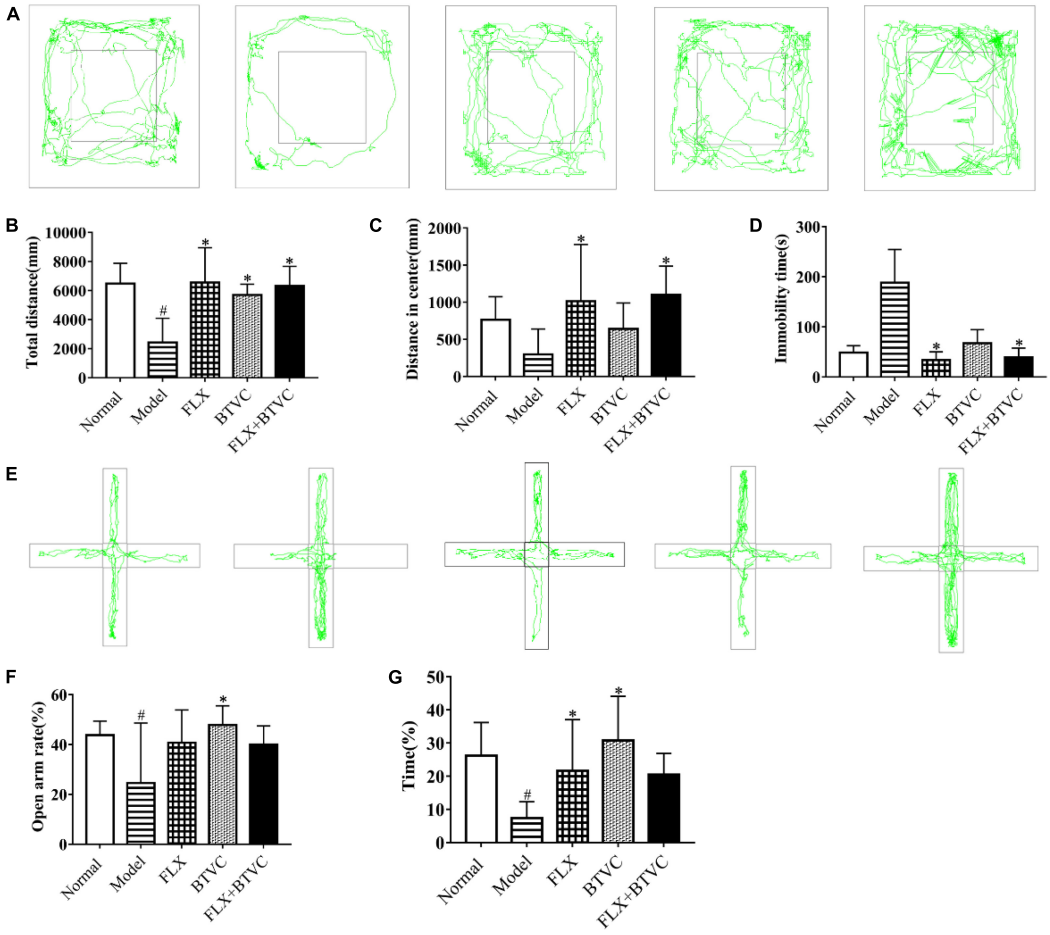

FIGURE 2
Behavioural test, **(A–D)** the open field test, **(E–G)** the elevated plus-maze test. Representative figure of rats' tracks **(A)**, the total distance travelled **(B)**, the distance travelled in the central area **(C)**, immobility time in the open field test **(D)**. The movement trajectory of each groups in the elevated plus-maze, the vertical arms are closed arms, the horizontal arms are open arms, and the middle is the central area **(E)**, the ratio of the number of times the rats in each group enter the open arm in the elevated plus-maze **(F)**, the time ratio of staying in the open arm in the elevated plus-maze **(G)**. Compared with the Normal group, $^{\#}P < 0.05$; compared with the Model group,$^{*}P < 0.05$.

The results of Nissl staining are shown in Figures 3C,D. Compared with those of the Normal group, Model group displayed more pyramidal cells, ambiguous edges, loose and disordered cell arrangement; disappeared nuclei, reduced and diffused nissl bodies, and lighter staining (black arrows). Compared with the Model group, the hippocampal pyramidal cells of the drug treatment groups were neatly arranged, had distinct edges and nucleus, and nissl bodies were observed with the number recoveries (Figure 3D) and normal cytoarchitecture (black arrows).

Metabolomics analysis

Multivariate data analysis

A metabolomics analysis of all samples was performed by principal component analysis (PCA) in this experiment. The PCA score plot in positive ion mode indicated that the Model group was distinct from the Normal group (Figure 4A). The negative ion mode PCA score plot is shown in Figure 4B. Although the Model group overlapped with the Normal group and the drug treatment groups, a trend toward separation was observed, indicating that the establishment of the CUMS model changed rat serum metabolites, and thus the model was successfully established. The QC sample contained an equal amount of all tested samples, as shown in Figures 4A,B. QC samples were tightly gathered, indicating that the instrument was stable and had good repeatability.

The OPLS-DA model of each group was established, and Figures 4C,D show that all experimental groups had distinct LC–MS metabolic profiles in positive and negative ion modes. This result showed that BTVC treatment was beneficial for the rat CUMS model. A permutation test was used to validate the PLS-DA model. The OPLS-DA in the positive ion mode

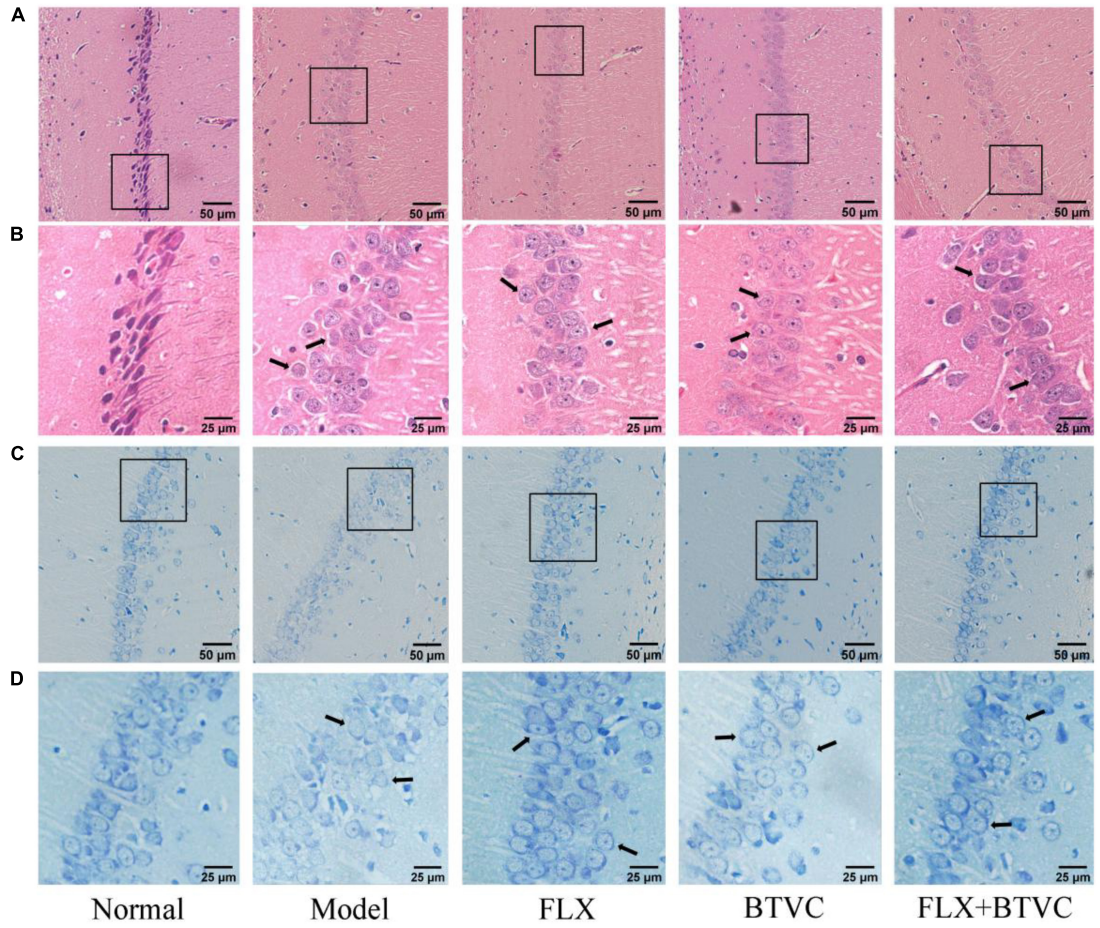

FIGURE 3
Hematoxylin and eosin staining of rat hippocampus region of each group, **(A)** 100× magnification, scale bar = 50 μm, **(B)** 200× magnification, scale bar = 25 μm; Nissl staining of rat hippocampus region of each group, **(C)** 100× magnification, scale bar = 50 μm, **(D)** 200× magnification, scale bar = 25 μm.

of this experiment was $R^2X = 0.754$, $R^2Y = 0.651$, and $Q^2Y = 0.388$, while the negative ion mode was $R^2X = 0.968$, $R^2Y = 0.358$, and $Q^2Y = 0.23$, and the group information in the model was credible.

Identification of differences metabolites

Through the PCA and OPLS-DA score plots, we detected changes in serum metabolites in rats exposed to CUMS. Differential metabolites were identified by a VIP > 1.0 or VIP < 0.5, and $P < 0.05$. A total of 20 differential metabolites were detected (Table 1). Compared with the Normal group, in the Model group PC [14:0/20:1 (11Z)], lysoPC (24:0), taurohyocholate, 3-dehydro-2-deoxyecdysone, PC (O-14:0/O-1:0), glycocholic acid hydrate, Sclareol, mesterolone, oleoylethanolamide, eicosapentaenoic acid, and vetiveryl acetate showed significant changes. BVTCs regulated 6 metabolites: D-phenylalanine, methoxyeugenol, (±)-myristoylcarnitine, 18:3 (6Z, 9Z, 12Z)/P-18:1 (11Z), propionyl-L-carnitine, and arachidonic acid (AA). FLX regulated 6 metabolites: D-phenylalanine, methoxyeugenol, (±) myristoylcarnitine, PE [18:3 (6Z, 9Z, 12Z)/P-18:1 (11Z)] picotamide monohydrate 80530-63-8, and PA (13:0/22:0). FL+BTVCs regulated three metabolites: methoxyeugenol, (±)-myristoylcarnitine, and kalihinol A. Kalihinol A was only found to be a differential metabolite in the FLX+BTVCs group (Table 1).

Metabolic pathway analysis

The 20 KEGG-annotated differential metabolites were imported into MetaboAnalyst to analyse the metabolic pathway.[2] The pathway analysis is summarised in Figure 5, showing the biosynthesis of unsaturated fatty acids, glycerophospholipid metabolism, linoleic acid metabolism, and AA metabolism. Biosynthesis of unsaturated fatty acids and AA metabolism pathways could be mediated by BTVC.

2 https://www.metaboanalyst.ca/

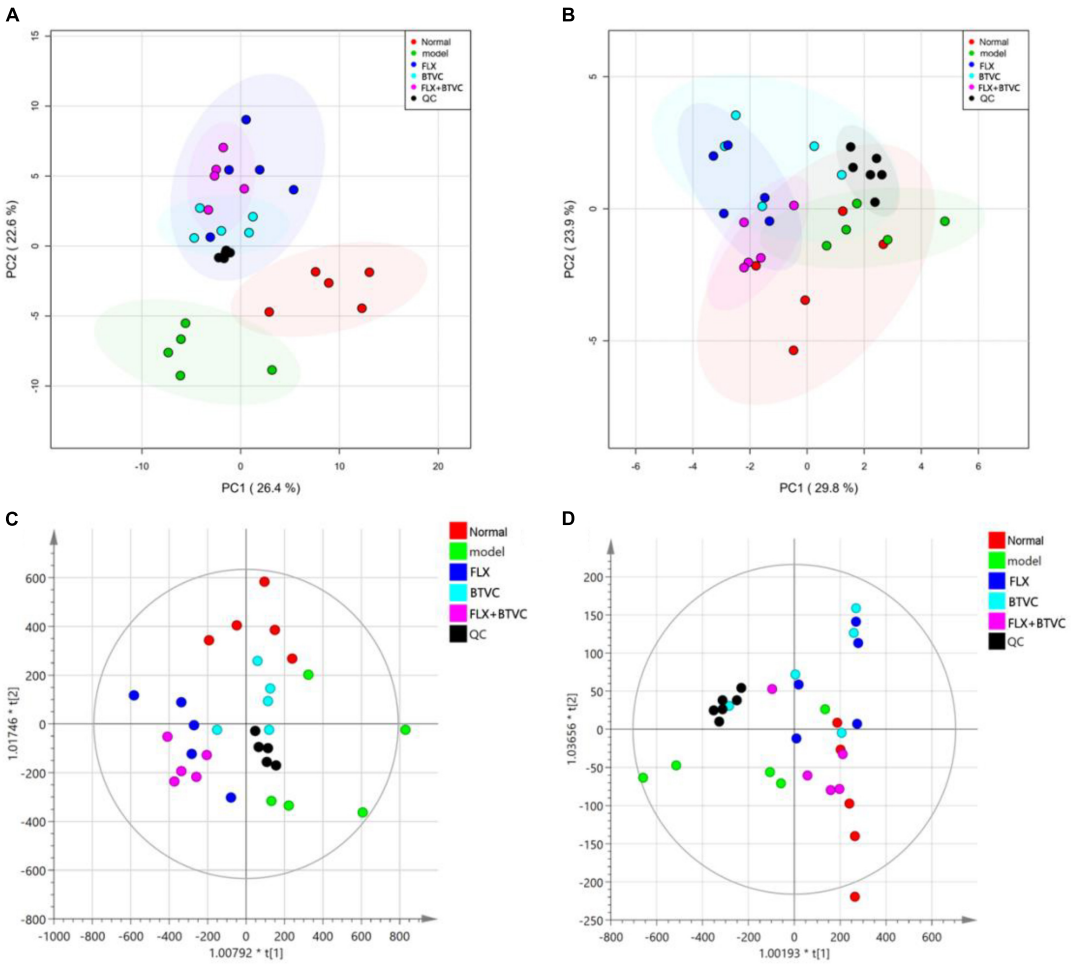

FIGURE 4
(A) Positive ion mode PCA score map; **(B)** negative ion mode PCA score map, **(C)** positive ion mode OPLS-DA score; **(D)** negative ion mode OPLS-DA score.

The network function analysis by ingenuity pathway analysis

Ingenuity pathway analysis was applied to discover potential biomarkers of depression. In the network function analysis, the related metabolites tended to gather into a single network (Figure 6). The pathways were cyclic adenosine monophosphate response element binding protein (CREB) signalling, phagosome formation, endothelin-1 (ET-1) signalling, eicosanoid signalling, and insulin secretion signalling pathways in neurons. The results indicate that these altered pathways may be involved in depression development.

Discussion

As previously reported by others, CUMS is currently a well-recognised animal model of depression, and the animal model established can stably simulate the symptom of human depression (23). In the present study, the CUMS depression model was constructed by stimulating animals with chronic random stressors to induce a depressive state, leading to prolonged immobility and reduced exploration. The drug intervention ameliorated these effects; the distance travelled and exploration increased. HE and Nissl staining were used to observe the morphology of hippocampal tissue in each group and evaluate the therapeutic effect of the intervention. A grate number of neurons in CUMS rats were damaged, and Nissl-positive cells were remarkably reduced in the hippocampi. Compared with the Model group, we found that the severity of hippocampal pyramidal cell and nissl body damage induced by CUMS was ameliorated by BTVCs treatment. All of the results above indicate an antidepressant effect of BTVCs. Recent studies demonstrated that psychobiotics or prebiotics may be more specific and beneficial to depression. In a placebo-controlled trial, Pinto-Sanchez et al. discovered that the probiotic *Bifidobacterium longum* reduced depression scores

10.3389/fnut.2022.947697

TABLE 1 Serum metabolites with significant changes in treated rats.

Metabolite	Normal compared with model	FLX compared with model	BTVCs compared with model	FLX + BTVCs compared with model
PC [14:0/20:1 (11Z)]	↑*	↓	↓	↓
LysoPC (24:0)	↓*	↓	↑	↑
Taurohyocholate	↑*	↑	↑	↑
3-Dehydro-2-deoxyecdysone	↑*	↑	↑	↓
PC (O-14:0/O-1:0)	↓*	↓	↓	↓
Glycocholic acid hydrate	↓*	↓	↓	↓
Sclareol	↓*	↓	↓	↓
Mesterolone	↓*	↓	↓	↓
Oleoylethanolamide	↓*	↓	↓	↓
Eicosapentaenoic acid	↓*	↓	↓	↓
Vetiveryl acetate	↓*	↓	↓	↓
D-Phenylalanine	↑*	↑*	↑*	↑
Methoxyeugenol	↑*	↑*	↑***	↑***
(±)-Myristoylcarnitine	↓*	↓*	↓***	↓***
PE [18:3 (6Z, 9Z, 12Z)/P-18:1 (11Z)]	↑*	↑**	↑**	↑
Propionyl-L-carnitine	↑*	↑	↑*	↑
Arachidonic acid	↓*	↓	↓*	↓
Picotamide monohydrate	↑*	↑**	↑	↑
PA (13:0/22:0)	↓*	↓*	↓	↓
Kalihinol A	↓*	↓	↓	↓*

Compared with the model group, "↑," the expression level is up-regulated; "↓," the expression level is down-regulated.
*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

and increased the quality of life in patients with Irritable Bowel Syndrome (26). However, this probiotic is now not available on the market. In another randomised clinical trial, Bifidobacterium *breve* CCFM1025 showed an antidepressant effect in patients with major depression disorder (27, 28). Similarly, Yang et al. demonstrated that supplementation of Bifidobacterium reversed depression-like behaviours, social interaction and sucrose preference, in mice with chronic social defeat stress (29). In this regard, intake of Bifidobacterium may prevent the onset of depression and relapse in depressed patients and animals.

Changes in serum metabolites were identified by Untargeted Metabolomics techniques based on LC–MS to determine the potential mechanism of BTVCs in the treatment of depression. We identified 20 depression-related biomarkers by serum metabolomics, six of which were significantly regulated by BTVC. The serum-differentiated metabolites were enriched in four metabolic pathways that include the glycerophospholipid metabolism, AA metabolism, linoleic acid metabolism, and biosynthesis of unsaturated fatty acids.

Glycerophospholipid metabolism and AA metabolism are the most profound pathways ($p < 0.05$, impact >0.1) in the pathway analysis. Glycerophospholipids are important constituents of the brain and regulate brain function. Zheng et al. demonstrated that glycerophospholipid might participate in the onset of depression-like behaviours in female cynomolgus macaques (30). Tian et al. identified glycerophospholipid metabolism as a vital cause of depression-like behaviours in mice (31). Additionally, Zhang et al. elucidated that dysfunctions of glycerophospholipid and the related metabolic enzymes may account for the depression in CUMS rat model (32). Consistent with the literature, this research found that PE [18:3 (6Z, 9Z, 12Z)/P-18:1 (11Z)] descended in the model group compared to the Normal group, but was reversed after BTVC intervention.

PE [18:3 (6Z, 9Z, 12Z)/P-18:1 (11Z)], in particular, consists of a γ-linolenic acid chain at the C-1 position, a plasmalogen 18:1n7 scaffold at the C-2 position, and a phosphorylethanolamine moiety at the C-3 position. γ-linolenic acid that differs from the saturated fatty acid on C-1 of most phospholipids belongs to an omega-6 fatty acid. Its elongation product in the human body, Dihomo-γ-linolenic acid, may contribute to a lower risk of depression (33). Phosphatidylethanolamine, a major constituent of cell membranes in the brain, is associated with depression (34) and chronic stress (35).

Glycerophospholipid can be catalysed by phospholipase A2 to produce AA (36). AA metabolism has also been associated with depression severity (37, 38). AA is one of the most abundant polyunsaturated fatty acids (PUFAs) in vertebrates, and can affect depression severity through modulating 5-HTT binding potential (37). AA is metabolised to potent signalling molecules, including leukotrienes and prostaglandins, that mediate responses to physiological stresses, such as

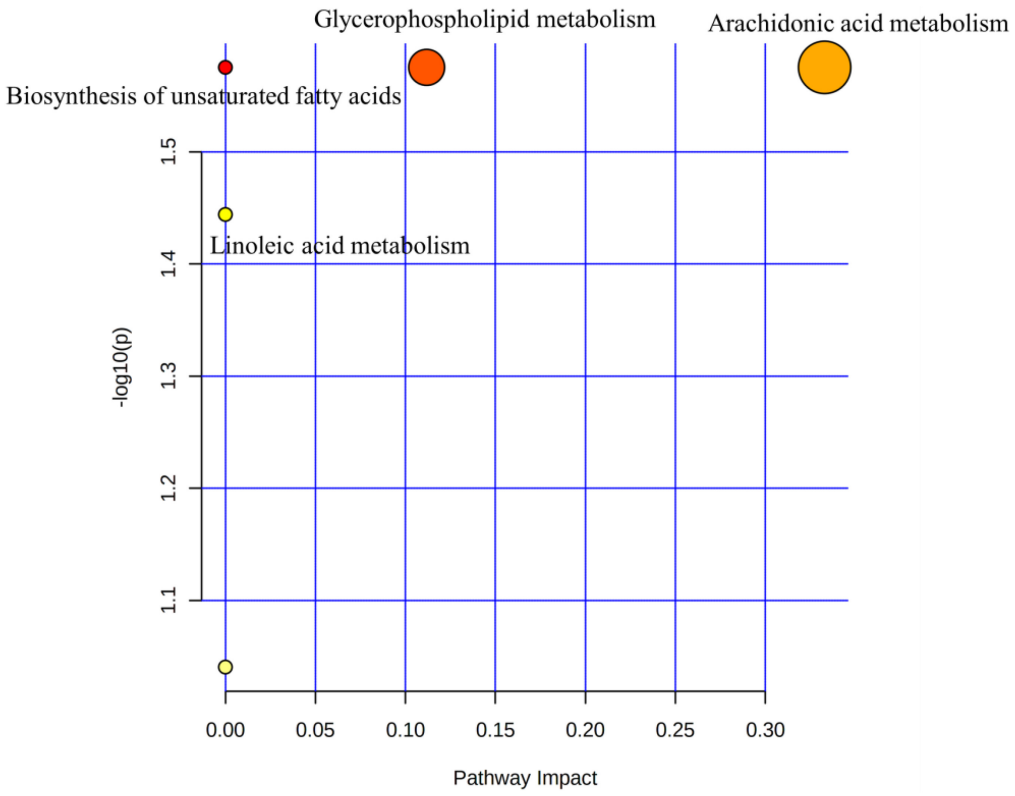

FIGURE 5
Metabolic pathway analysis.

inflammation, and play an important role in the immune inflammation mechanism of depression (39, 40). In our study, the concentration of AA in the serum samples of the Model group and intervention groups was higher than that of the Normal group, and the inflammatory reaction of the Model group was more intense than that of the other groups.

Among the serum-differentiated metabolites, phenylalanine is a precursor of catecholamine that can act as a neurotransmitter and an epinephrine-like substance and plays a pivotal role in depression (41). D-phenylalanine administration has been reported to rapidly activate extracellular signal-regulated kinase (ERK) pathways, a critical step for memory formation, in the cortex and hippocampus, two brain areas involved in memory processing (42). In our study, the serum D-phenylalanine concentration in the Model group decreased after CUMS and was significantly improved after probiotic intervention, indicating that BTVCs may improve depressive symptoms by modulating D-phenylalanine metabolism.

L-carnitine is an endogenous substance that acts as a carrier for fatty acids across the inner mitochondrial membrane, and propionyl-L-carnitine (PLC) is an ester of L-carnitine required for the transport of fatty acids into the mitochondria (43). PLC is a member of the most abundant group of carnitines in the body, comprising more than 50% of all acylcarnitines quantified in tissues and biofluids (44). PLC attenuates forebrain ischaemia-induced neuronal injury, oxidative stress, and energy depletion in the hippocampal CA1 region (45). Changes in acylcarnitine metabolites represent the metabolic status in the brain (46). The biological function of myristoylcarnitine is unclear, yet studies showed that myristoylcarnitine significantly increased when the hippocampus was damaged (47). A significantly increased concentration of myristoylcarnitine in the serum of Model group rats was observed in this experiment, which is consistent with earlier findings.

To further explore the mechanism of BTVCs, this study used IPA to establish a biomolecular interaction network. CREB signalling in neurons, phagosome formation, endothelin-1 signalling, eicosanoid signalling and insulin secretion signalling pathways in other cells were highlighted by the IPA. ET-1 has been reported to evoke necrotic neuronal damage and cause reactive nitrogen species-mediated tissue injury (48). ET-1 in CA1 was critical for regulating the excitability of CA1 pyramidal neurons. Upregulation of ET-1 expression reduced the excitability of CA1 pyramidal neurons and decreased excitatory neurotransmission (49). CREB is a nuclear regulatory factor in eukaryotic cells. The impaired CREB signalling pathway is associated with the progression of depression (50). The activity and expression of CREB in brain tissues are

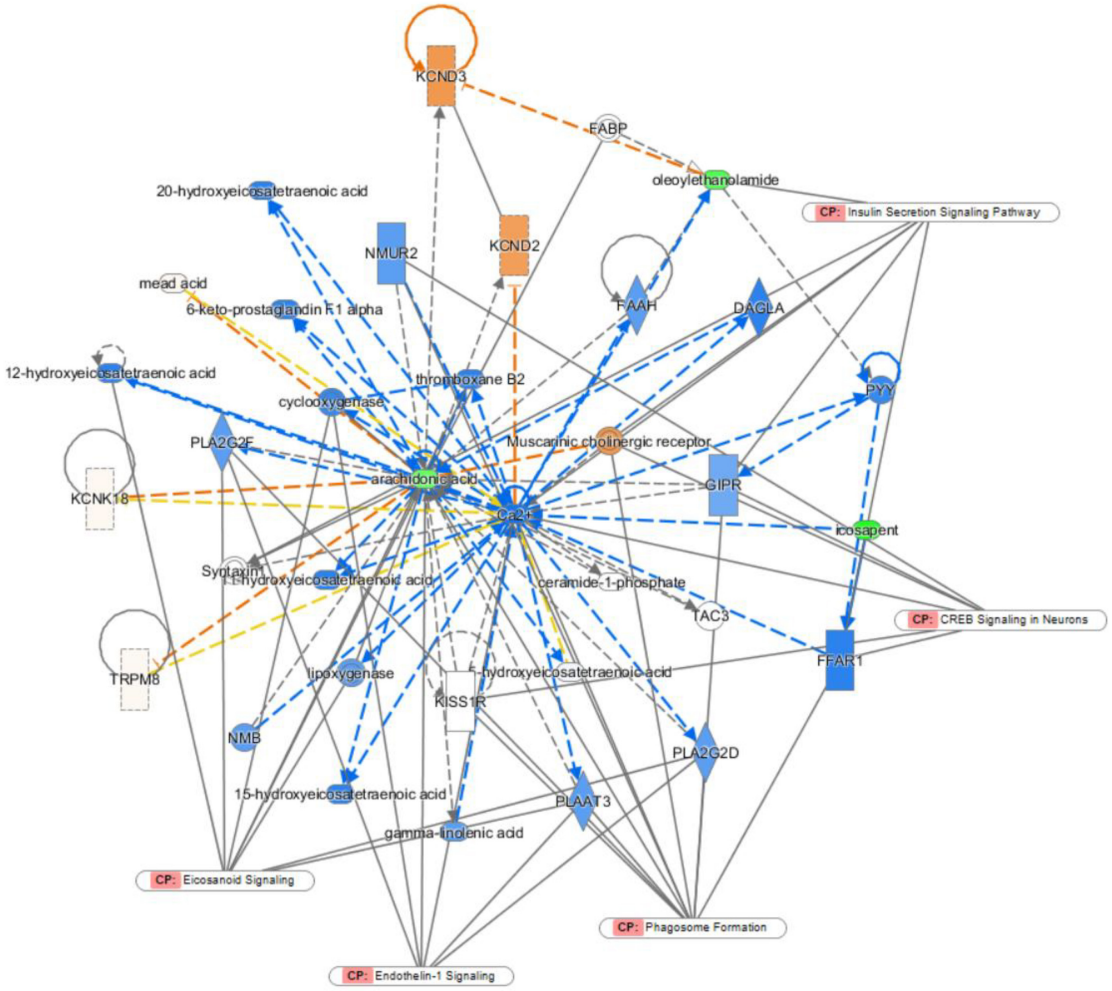

FIGURE 6
Ingenuity pathway analysis (IPA) analysis.

markedly reduced, and its restoration may be responsible for the therapeutic effect of antidepressants (51). In the hippocampus, CREB is crucial mediator of antidepressant effects. A wide variety of standard antidepressant treatments increase CREB activity within the hippocampus, and accumulating evidence suggests roles for CREB-regulated expression of neural growth factors (52).

Bifid triple viable capsules may be a potential adjuvant therapy for depression. This study provides a new insight into the development of antidepressants with fewer side effects than traditional treatments, as it revealed the interconnected metabolic influence of BTVCs in CUMS rats and provided vital evidence for the antidepressant efficacy of BTVCs *via* the Biosynthesis of unsaturated fatty acids and AA metabolism pathways. CREB signalling in neurons and endothelin-1 signalling in neurons may be involved in the development of depression. In addition, further studies that examine the therapeutic effect of BTVCs in humans may be merited. According to the results of IPA, further molecular biology experiments should be carried out.

Conclusion

The present study demonstrated that BTVCs can significantly alleviate depression-like behaviours and decrease hippocampal structural damage. In addition, our results elucidated that BTVCs modulated glycerophospholipid metabolism, linoleic acid metabolism, AA metabolism, and biosynthesis of unsaturated fatty acids in CUMS rats. Furthermore, IPA analysis showed that BVTCs alleviate depression by regulating endothelial-1 signalling and CREB signalling pathways. This study has provided a deeper understanding of microbiota-related metabolic processes in the treatment and prevention of depression and has, at least partially, elucidated the anti-depressant mechanism of BTVCs.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Ethical Committee in Research Medical College of China Three Gorges University of Medical Sciences.

Author contributions

QB and JZ: investigation, formal analysis, data curation, and writing—original draft. XG: software and visualisation. YF and HY: writing—original draft. WC: resources. ZF: conceptualisation, supervision, and writing—review. MC: conceptualisation, supervision, writing—review, and funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This project was supported by grants from Shenzhen Science and Technology Programme (No. JCYJ20180508152437368).

Acknowledgments

Special thanks American Journal Experts for English language 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.947697/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Review
PUBLISHED 15 November 2022
DOI 10.3389/fnut.2022.947567

OPEN ACCESS

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

RECEIVED 18 May 2022
ACCEPTED 13 October 2022
PUBLISHED 15 November 2022

CITATION
Saris CGJ and Timmers S (2022) Ketogenic diets and Ketone suplementation: A strategy for therapeutic intervention. *Front. Nutr.* 9:947567. doi: 10.3389/fnut.2022.947567

Ketogenic diets and Ketone suplementation: A strategy for therapeutic intervention

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Ketogenic diets and orally administered exogenous ketone supplements are strategies to increase serum ketone bodies serving as an alternative energy fuel for high energy demanding tissues, such as the brain, muscles, and the heart. The ketogenic diet is a low-carbohydrate and fat-rich diet, whereas ketone supplements are usually supplied as esters or salts. Nutritional ketosis, defined as serum ketone concentrations of $\geq$ 0.5 mmol/L, has a fasting-like effect and results in all sorts of metabolic shifts and thereby enhancing the health status. In this review, we thus discuss the different interventions to reach nutritional ketosis, and summarize the effects on heart diseases, epilepsy, mitochondrial diseases, and neurodegenerative disorders. Interest in the proposed therapeutic benefits of nutritional ketosis has been growing the past recent years. The implication of this nutritional intervention is becoming more evident and has shown interesting potential. Mechanistic insights explaining the overall health effects of the ketogenic state, will lead to precision nutrition for the latter diseases.

KEYWORDS

nutritional ketosis, ketogenic diet, ketone bodies, precision nutrition, ketone supplementation

The ketogenic diet and its metabolic effect

Caloric restriction and various forms of fasting (intermittent fasting, time restricted eating, periodic fasting) showed extension of lifespan in animals and reduced rates of several diseases, especially metabolic diseases, and cancers (1, 2). Fasting elicits evolutionarily conserved, adaptive cellular responses that reduce free-radical

Abbreviations: AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; APOE, Apolipoprotein E; ATP, adenosine triphosphate; βHB, beta-hydroxybutyric acid; FA, fatty acids; GABA, gamma-amino butyric acid; KB, ketone bodies; KD, Ketogenic diet; MAD, Modified Atkins diet; MCT, medium-chain triglyceride; MS, multiple sclerosis; PD, Parkinson's disease; PEO, progressive external ophtalmoplegia; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acids; RCT, randomized controlled trial; ROS, reactive oxygen species; SGLT2, sodium-glucose cotransporter-2; UCP, uncoupling proteins; VLCKD, very-low-carbohydrate ketogenic diet.

production, improves glucose regulation, increases stress resistance, suppresses inflammation, and causes weight loss. In recent years carbohydrate restriction is gaining popularity and attention. Benefits for general health can be weight loss and improved glucose tolerance, but also better blood pressure control and cholesterol profile (3). It is still unclear whether (neuro) protective effects are associated with an overall reduction in calories (hypocaloric diet) or rather by specific nutrient restriction (diet restriction).

The human body is adapted to survive these periods with no or limited food availability by adapting its substrate preference to sustain bodily functions. During fasting and starvation, but also with carbohydrate restriction, or prolonged intense exercise, the body is stimulating lipid utilization from lipid reserves by mitochondrial beta-oxidation. Whole-body fat oxidation rates increase twofold to threefold (4). The mitochondria of the liver start producing ketone bodies as an alternative fuel source to replace glucose. These ketone bodies (KB), including acetoacetic acid (acetoacetate), acetone and beta-hydroxybutyric acid (βHB), can be utilized by the brain, heart and skeletal muscle through the citric acid cycle (Krebs cycle) and act as an energy source when glucose is not readily available (see **Figure 1**) (5–7). β-hydroxybutyrate provides more adenosine triphosphate (ATP) per mole of substrate than pyruvate for brain and muscle tissue [for more details on the biochemical signaling, we recommend ref (5)]. Ketosis, referred to by Hans Krebs as "physiological ketosis," is defined as blood βHB concentrations of $\geq$ 0.5 mmol/L. The maximum concentration of KB is around 8 mmol/L in undisturbed glucose metabolism while blood pH remains unchanged. Upon refeeding, the presence of insulin and reduction of glucagon will reduce lipolysis and diminish the ketogenetic flux in the liver (8). Besides starvation, also diets low in carbohydrate content can be used to reach a state of ketosis (see **Table 1**). The latter carbohydrate restricted diets vary in their proportion of carbohydrates. The challenge, however, has been to raise circulating KB levels by using a palatable diet without significant elevated concentrations of plasma cholesterol and free fatty acids (FA). The classic or standard long-chain triglyceride ketogenic diet contains high fat vs. low carbohydrate and low protein content with a 4:1 ratio of fat to protein plus carbohydrate (in grams). Lower ratios can be used to improve adherence. Another strategy to improve adherence is to include days in which more carbohydrates are added to the diet (cyclical ketogenic diet). Targeted ketogenic diet is similar to a standard ketogenic diet except that carbohydrates are consumed just before exercise. Lower ratio with increase in protein content (high protein ketogenic diet) can also be helpful to increase adherence. A very-low-carbohydrate ketogenic diet (VLCKD) is usually referred to as a standard ketogenic diet. Modified Atkins diet (MAD) is adapted from Atkins weight reduction diet in which carbohydrate intake is restricted to 10–20 g/day. Ratio's between fat to protein plus carbohydrate range typically between 1:1 and 1.5:1, but can reach 4:1. The third type ketogenic diet is the medium-chain triglyceride (MCT) diet. It follows the outline of standard ketogenic diet, but instead of using long-chain triglycerides, medium chain (C6–C12) triglycerides are used since these triglycerides are more ketogenic. Calorie intake is calculated based on the percentage of energy derived from MCT. A fourth strategy is to minimize glycemic increases by higher amounts of carbohydrates with low glycemic index ($<$ 50). This low glycemic index (LGI) diet allows more carbohydrate than either the classic ketogenic diet or the modified Atkins diet and causes more stable glucose levels.

Adverse reaction during initiation of the diet as a result of the low glucose levels is termed "keto-flu" and consists of headache, weakness, irritability, constipation, nausea, and vomiting. Symptoms can vary and often diminish after a week of diet. Due to reduction of total calorie intake, since high fat diet is less palatable and fat has a weak effect on satiation, weight loss and anorexia often occur. Polyunsaturated fatty acid intake and reduced intake of cholesterol is important to prevent elevated cholesterol and triglycerides levels associated with atherogenic risk, especially during long-term use of the ketogenic diet (9). Supplementation of omega 3-fatty acids can prevent hyperlipidemia and improve fatty acid profile (10). Gut microbiome alterations in its composition can result in gastrointestinal discomfort, and certain species (Akkermansia, Bacteroidetes, Firmicutes, Muciniphila, Lactobacillus) increase production of short-chain FAs like acetate, propionate and butyrate (11). It is important to avoid malnutrition and include an adequate intake of macro- and micronutrients. Low-carbohydrate diets are often low in thiamin, vitamin B6, folates, vitamin A, vitamin E, calcium, magnesium, iron or vitamin K. There is a reduced bone- and calcium (Ca) metabolism and a risk for developing hypercalciuria and kidney stones (12). Urine analysis and urine calcium creatinine ratio should be analyzed during KD. Good hydration minimizes the risk of stone formation. Mild carnitine depletion occurs in the first months of diet treatment and stabilize or restores slightly with long term treatment (13).

KB can also arise in pathological conditions without carbohydrate restriction. In relative or absolute insulin deficiency, as can occur in diabetic patients, glucose cannot be transported into liver cells, resulting in KB production in the liver (diabetic ketoacidosis). With excessive long-term alcohol consumption an alcoholic ketoacidosis can occur. Hepatic glycogen stores are depleted by malnutrition and ethanol metabolism further impairs gluconeogenesis (14).

In recent years, the effectiveness of exogenous KB supplementation has been explored (15). Oral administered exogenous KB supplementation rapidly elevate plasma levels of KB during a ketogenic diet, but can also be taken as a supplement on top of a normal diet (see **Table 1**). The metabolic response, due to the presence of glucose, is however different. Exogenous KB lowers fasting glucose concentrations

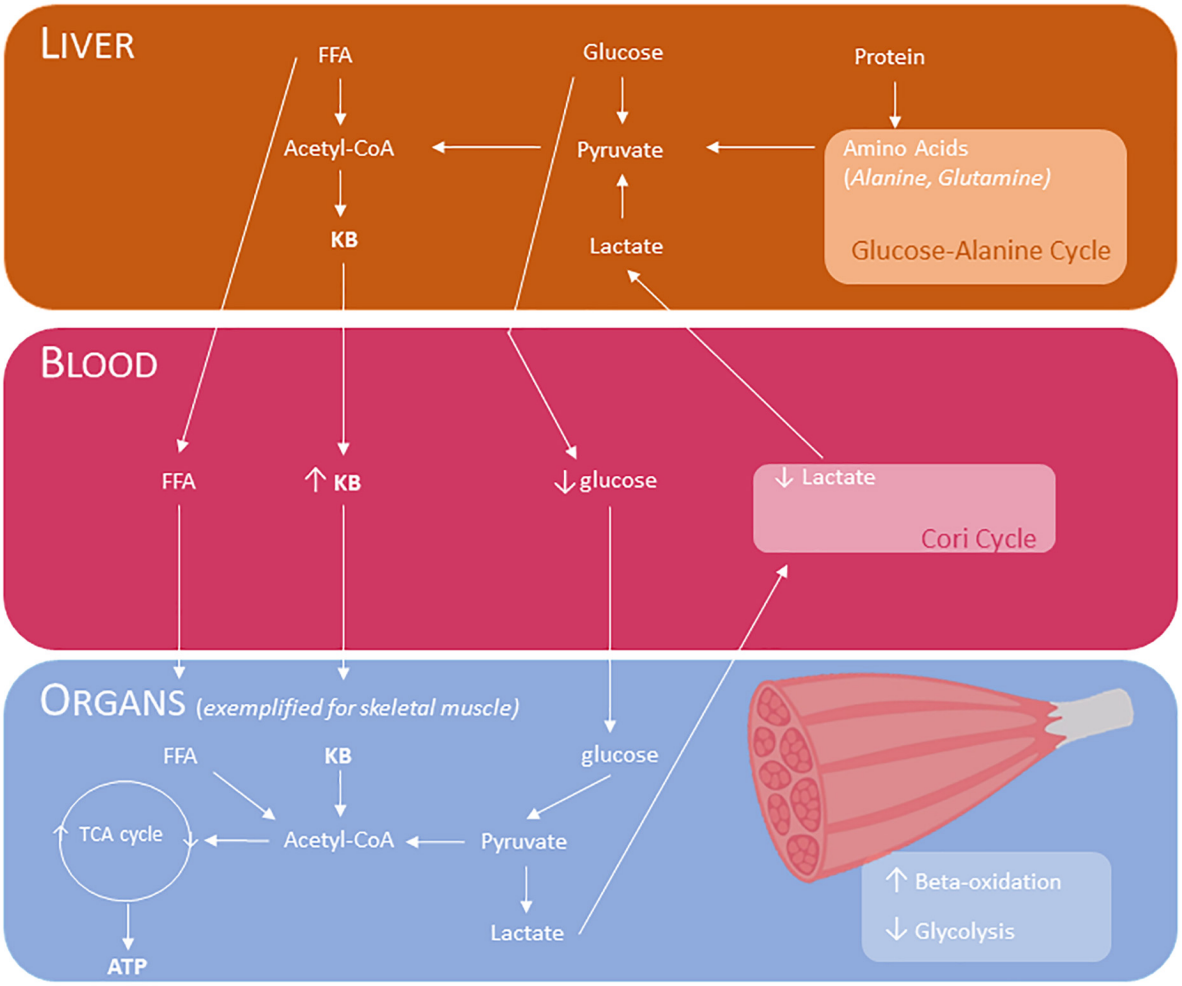

FIGURE 1
Effects of ketogenic diet and ketone body supplementation and downstream metabolism, exemplified for skeletal muscle. The liver produces ketone bodies during fasting, starvation, in carbohydrate restriction, or prolonged intense exercise. These ketone bodies can be used for input in the TCA cycle in various tissues, including the muscle to generate ATP. The Cori cycle, also known as lactic acid cycle, is the pathway in which lactate is transported to the liver and converted into glucose. Glucose-alanine cycle is the pathway in which alanine is transported to the liver and converted into glucose. KB, ketone bodies (acetoacetic acid/acetoacetate, acetone and beta-hydroxybutyric acid); TG, triglycerides; FFA, free fatty acids; Acetyl CoA, Acetyl coenzyme A; TCA, tricarboxylic acid; ATP, Adenosine triphosphate.

via attenuated hepatic glucose output (without increase in skeletal muscle glucose uptake). Dairy products are a natural source of β-hydroxybutyrate with concentration ranging from 10 to 631 μM (16–18). Beta-hydroxybutyrate and acetoacetate salts and/or amino acids, and esters, and MCT supplementation is commercially available and generally considered as safe. Ketone salts, consumed as sodium, potassium and/or calcium βHB, result in high levels of inorganic ion consumption. Ketone esters decrease blood pH and ketone salts increase urinary pH (19). Ketone formulations are racemic mixtures with equal amount of left- and right-handed enantiomers (D-βHB and L-βHB). In the body, D-enantiomers are the predominant circulating KB and is better oxidized than L-enantiomers. It is important to take into account when evaluating these effects of diets or supplementation, that commercial ketone meters and laboratory analysis only detect D-βHB and most do not test for acetoacetate. The combination of KB and MCT supplementation can augment the plasma KB levels to a higher extent and keep them elevated during a more prolonged period (20).

There is no systematical evaluation of cardiovascular risks associated with long term use of KD. Currently, there are no recommendations for routine evaluations such as ECG, cardiac or carotid artery ultrasound (21). Reduced levels of insulin-like growth factor 1 cause growth and bone problems in children. Routine weight and height measurements during KD help to assess growth. Dual energy X-ray absorptiometry (DEXA) and vitamin D as routine examination is recommended during

TABLE 1 Types of diet to reach nutritional ketosis.

Diet	Specifics	Advantages	Disadvantages
Classic KD, VLCKD	4:1 ratio fat to protein plus carbohydrate	Faster in ketosis, more effective?	Less palatable, difficult to adhere
MAD	Carbohydrate intake restricted to 10–20 g/day (ratio 1:1 to 1.5:1)	More palatable, better adherence	Less effective compared to classic KD?
MCT	Medium chain triglycerides as fat source	MCT are more ketogenic	Not all fat sources can be used.
LGI	Carbohydrates with low glycemic index (<50).	More carbohydrates are allowed	Less production of ketone bodies, less effective?
KB supplementation	Oral ingestion of KB	Better adherence	Only direct effects of KB, no metabolic shift.

KD and calcium and vitamin D supplementation should be maximized and exercise strongly encouraged. When growth retardation occurs, protein intake should be maximized.

Physical performance and fatigue are dependent on substrate availability to working muscles. As exercise intensity increases, there is a shift in the contribution of different substrates to energy provision from free fatty acids and glucose in the blood toward intramuscular triglycerides and glycogen. At moderate-to high intensity exercise (> 75% of maximal oxygen uptake, VO2max), muscle glycogen is the main energy source (22). Hence, much focus has been directed toward advancing nutrition strategies that might spare glycogen reserves as this could support or even optimize sports performance (23–25).

KB have the potential to be used as an alternative fuel source to carbohydrates and fat during endurance exercise and can lower the exercise-induced rise in plasma lactate (see **Figure 1**) (26), amongst other metabolic effects (6). Moreover, the capacity to take up and utilize KB during exercise seems higher in exercise-trained skeletal muscle (27).

The potential ergogenic effects of KB supplementation has therefore attracted a lot of scientific interest. Habitual consumption of a ketogenic diet, which is high in fat (∼80% total kcal), very low in carbohydrate (∼5% total kcal), and moderate in protein (∼15% total kcal), augments circulating KB and enhances fat oxidation while sparing carbohydrate oxidation during endurance exercise (28–30). Yet benefits of ketogenic dieting for performance remain equivocal, possibly attributed to a low muscle glycogen content at the start of exercise due to severe carbohydrate restriction and impaired glycolytic flux during high-intensity exercise (31–33). This has led to the exploration of means to achieve acute nutritional ketosis that bypass the undesirable effects of carbohydrate restriction. Acute exogenous ingestion of KB in the form of βHB salts or ketone esters has sparked the interest to enhance performance and recovery in athletes (34, 35). The latter ketone supplements can induce acute ketosis as evidenced by > 0.5 mM βHB in the blood for up to 3 h after consumption of an acute dose, without the necessity to modify dietary intake (19, 36). Hence, they could serve as an attractive strategy to supply the working muscle with extra energy, while sparing muscle glycogen stores (35). In addition, ketone bodies are in essence a more efficient energy substrate than glucose or fatty acids because its conversion into an oxidizable form does not require ATP, thereby enabling more muscle work for a given energy cost (34).

From a performance point of view, the effect of exogenous KB supplementation as an ergogenic aid, especially in the acute form, is not ambiguous. A recent meta-analysis (13 studies) (37) and systematic review (10 studies) (38) both failed to show consistent effects of exogenous KB supplements on physical performance (both endurance and power outcomes). Except for the seminal study of Cox et al. (39), the remainder of the studies failed to show any benefit of acute KB supplementation on performance (40–46), and some even reported detrimental effects (47, 48). Also, on secondary outcome measures like plasma lactate and glucose, respiratory exchange ratio, or perceived exertion the efficacy of KB supplementation remains inconclusive.

The various KB supplements in combination with their pharmacokinetics as well as the variation in gastrointestinal distress between supplements is likely to affect the performance outcome measures. That is, the type of supplement (ketone ester vs. ketone salt) and the nutritional status (fed vs. fasted state) seems to determine the level of circulating βHB concentrations, and hence explain a lot of the variance between studies (37, 38). Providing ketone monoesters in the fasted state maximizes the state of ketosis, reaching peak circulating βHB concentrations above 2 mM (19, 39, 40, 49), which are blunted in the fed state (19). Ingestion of ketone salts (46, 48), ketone diesters (47), ketone precursors (44, 45), or ketone monoesters (41) in the fed state induce acute ketosis (βHB > 0.5 mM), yet fail to reach the 2 mM cutoff. The difference in peak βHB concentrations between ester and salt supplements are likely mediated by the βHB isoform present in the drinks. In contrast to ketone esters, which are composed of D-βHB, ketone salts are often a racemic mixture of both D-βHB and L-βHB isoforms of βHB, even though the metabolism and metabolic fate of L-βHB being less well understood (50, 51). The L-βHB isoform seems less readily oxidized and does not seem to contribute a lot to energy supply, and hence may accumulate upon repeated ketone salt drinks (19). Furthermore, it is documented that food in the gut can delay or prevent the uptake of small hydrophilic carbons such as βHB (52, 53), probably explaining the lower circulating βHB following food consumption (19). KB supplementation is also associated with symptoms of gastrointestinal distress, which can range in

severity. Symptoms including nausea, diarrhea, constipation, vomiting and abdominal pain have been reported (36). Another factor that may likely influence the performance benefit is the type of test chosen. As KB have been suggested to exert antiglycolytic effects (39), KB may especially work advantageous during low-to-moderate intensity endurance exercise (35). That is, KDs have shown to be beneficial during a long duration ($\geq$ 12 h), low-intensity ultra-endurance-type event, where mitochondrial beta-oxidation can match lower rates of ATP demand, whereas the combination of nutritional ketosis and high carbohydrate intake may confer performance benefits in endurance events of moderate intensity and durations of $\geq$ 8 h (54). In summary, even though there is a clear biological rationale to support a performance enhancing effect of acute exogenous KB supplementation, to date there is not sufficient evidence to support a clear ergogenic effect. Due to divergent effect across subjects, this might also suggest responders and non-responders (55).

Overall, nutritional ketosis seems an interesting and promising strategy to increase the health status of healthy individuals and to serve as a therapeutic intervention in several diseases. Insights in these mechanisms will lead to precision nutrition for these diseases (56). Below, the effects on KB on epilepsy, heart diseases, mitochondrial diseases, and neurodegenerative disorders will be summarized.

Ketogenic diet in (intractable) epilepsy

KD is a well-established, effective non-pharmacologic treatment for intractable childhood epilepsy. Its history dates back to 500 years BC when fasting was already was recognized as an effective therapy against epilepsy; a finding that was also mentioned in Hippocrates Collection, Hippocrates' medical theories on the treatment of various illnesses (57). Since then, many different diets have passed the revue and in the recent years, KD has gained more popularity in both pediatric and adult patients. Both the classic and the modified KD are effective for the treatment of (Intractable) childhood epilepsy (58, 59). Furthermore, both carbohydrate restricted diets as well as calorie restriction reduce seizures (60) since the reduction of glycolysis is a key element to suppress seizures (61). In a randomized controlled trial (RCT) in children with refractory epilepsy, KD was compared to MAD and LGI (62). All 3 diets showed a similar reduction in seizures (66% for the KD, 45% for the MAD, and 54% for the LGI). While LGI showed relatively fewer adverse events, neither the MAD nor LGI were non-inferior to KD. Of notice is that seizure decline was rapid for KD and MAD, while more gradual for LGI.

An important antiepileptic effect of KD occurs through direct inhibition of receptors by FAs. One of the antiepileptic drugs that interacts with sodium channels, valproic acid, is actually a branched short-chain fatty acid made from valeric acid. Antiepileptic action of a number of PUFAs was also observed on isolated hippocampal neurons. Decanoic acid, an MCT, can directly and selectively inhibit AMPA receptors non-competitively in animal models (63). Decanoic acid is also a peroxisome proliferator activated receptor (PPAR) γ agonist, which elicits neuronal mitochondrial biogenesis (64–66).

Many hypotheses for indirect metabolic antiepileptic effect of KD in the brain have been postulated as well. Increasing ATP availability, reducing reactive oxygen species (ROS) generation by mitochondrial complex I, inhibiting the mitochondrial permeability transition pore, and stimulating mitochondrial biogenesis, all seem to stabilize synaptic functions. That is, βHB can be produced in astrocytes and is metabolized in the mitochondria of all brain cell types (67). KB serve as an efficient mitochondrial fuel, where it can alter the NAD+/NADH and Q/QH2 couples and reduce production of mitochondrial ROS. Evidence for mitochondrial dysfunction in acquired epilepsy comes from the observation that metabolic and bioenergetic changes occur following acute seizures and during different phases of chronic epilepsy (68). Stimulation of mitochondrial biogenesis is important to improve and stabilize synaptic transmission, for which about 30% of the energy in the human brain is spent (69). Ketogenic diet causes a reduction of glycolytically derived lactate in the brain due to a better tolerance to *in vivo* hypoxia (70). Other indirect ways to stimulate the antiepileptic effects of KD may lay in the alterations of the metabolism of neurotransmitters such as glutamate and gamma-amino butyric acid (GABA), and the activation of energy-sensing signaling pathways such as the PPAR, mammalian target of rapamycin (mTOR), and adenosine monophosphate-(AMP)-activated kinase (AMPK) pathways (71, 72). Indeed, KB are able to change GABA and glutamate aminoacid metabolism thereby inhibiting glutamatergic excitatory transmission and mitigate neuronal hyperexcitability (73). These effects were also noted after brain injury in rats with an age-dependent decreased cortical contusion volume with ketogenic neuroprotection (74).

Another system in which KD has influence on is the gut-microbiome (75). Mice treated with antibiotics or reared germ free are resistant to KD-mediated seizure protection, whereas enrichment of *Akkermansia muciniphila* and *Parabacteroides* populations restores anti-epileptic effect of KD (76). Efficacy of KD in seizure reduction in children with refractory epilepsy was associated with changes in intestinal microbiota and specific microbiomes may serve as an efficacy biomarker (77). Addition of probiotics can reduce fat accumulation in the liver caused by ketogenic diet in rats (78).

Intestinal dysbiosis might also be a possible etiopathogenic factor in drug-resistant epilepsy (79). Gut bacteria can release neuropeptides and neurotransmitters, such as serotonin, GABA

and glutamate, or their precursors (tryptophan and its metabolites) (80). They are also important for the biosynthesis of short-chain fatty acids. An additional mechanistical connection between the gut and brain is the stimulation of afferent neurons of the enteric nervous system (ENS) by bacteria. Enteric afferent neurons communicate intestinal conditions to the brain through the vagus nerve.

In drug-resistant epilepsy, KD has shown anti-epileptic effect and has a major benefit compared with standard epilepsy treatment with AEDs in children and adolescents due to less long-term adverse effects (81). A meta-analysis showed that treatment with KD in refractory epilepsy in children gives a 5.6 times more likely chance than the control group to have a 50% reduction of seizures after three months of the diet or earlier (82).

Even in infants < 2 years of age, KD can be considered as a non-pharmacological treatment and is currently used in infants with refractory epilepsy syndromes (83). In super-refractory status epilepticus (status epilepticus that persists or recurs 24 h after anesthetic therapy onset or after its withdrawal) KD is recommended as an alternative therapeutic strategy (84). Evidence for KD in refractory epilepsy or status epilepticus in adults is limited, with only one RCT. In comparison with a control group, cotreatment with MAD in adults with refractory epilepsy decreased seizure frequency 2.19 times (85, 86). Effect of KD in epilepsy is thus well-established and has been used for centuries. However, the quality of evidence is low due to the limited number of randomized controlled studies, small sample sizes and the limited studies in adults (87). Large-scale RCTs, refinement of more palatable diets and other nutritional ketogenic strategies are needed to optimize nutritional ketosis in clinical practice as a non-pharmacological treatment strategy in epilepsy.

Ketogenic diet in cardiology

Energy source of heart tissue is 60–90% oxidation of fatty acids, 10–30% of glucose, and 5% of KB of total ATP under normal conditions, respectively. Per unit mass, the myocardium consumes most of the KB (88, 89). Under hemodynamic stress the ventricles of the heart secrete B natriuretic peptide (BNP) to stimulate the release of free fatty acids from adipose tissue and ketogenesis in the liver to provide heart muscle tissue an additional energy source (90). Atrial natriuretic peptide (ANP) exert potent lipolytic effects by activating hormone-sensitive lipase in adipocytes, however the role of ANP in ketogenesis remains unknown (91).

In the hypertrophied and failing heart, decreased myocardial capacity for fatty acid oxidation occurs, leading to enhanced cardiac glucose utilization through anaerobic glycolysis, pyruvate accumulation and lactate production. This remodeling of mitochondrial energy metabolism is suggested to be a fetal shift resulting in an increase in cardiac efficiency (92). Intramyocardial lipid accumulation and myocardial insulin resistance occur. KBs are able to provide an additional energy source and may therefore improve energetics in the failing heart.

In a mouse model were pressure overload-induced heart failure was established by transverse aortic constriction, continuous KD showed no significant effects on cardiac systolic function and fibrosis but aggravated cardiac diastolic function (93). In the same study, an alternate-day KD, however, exerted potent cardioprotective effects against heart failure after 8 weeks of diet. Continuous KD for 8 weeks also transiently increased endothelial cell proliferation in the heart and prevents capillary rarefication in these mice (94).

A new class of treatments in chronic heart failure are sodium-glucose cotransporter-2 (SGLT2) inhibitors. SGLT2 inhibitors were initially designed as antidiabetic drugs by blocking glucose reabsorption in the proximal renal tubules. As a result, glucose renal excretion will be increased, thereby lowering serum glucose levels and stimulating ketogenesis. These KB improve cardiac energy supply and reduce pathological ventricular remodeling, and inflammation in patients with heart failure (95). Animal studies further support the improvement of heart function in ketogenic therapy. In type 2 diabetic mice, KD reduces mitochondrial fission and hence improves mitochondrial function in the heart (96). Rats fed for 5 days with a ketone ester supplementation diet improved running performance on a motorized treadmill (97). The isolated hearts from these rats, treated with KB supplementation for 66 days, had greater free energy available from ATP hydrolysis during increased work. In another study on rat hearts, addition of both insulin and KB to isolated perfused working rat hearts led to a 35% increased efficiency of cardiac hydraulic work (98).

Besides KB, the KD can also provide a significant source of polyunsaturated fatty acids (PUFAs). These PUFAs can inhibit voltage-regulated sodium and calcium currents, thereby exerting an antiarrhythmic action on the heart (99, 100). In addition, KD lowers heart rate and increases heart rate variability due to reduced sympathetic activity (101).

However, long-term use of KB might promote side effects in the heart. When treated for 16 weeks, KD-fed rats showed increased heart rates and impaired cardiac function. Additionally, increased cardiac fibrosis was found, potentially mediated by βHB-induced effects on the Sirtuin 7 promotor (102).

In summary, nutritional ketosis may serve as a cardioprotective alternative energy supply in the failing heart and reduce pathological ventricular remodeling and could serve as a supportive, non-pharmacologic treatment in people with heart failure along current treatments, such as SGLT2 inhibitors (103, 104). Long-term use of ketosis should however be tested for safety.

Ketogenic diet in mitochondrial diseases

Mitochondrial diseases are systemic and heterogenic diseases caused by underlying pathogenic variants in nuclear or mitochondrial DNA (mtDNA). Oxidative phosphorylation and ATP production are impaired and energy demanding organs most often show symptoms. Reason for initiation of KD in patients with a mitochondrial disease is intractable epilepsy and similar positive effects on epilepsy compared to patients without a primary mitochondrial disease (105). Few patients have been treated with KD for other reasons (muscle involvement, movement disorder, and intellectual disability) with some reports on improvement of clinical symptoms.

In mitochondrial dysfunction, increased anaerobic glycolysis causes excess amounts of pyruvate that accumulate and is converted into lactate. To prevent acidification of the cell, pyruvate is also transaminated into alanine and lactate is transported out of the cell thereby acidifying the extracellular environment. Lactate and alanine are transported to the liver to be converted again into glucose (gluconeogenesis), *via* the Cori cycle and the glucose-alaninecycle, respectively (see **Figure 1**). When pyruvate is converted into acetyl coenzyme A, this step is irreversible and therefore carbohydrates can be converted into fats but not vice versa (106).

KD and KB are capable to increase mitochondrial respiration *via* an increase in ATP production and improve the efficiency of the mitochondrial respiratory chain complex with increased mitochondrial biogenesis (69). βHB also reduces the production of ROS thereby improving mitochondrial respiration and bypassing the complex I dysfunction (67, 107). After oxidative stress in neocortical neurons, KB are neuroprotective by inducing expression of mitochondrial uncoupling proteins (UCP) which reduce ROS production (69, 108). In acidic conditions and high levels of ATP and NADH, the mitochondrial membrane permeability transition is further reduced (109). In brown adipose tissue of mice, mitochondrial biogenesis and UCP1 expression was shown to increase with a ketone ester diet (110). Increase in antioxidant activity was accomplished during KD by an increase of glutathione and glutathione peroxidase activity (111, 112). In addition, rats given a diet including decanoic acid-containing triglycerides had increased brain mitochondrial function and ATP synthesis capacity (113). In MCT-fed aged rats mitochondrial density and function in cerebellar Purkinje cells were significantly increased and age-related mitochondrial dysfunction was recovered (114). Also, a mouse model for late-onset mitochondrial myopathy due to overexpression of mutated Twinkle protein, a nuclear-encoded replicative helicase of mtDNA, showed clear health improvements after 10 months of KD. That is, markers for disease progression improved in these mice (115). The amount of cytochrome c oxidase negative muscle fibers in treated mice decreased by 30%, the citrate synthase activity in muscle as a marker of mitochondrial biogenesis was doubled, liver lipid levels were restored and muscle mitochondrial ultrastructure was normalized (no ragged red fiber-like muscle fibers or mitochondria with distorted structure and cristae on electron microscopy).

Few trials have been carried out in patients with mitochondrial myopathy, or on patient-derived material. In five patients with mtDNA deletions with a progressive external ophtalmoplegia phenotype (PEO), the MAD induced progressive muscle pain and muscle fiber necrosis after 2 weeks of diet (116). Muscle biopsy showed lysis of muscle fibers with the most mitochondrial abnormalities (ragged-red fibers). Transcriptomic analysis showed increase in mitochondrial biogenesis, and oxidative phosphorylation. Follow-up analysis after 2, 5 years suggested that muscle regeneration and mild improvement in muscle strength occurred. Lysis of ragged-red fibers might actually be beneficial by inducing satellite cell fusion. In another study, cell cultures of patients with PEO/Kearns-Sayre syndrome grown in a medium containing KB, hence replacing glucose as the carbon source, showed reduced heteroplasmy mtDNA mutation levels (117).

In summary, the above experiments suggest that nutritional ketosis can positively impact on mitochondrial bioenergetics, mitochondrial ROS/redox metabolism and mitochondrial dynamics (118). In human trials, however, the risk for muscle fiber necrosis should be monitored carefully and explored further.

Ketogenic diet in neurodegenerative diseases

Central features of neurodegenerative diseases are neuronal oxidative stress and mitochondrial dysfunction. The brain hypometabolism in neurodegenerative diseases is associated with reduced glucose utilization as detected by fluorodeoxyglucose positron emission tomography (FDG-PET). The brain preferentially uses KB over glucose (119, 120). A ketogenic diet is able to increase KB substrate and thereby increase brain phosphocreatine (PCr) levels (121). In addition, the KD is able to abate apoptosis of neurons (122).

It is unclear whether neuroprotective properties arise from the combination of elevated concentration of KB with low availability of carbohydrates, or solely from elevated concentration of KB. Hence, it is plausible that caloric restriction is already sufficient for neuroprotection (123, 124). KD and caloric restriction both result in reduction of blood glucose and a reduced glycolytic flux. The restriction of calories will improve mitochondrial functions, leading to reduced ROS production and increased energy output. Both caloric restriction and KD decrease inflammatory and pro-apoptotic activities through activation of PPAR (125, 126). It has also been shown in

numerous species, including primates, that caloric restriction prolongs the lifespan (127, 128).

From a brain-function point-of-view, ingesting a single MCT meal improves task performance in healthy elderly. This was accompanied by decreased regional blood flow in the dorsolateral prefrontal cortex, the brain region responsible for executive functions, indicative of extra energy source availability (129). Decanoic acid is able to modulate astrocyte metabolism directly, leading to activation of the astrocyte-neuron lactate shuttle and providing fuel to neighboring neurons in the form of lactate (130, 131). Healthy participants between 55 and 80 years had no effects on cognitive function with supplementation of 30 g MCT for a period of 2 weeks (132).

Most trials in neurodegenerative diseases on KB and KD have been investigated in Alzheimer's (AD) and Parkinson's disease (PD).

Alzheimer's disease

There is growing evidence that the KD may be an effective treatment for AD largely through enhanced mitochondrial functioning, however, clinical studies to date have been equivocal (133–135). Consumption of monounsaturated, polyunsaturated and omega 3 fatty acids is related to a decreased risk for AD (136). A direct effect of PUFAs is blocking voltage-gated sodium and calcium channels (137). On the contrary, intake of high saturated fat may possibly be associated with an increased risk in AD (138). Also, high glycemic diet can deteriorate the brain, as 1 year of high glycemic dieting was related to precuneal amyloid accumulation in the lateral temporal lobe and posterior cingulate gyrus (139).

In a mouse model of AD, long-term administration of ketone esters lessened amyloid β-peptide and hyperphosphorylated tau deposition, which decreased levels of anxiety and improved cognition (140). The KD was also found to reduce the volumes of soluble amyloid-beta in homogenates of murine brains (141). Four months of KD improved spatial learning, spatial memory and working memory in a mouse model of AD (the 5XFAD mic) (142). At a histopathological level, reduced amyloid plaque deposition and microglial activation was seen, resulting in reduced neuroinflammation. Initiation at a late stage showed no effect on cognitive improvement.

In patients with mild cognitive impairment, 6 weeks of very low carbohydrate diet improved verbal memory performance (143). Urinary ketone levels were positively correlated with memory performance. Consumption of 56 g/day of MCT supplementation for 24 weeks also increased serum KB concentrations and improved memory in subjects with mild cognitive impairment (144). A single oral dosage of MCT led to the elevation of plasma KB levels in AD patients, and to increased cognitive performance for Apolipoprotein E (APOE) ε4 negative, but not for APOE ε4 positive subjects (120). Apolipoproteins are involved in the metabolism of fats, and the epsilon subunit is associated with risk for Alzheimer's disease. APOE-ε4 status may possibly influence βHB consumption efficacy and may therefore be efficient in APOE ε4 negative patients. A randomized placebo controlled trial in which MCT was given to subjects with mild and moderate AD for 90 days increased serum concentrations of KB and improved cognitive functioning (145). Effects were again most notable in APOE ε4 negative patients who were dosage compliant. In a study in Japan MCT was administered to 20 Japanese patients with mild-to-moderate AD (146). At 12 weeks they showed significant improvement in the digit-symbol coding test and immediate logical memory tests compared to the baseline.

In the Ketogenic Diet Retention and Feasibility Trial, 15 patients with AD maintained an MCT-supplemented KD for 3 months (147). They observed that upon achieving complete ketosis, the mean of the Alzheimer's Disease Assessment Scale cognitive subscale score was significantly improved, but this reverted back to baseline after the washout. After a 12-week trial with modified KD in 21 AD patients, daily function and quality of life improved (148). Changes in cardiovascular risk factors were mostly favorable, and adverse effects were mild in this study.

Therapeutic hyperketonemia was achieved in a patient with Alzheimer's disease dementia during oral administration of 28.7 grams βHB thrice daily. After 20 months of treatment, improvements in behavior and cognitive and daily activity performance were observed (149).

Parkinson's disease

Nutrition plays an important role in risk for developing PD and as modifier during the disease progression (150). High prevalence of insulin resistance is suggested in patients with PD ranging from 50 to 80% and type 2 diabetes is associated with an increased risk of PD (151). Insulin receptors are abundant in the brain, however, concentrated in some areas such as the substantia nigra and basal ganglia. Reduced insulin-mediated glucose uptake was found in newly diagnosed untreated adults with PD (152). Yet, dietary glycemic index is inversely associated with the risk of Parkinson's disease (153). So, high glycemic index carbohydrates might decrease the risk of Parkinson's disease (PD) by an insulin-induced increase in brain dopamine. However, epidemiological studies about carbohydrate consumption and PD remain inconclusive.

Mitochondrial dysfunction is a fundamental and complex hallmark in many neurodegenerative disorders, including PD. KB can enhance mitochondrial oxidative phosphorylation and mitochondrial biogenesis in the substantia nigra and bypass complex I deficiency (154, 155). Nutritional supplements coenzyme Q10 and fish oil have also been associated with reduced PD progression (156).

Animal and *in vitro* studies have demonstrated beneficial effects of KB on the course of PD. It was shown that βHB acts *in vitro* as a neuroprotective agent against the toxicity of MPTP, which induces a defect in the mitochondrial complex I of dopaminergic neurons (157). Infusion of βHB in mice treated with the MPTP neurotoxin confers partial protection against dopaminergic neurodegeneration possibly mediated by βHB effects on complex II and by improvement of cellular respiration and ATP production (107). KB also show prevention of synaptic dysfunction induced by mitochondrial respiratory complex inhibitors (Rotenone and 3-nitropropionic acid) in rat brain slides. The protective effects of KB could result from possible antioxidative activity, improved ATP synthesis, and from the effect on the ATP-sensitive potassium channel (KATP) (158, 159).

Human trials conducted being limited, heterogeneous and lacking PD-specific outcomes (160). In a first clinical pilot in 5 patients with PD, improvements in motor score was noticed after 4 weeks of KD (161). A possible (additional) reason for improvement could be the low intake of proteins (8% in this study) which increases the bioavailability of levodopa, a drug used to treat motor symptoms. In a 8-week, randomized, controlled trial to assess the effect of a low-fat vs. ketogenic diet in 38 PD patients, an improvement in motor and non- motor symptoms was found in both groups (162). The ketogenic group however, showed greater improvements in non-motor symptoms. This is an important finding, since treatment with levodopa mainly addresses motor symptoms and does not adequately control many non-motor symptoms. Protein intake was kept at approximately 1 g per kg of body weight per day within each diet group to prevent increased bioavailability of levodopa. In some patients, KD exacerbated the PD tremor and/or rigidity. Perhaps the abrupt increase in fat intake augmented dopamine depletion and/or oxidative stress in the substantia nigra (163). Whether cognitive gains would be maintained upon discontinuation of the KD (or KB supplementation) remains unknown so far.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder in which spinal and cortical motor neurons progressively degenerate. Mutations in the gene encoding Cu/Zn superoxide dismutase 1 (*SOD1*) is found in a small percentage of familial ALS. Mutant *SOD1* has been localized in the mitochondria. KD fed *SOD1-G93A* transgenic ALS mice lost 50% of baseline motor performance 25 days later than the disease controls through the gain in mitochondrial energy production (164). In line, administration of caprylic triglyceride, a medium-chain triglyceride, to mutant *SOD1* mice resulted in delayed motor function and better preserved spinal cord motor neuron counts (165).

Within the PatientsLikeMe Community, seven ALS patients reported using ketogenic diets of whom two reported "moderate" effectiveness (166). A small randomized, double-blind trial that compared a high-fat with a high-carbohydrate hypercaloric diet and an isocaloric control diet reported more adverse events during the 6-months high-fat hypercaloric diet, including weight loss (167). Adequate caloric intake is essential in amyotrophic lateral sclerosis and future studies should monitor malnutrition closely (168).

Multiple sclerosis

Nutritional ketosis has anti-inflammatory properties that can be beneficial to patients with Multiple Sclerosis (MS) (169). In mice experimental autoimmune encephalitis, a mouse model for MS, calorie restriction 3 days a week for 5 weeks was effective in ameliorating symptoms and completely reversed disease progression in a portion of animals (170). KD had more modest effects and did not reverse EAE progression in mice.

In a RCT with 60 relapsing-remitting MS patients, a diet low on carbohydrates (<50 g carbohydrates) for 6 months or a 7 days low caloric intake (10–18% of normal) followed by a Mediterranean diet for 6 months showed improved quality of life compared to regular diet with a mild reduction in expanded disability status scale (EDSS) scores (170). After 6 months on the low carbohydrates diet, serum levels of neurofilament light chain, a biomarker of neuroaxonal damage, were significantly reduced compared with regular diet in patients with relapsing-remitting MS (171). Also the expression of enzymes involved in the biosynthesis of pro-inflammatory eicosanoids was reduced (172). A comparison of 15 patients on a MCT diet, a modified paleolithic diet or usual diet showed only a significant reduction on fatigue scores and maintained cognitive function scores for the modified paleolithic diet compared to the control group (173). A pilot study with twenty subjects and a phase II trial with 65 patients with relapsing MS were enrolled into a 6-month prospective MAD intervention. In these MS patients, KDs were reported to be safe and tolerable. Improvements were seen in body composition, fatigue, depression, quality of life, and neurologic disability. Reduced pro-inflammatory adipokines and elevated anti-inflammatory adipokines were found in the serum (174, 175).

Studies of a KD in neurometabolic degenerative disorders, and more specifically AD and PD consistently demonstrated improved learning and memory. Especially in the prodromal stage of the diseases, nutritional ketosis might delay start of symptoms. Its role in disease progression needs further evaluation. For other neurodegenerative diseases like ALS and MS, the evidence for the therapeutic value of KD or KS are limited, yet promising. In ALS mouse models delayed motor function is observed and in MS anti-inflammatory properties

of nutritional ketosis can result in improvements in fatigue, depression, quality of life, and neurologic disability (176).

Conclusion/future directions

Nutritional ketosis is a well-established strategy for treating epilepsy and has plausible mechanisms for treating neurodegenerative and heart diseases as well. As a performance enhancing strategy, the role of KB supplementation is equivocal, and it might be of added value especially in long duration, low-intensity (ultra) endurance exercise. Both KD and orally administered exogenous KB supplementation elicit widespread physiological changes at both a systemic and cellular level. Unraveling the exact mechanisms for the different nutritional strategies will lead to precision nutrition. International recommendations for the management of children (21) and adults (177) treated with KD have been composed.

Author contributions

CS and ST wrote and revised the manuscript. Both authors contributed to the article and approved the submitted version.

Funding

CS was member of the Radboudumc Center of Expertise for Neuromuscular Disorders (Radboud-NMD), the Netherlands Neuromuscular Center (NL-NMD), and the European Reference Network for rare Neuromuscular Diseases (EURO-NMD).

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

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frontiers | Frontiers in Nutrition

TYPE Clinical Trial
PUBLISHED 17 November 2022
DOI 10.3389/fnut.2022.951000

OPEN ACCESS

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

RECEIVED 23 May 2022
ACCEPTED 31 October 2022
PUBLISHED 17 November 2022

CITATION
Grundler F, Viallon M, Mesnage R, Ruscica M, von Schacky C, Madeo F, Hofer SJ, Mitchell SJ, Croisille P and Wilhelmi de Toledo F (2022) Long-term fasting: Multi-system adaptations in humans (GENESIS) study–A single-arm interventional trial.
Front. Nutr. 9:951000.
doi: 10.3389/fnut.2022.951000

Long-term fasting: Multi-system adaptations in humans (GENESIS) study–A single-arm interventional trial

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Fasting provokes fundamental changes in the activation of metabolic and signaling pathways leading to longer and healthier lifespans in animal models. Although the involvement of different metabolites in fueling human fasting metabolism is well known, the contribution of tissues and organs to their supply remains partly unclear. Also, changes in organ volume and composition remain relatively unexplored. Thus, processes involved in remodeling tissues during fasting and food reintroduction need to be better understood. Therefore, this study will apply state-of-the-art techniques to investigate the effects of long-term fasting (LF) and food reintroduction in humans by a multi-systemic approach focusing on changes in body composition, organ and tissue volume, lipid transport and storage, sources of protein utilization, blood metabolites, and gut microbiome profiles in a single cohort. This is a prospective, single-arm, monocentric trial. One hundred subjects will be recruited and undergo 9 ± 3 day-long fasting periods (250 kcal/day). We will assess changes in the composition of organs, bones and blood lipid profiles before and after fasting, as well as high-density lipoprotein (HDL) transport and storage, untargeted metabolomics of peripheral blood mononuclear cells (PBMCs), protein persulfidation and shotgun metagenomics of the gut microbiome. The first 32 subjects, fasting for 12 days, will be examined in more detail by magnetic resonance imaging (MRI) and spectroscopy to provide quantitative information on changes in organ volume and function, followed by an additional follow-up examination after 1 and 4 months. The study protocol was approved by the ethics board of the

State Medical Chamber of Baden-Württemberg on 26.07.2021 and registered at ClinicalTrials.gov (NCT05031598). The results will be disseminated through peer-reviewed publications, international conferences and social media.

Clinical trial registration: [ClinicalTrials.gov], identifier [NCT05031598].

KEYWORDS

organ size, lipoprotein metabolism, metabolomics, microbiome, long-term fasting, magnetic resonance imaging (MRI), protein utilisation

Introduction

The health-promoting effects of long-term fasting (LF), lasting more than 4 days and up to several weeks, are increasingly documented (1–3). Our group has shown that LF improves cardiovascular (CV) risk factors such as hypertension even in medicated subjects, lipoprotein distribution, non-alcoholic fatty liver symptoms, inflammatory parameters, oxidative stress, and gut microbiota profiles (4–9). A large observational study of 1,422 subjects underlined the safety, tolerability, and therapeutic efficacy of LF from 4 to 21 days (10). However, the precise physiological consequences of acute LF periods and subsequent food reintroduction phases are still not comprehensively understood.

A key molecular mechanism of fasting-mediated health benefits is the metabolic switch from glucose, as the main energy source, to the utilization of fat-derived lipids and ketones (11). During the first 48 fasting hours, the initial glycogen depletion is accompanied by fat and–to a lesser extent–protein utilization. The proportion of protein usage then decreases after the activation of protein-sparing mechanisms (12). In the 1980s, concerns about protein loss–and subsequent muscle decline–were raised for zero-calorie diets lasting more than 100 days, based on nitrogen balance measurements (13, 14), while the origin of protein breakdown was not determined. Conversely, in a recent study investigating 10 days of fasting, including daily moderate physical activity in 16 healthy men, muscle strength (grip and leg strength) was observed to be maintained and even increased (15).

Due to substrate mobilization in several metabolically active tissues, the size and weights of organs changes. Liver, spleen, kidneys and skeletal muscle mass decrease in rats after long periods of severe 50% calorie restriction (CR), while the brain and testes seem unaffected (16). Multiple cycles of fasting-mimicking-diet (FMD; 10% of normal daily calorie intake) in mice restored insulin-generating β-cells, promoted stress resistance, self-renewal division of stem cells, and hematopoietic lineage-balanced regeneration (17–19). Few clinical studies of long-term CR are available in humans. A 12-week semi-starvation intervention in 32 men reduced the heart's overall size proportionally to the body weight and showed a trend of recovery after food reintroduction (20). Fat loss plays a role in the shrinkage of organ volume. After a 10-day fast in men, enhanced lipid oxidation was shown in muscles (15).

Furthermore, fasting-induced autophagy and apoptosis are also thought to contribute to organ shrinkage (21). Decreased protein content during fasting is restored upon food reintroduction, as documented by the switch to a positive nitrogen balance (22, 23). Consequently, organ size can be rebuilt due to *de novo* synthesis and stem cell activation, possibly conferring beneficial effects on tissue functionality. To the best of our knowledge, no extensive human studies exist that systematically analyzed the body composition after a LF period using state-of-the-art magnetic resonance imaging/spectroscopy (MRI/MRS) approaches. One case report outlined the effects of a 14-day fasting period in a healthy man by MRI/MRS, showing changes in adipose tissue distribution and fatty acid composition in multiple organs (24).

Thus, in addition to body and organ composition, we will investigate lipid metabolism during LF. Serum triglycerides, low-density-lipoprotein (LDL) and atherogenic subfractions (e.g., small dense LDL) significantly decreased after 2 weeks of fasting, pointing to a reduced lipid-associated atherogenic risk (9). Of note, high-density lipoprotein cholesterol (HDL-C) is inversely associated with both CV disease and mortality (25) and pharmacological interventions aiming at raising HDL-C levels were not successful thus far (26, 27). Hence, there is an increasing interest in measuring high-density lipoprotein (HDL) function, as reflected by cholesterol efflux capacity (CEC) and the serum cholesterol loading capacity (CLC), which are both indices of CV risk (28, 29). This study will investigate whether LF influences the reverse transport of cholesterol from the periphery to the liver. In addition, erythrocyte membrane fatty acid composition reliably predicts total mortality and clinical events (30–32). Therefore, we will determine the fatty acid composition of erythrocytes and the omega-3/omega-6 ratio during LF.

The exact cascade of molecular mechanisms leading to therapeutic benefits of different fasting regimes remains elusive, at least in humans (33). Interestingly, organisms maintain cellular and organismal homeostasis during fasting, *inter alia*, by well-orchestrated biochemical and (epi) genetic mechanisms

(11, 34). The progress of precision metabolomics technologies recently opened new avenues to study the changing metabolic landscape during the fasting state in a spatiotemporal manner (35). Thus, this study will shed light on the intracellular metabolic consequences of LF in peripheral blood mononuclear cells (PBMCs), focusing on lipids and polyamines, which are essential for diverse cellular functions (36). Another aspect of LF-induced metabolic effects is increased endogenous hydrogen sulfide (H2S) production, a physiological gasotransmitter (37, 38) *via* sulfhydration/persulfidation (39) with health-promoting properties (REF). In mice, 40% dietary restriction increased H2S levels, which is required for the geroprotective effects of CR (37). Thus, we will investigate the impact of LF on H2S-mediated signaling in humans.

Finally, we will explore gut microbiota changes during LF. LF does not eliminate the gut microbiota, but elicits profound changes in its composition (6, 40). In our recent study, changes in bacteria profiles caused by fasting were associated with serum glucose and fecal branched-chain amino acids (6), suggesting that the gut microbiota can influence fasting-induced changes in energy metabolism. A more recent study even identified a bacteria which abundance correlates with serum concentrations of 3-hydroxybutyrate (41).

In light of these incognita, we designed a multi-stage single-arm interventional trial to apply state-of-the-art methods to elucidate the fasting-induced changes in metabolism, body composition, organ size and function, and the gut microbiome (**Figure 1**).

Methods and analysis

Aims

First, the GENESIS study aims to document changes in body composition and the contribution of the main metabolically active tissues (skeletal muscular tissues, adipose tissues, liver, heart, spleen, kidneys, and brain) to the metabolic switch during a 12-day fasting period. Changes in the size and mass of organs will be quantified. We hypothesize that a re-expansion and regeneration will follow a transient decrease of organ volume during fasting up to 4 months after food reintroduction. Protein resynthesis upon food reintroduction might correspond to an acceleration of the physiological protein turnover, which will be evaluated by measuring nitrogen excretion. Additionally, the organs' specific compositions, especially the fat components, and their function will be documented along the fasting process. We hypothesize that these multi-system changes are safe, and that they could also be reflected by an improved cardiac and skeletal muscle metabolism and mitochondrial oxidative capacity which will be investigated by spiroergometry.

Second, the study focuses on fat storage, function, and exchange in adipose tissue, liver, spleen, kidney, and splanchnic tissue. Among the lipids studied, we focus on cholesterol metabolism during fasting, particularly sub-types of HDL, but also CEC and fatty acid profiles in the erythrocyte membrane.

Third, we will measure PBMC metabolome profiles to reveal cellular metabolic changes in circulating, easily accessible immune cells.

Fourth, we will determine whether persulfidation, which contributes to the maintenance of cellular oxidative functions, changes during fasting.

Last, we will measure if various metabolic and physiological changes, as described above, correlate to individual gut microbiota profiles. The composition and function of the fecal microbiota will be evaluated during LF using shotgun metagenomics. This will help us understand how intestinal microorganisms are linked to human physiology during fasting.

Study design

The GENESIS study is a prospective, monocentric, single-arm interventional study using a two-stage design. A total of 100 subjects will be included (**Figure 2**). The first 32 participants will undergo an augmented examination plan, including MRI/MRS scans to evaluate body composition changes at four time points: prior to and at the end of 12 fasting days, 1 and 4 months post fasting. This sub-cohort is statistically powered for detecting changes in whole body composition. Sixty-eight additional subjects will then be included for gut microbiota and lipid profile analyses. The study follows the STROBE guidelines (42).

Recruitment

The study site is a specialized center for long-term therapeutic fasting under medical supervision. Detailed information about the GENESIS study will be provided orally and in a written manner to potential participants. For the recruitment of the first phase (32 participants), participation calls will be distributed in the study center, on social media channels of the Buchinger Wilhelmi clinic and in institutions working on fasting and nutrition. Informed consent will be collected prior to the start of the fasting period and any baseline measurements. Since participants of the first stage need to be physically present at four time points (prior and post fasting, follow-up visits after 1 and 4 months), they will stay at the Buchinger Wilhelmi clinic without charges. The remaining 68 participants will be recruited prospectively during 1 year from the regular pool of customers voluntarily undergoing fasting in the Buchinger Wilhelmi clinic in Überlingen. These participants will not receive any financial incentives. The recruitment started in September 2021. Thus, the last follow-up will be prospectively performed in January 2023. Study participants will be informed by email about their results and publications based on data collected in the GENESIS trial.

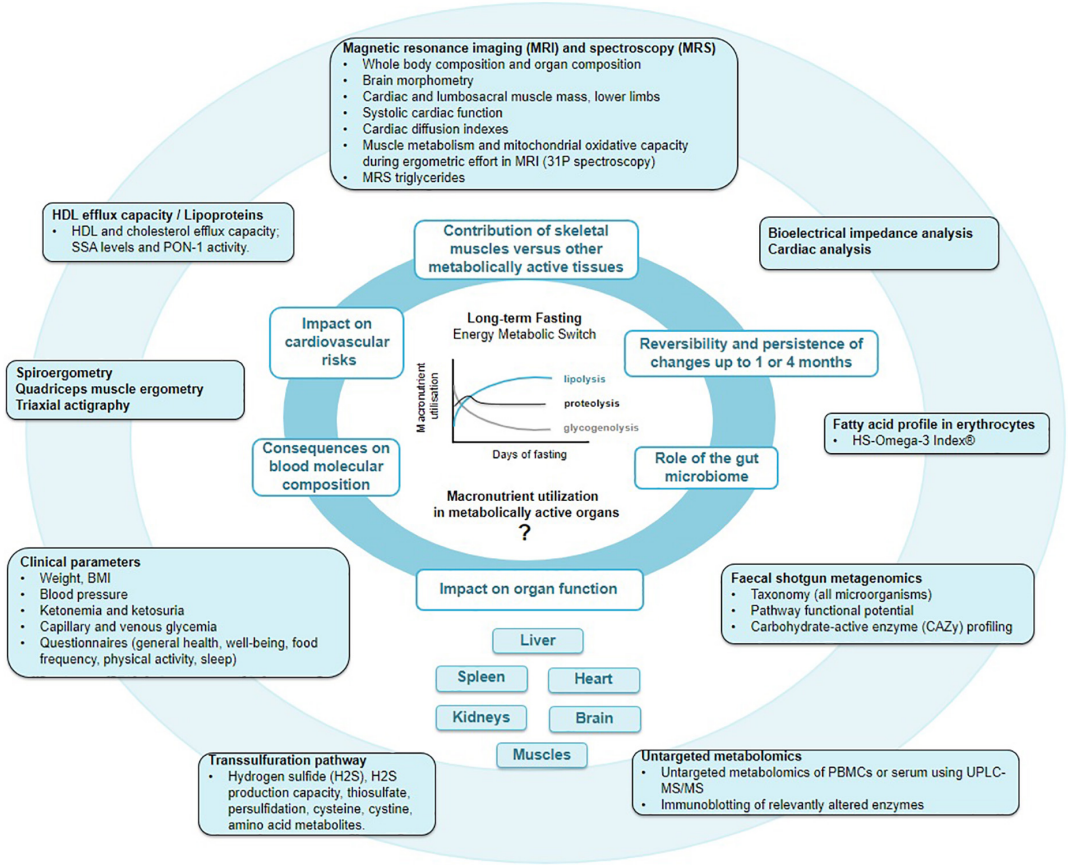

FIGURE 1
Main outcomes of the GENESIS study. By pooling knowledge acquired by macroscopic multi-organ fat distribution and composition provided by magnetic resonance imaging (MRI), with erythrocyte fatty acids, lipoprotein, peripheral blood mononuclear cell (PBMC) metabolome, protein persulfidation, and gut microbiome profiles, we aim to gain a deeper comprehension of molecular and physiological changes during fasting and food reintroduction.

Participants

Men and women aged 20–75 years with BMIs between 22 and 35 kg/m^2 are eligible to participate if they match further eligibility criteria, as listed in Table 1. Any contraindication to fasting, as defined in the guidelines of fasting therapy including kidney, liver or cerebrovascular insufficiency (43) automatically leads to exclusion. Fulfilment of additional criteria related to contraindications for the MRI/MRS scans is required for participants of the MRI subgroup (Table 1).

Fasting intervention

The subjects will receive a plant-based, organic calorie-restricted diet (600 kcal/day) 1 day before the study begins. The 12-day fasting period will be initiated by emptying the intestinal tract *via* the intake of a laxative [e.g., sodium sulfate (Glaubers' Salt) in the morning or sodium picosulfate (Laxoberal®, Sanofi-Aventis, Germany) the night before]. During the fasting period, the energy intake is limited to 200–250 kcal/day (10), which will be achieved with 0.25 L freshly squeezed, organic fruit juice at noon, 0.25 L vegetable soup in the evening and 20 g honey. Participants will be asked to drink a minimum of 2 L of water or non-caloric, caffeine-free herbal teas that are purchased by certificated, organic companies (SONNENTOR Kräuterhandelsgesellschaft mbH, Sprögnitz, Austria; Ulrich Walter GmbH, Diepholz, Germany). Every second fasting day, the colon will be emptied by an enema or a laxative. On the last fasting day, plant-based organic food will be progressively reintroduced over 3–4 consecutive days (800–1,600 kcal/day).

Medical doctors and nurses will supervise the fasting intervention. An accompanying program consisting of physical exercise, mindfulness, and meditation will be provided and group interaction will be favored. The training program includes daily outdoor walks of 1.5 h, moderate-intensity fitness exercises and free access to the gym and swimming pool.

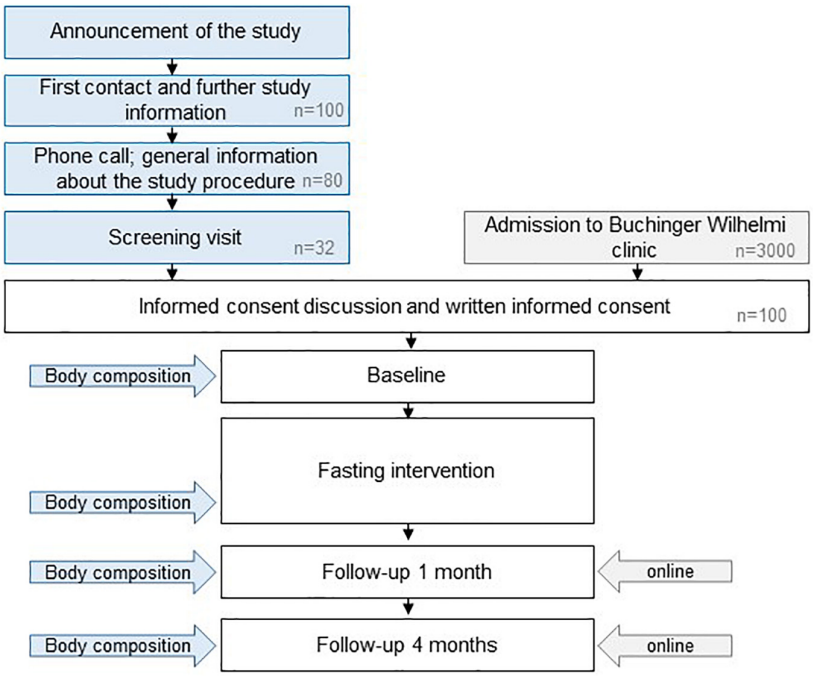

FIGURE 2
Study design. Specific sessions for the subjects enrolled in the first stage of the projects are indicated in blue. The estimated numbers of volunteers needed to recruit the sample size of 100 subjects are indicated in gray.

Outcome parameters

An overview of the main study outcomes is shown in **Figure 1**.

Body composition

The primary endpoints in this study are changes in whole body composition (fat mass, lean mass, and water) at the end of the fasting intervention as well as 1 and 4 months afterward compared to baseline measured by MRI (44–46) (**Figure 3**). Furthermore, changes in size and composition of heart (47–52), liver (53, 54), kidney, spleen, adipose tissue, lumbosacral muscle mass, and lower limbs (quadriceps, hamstrings, and calves) (55) over time as well as changes in brain morphometry (56, 57) will be assessed. Details of MRI measurements are provided as **Supplementary material**.

Changes in bio-electrical multifrequency impedance analysis will enrich the MRI/MRS scans (58–60) with information about global and segmental body composition (water, fat, and lean mass), liquid distribution (total, extracellular, and intracellular water), metabolic indexes [metabolic activity index (MAI)], and protein content (total and active cell mass fraction). Furthermore, the CV fitness will be assessed by measuring maximal oxygen consumption (VO2max) on cycloergometers (61). Triaxial actigraphy will allow a continuous recording of the physical activity and sleep quality during the whole study. Last, we will use standardized morning heart rate variability measurements (Polar H10 with Kubios HRV mobile app.) to assess the autonomous nervous system response along the protocol (62, 63).

TABLE 1 Eligibility criteria.

Inclusion criteria	Exclusion criteria
• Men and women • Age between 20–75 years • BMI between 22–35 kg/m^2 • Negative COVID-19 test • Available written declaration of consent	• Intake of medication (cardiovascular diseases, lipid, and glucose metabolism) • Chronic manifest psychical and psychiatric diseases • Participation in another study • Pregnancy or breastfeeding • Active uncontrolled gastrointestinal disorders including ulcerative colitis, Crohn's disease, indeterminate colitis, severe irritable bowel syndrome, persistent infectious gastroenteritis, persistent or chronic diarrhea of unknown etiology, and recurrent *Clostridium difficile* infection • Major surgery of the gastrointestinal tract, in the past 5 years. Any major bowel resection at any time • Intake of antibiotics in the last 2 months • In the MRI subgroup, any MRI contraindication (claustrophobia, pacemakers, MR-incompatible prosthetic valves, metallic implants, and foreign metallic body)

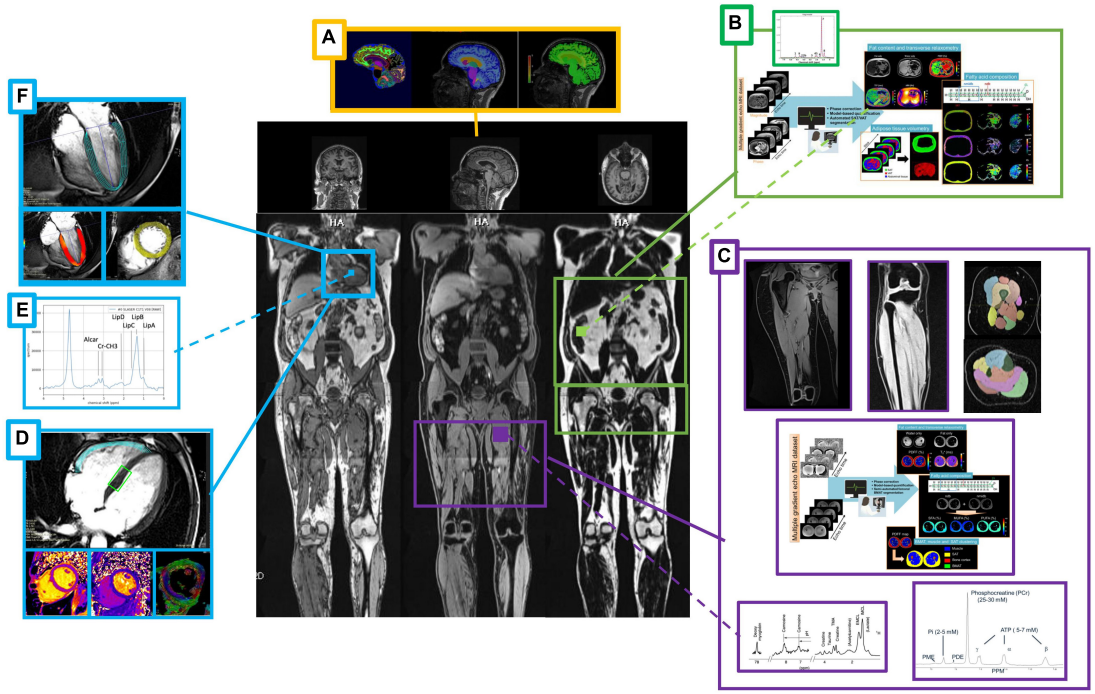

FIGURE 3
Summary of the magnetic resonance imaging/spectroscopy (MRI/MRS) measurements. Body composition after a 12 days fasting period by means of MRI/MRS scans with a focus on the brain **(A)**, liver **(B)**, skeletal muscle **(C)**, and myocardium **(D–F)**. Beyond brain morphometry **(A)**, myocardial mass, function and regional deformation **(F)**, and abdominal organs sizing **(B)** calculated from localized dedicated scans (side panels). Subcutaneous, visceral, extra-visceral and bone marrow fat quantification and total lean mass will be calculated from whole body acquisition (middle panel). MR spectroscopy of the liver **(B)**, leg muscle **(C)**, and heart **(E)** allows complementary fat decomposition, triglycerides and metabolites concentrations quantification. Advanced myocardium tissue characterization will include relaxometry and diffusion parameters mapping, as well as myocardial fiber orientation and local deformation **(D)**. Muscle legs volume and strength (maximum voluntary contraction) will be compared to MRS 31P data and extracted biomarkers of the oxidative metabolism **(C)**.

Lipid function

High-density lipoprotein, CEC, and serum CLC will be analyzed as previously described (64–66). Additional information will be gained through the analysis of chylomicrons (67), HDL and LDL subfractions, lipoprotein transfer enzymes (Cholesteryl Ester Transfer Protein activity) (68), proprotein convertase subtilisin/kexin type 9 (PCSK9) (69), paraoxonase (PON-1) activity (70), serum amyloid A levels, GlycA (71), apolipoproteins AI and B (71), lipoprotein(a) (72), and oxidized phospholipids (73). The fatty acid profile in erythrocyte membranes will provide additional insights at the molecular level (74).

Metabolic pathways

An analysis of the metabolome in PBMCs will be performed using combinations of untargeted and targeted LC/MS approaches (2).

Sulfur signaling

To address the transsulfuration pathway, sulfur compounds (amino acids, thiosulfate, and H_2S) will be analyzed in serum and urine at baseline, after three fasting days, at the end of fasting, as well as 1 month post food reintroduction (75).

DNA methylation profiling

Experimental surrogate indicators of biological age [so-called epigenetic aging clocks) will be analyzed using methylation arrays from blood DNA methylation (76)].

Gut microbiota composition

Stool samples will be collected for shotgun metagenomics to determine the fecal microbiota composition in all participants prior to fasting and from the first spontaneous stool after the fasting period as previously described (6). We will evaluate microbial composition by identifying species-specific marker genes and functional potential by profiling microbial metabolic pathways and other molecular functions as described in other published fecal microbiome studies (77). The study of carbohydrate metabolism will be complemented by a carbohydrate-active enzyme (CAZy) profiling method that is currently in development at european molecular biology laboratory (EMBL) Heidelberg. All raw microbial data will be made available on public repositories.

TABLE 2 Summary of measurements.

	Before	Transition	Fasting days													Food reintroduction	+1 month	+4 months
Measurements	**−5 to −2**	**−1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**			
Demographic data	x																	
Medical history	x																	
Anthropometric measurements	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Vital signs	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Blood sampling		x				x			x								x	
Capillary blood sampling	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
First morning urine sampling			x			x			x								x	
24 h urine sampling	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x		
MRI	x												x				x	x
MRS P31 and H1	x												x				x	x
Bioelectrical impedance analysis	x												x			x	x	x
Quadriceps muscle ergometry	x												x				x	x
Triaxial actigraphy	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x
Spiroergometry	x										x						x	
Stool sampling	x															x		
Visual scores: wellbeing, symptoms	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Questionnaires and life style		x											x				x	x
Adverse events	x	x	x	x	x	x	x	x	x	x	x	x	x			x	x	x

Measurements only conducted in the first stage of this study (n = 32) are highlighted in blue.

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Clinical data

Clinical data (e.g., body weight, body-mass-index, systolic and diastolic blood pressure, and heart frequency) will be documented at baseline, during fasting and food reintroduction as well as 1 and 4 months afterward. The abdominal circumference will be measured before and after fasting. Changes in ketonuria, capillary ketonemia, and capillary blood glucose levels will be measured during the fasting period and food reintroduction. Nitrogen balance, measured in 24-h urine samples (15), will be determined at baseline and during the fasting and food reintroduction period in the MRI subgroup only.

A clinical standard laboratory blood profile will be measured in each blood sample, during the transition day and after fasting (10). Additional blood samplings will be obtained from the first 32 subjects after three fasting days as well as 1 month afterward.

Questionnaires

Several validated questionnaires will be recorded at baseline, at the end of fasting as well as 1 and 4 months afterward to document mental wellbeing (Warwick-Edinburgh Mental WellBeing Scale) (78), global health (PROMIS Scale) (79), physical activity (Godin Leisure-Time Exercise Questionnaire) (80), sleep quality (Pittsburgh Sleep Quality Index) (81), and dietary behavior (short healthy eating index survey) (82). Lifestyle habits like smoking behavior, alcohol consumption, or physical activity in hours/week will be self-reported. Participants will document energy level, emotional and physical wellbeing, as well as symptoms including fatigue, muscle weakness, back pain, hunger, anxiety, headache, and sleep disturbances on visual scales (0–10) at baseline, daily during fasting and food reintroduction as well as 1 and 4 months afterward.

Adverse events will be documented continuously.

Data analysis plan

The data of all clinical endpoints will be collected before and after 9 ± 3 fasting days. Demographic data and each participant's medical history will be captured at baseline. The detailed timing of all measurements is shown in **Table 2**.

Further, the **Supplementary File** contains a detailed description of the anthropometric data, vital signs, blood, urine and stool samples, the MRI and MRS acquisition protocols, bioelectrical impedance analysis, quadriceps muscle ergometry, triaxial actigraphy, and spiroergometry.

Sample size calculation

The required sample sizes vary between the outcome parameters. Previously, the effect size in matched subjects was 0.81 for T2 relaxometry in muscles (55). Using these assumptions, the minimal sample size requirement is 14 to detect meaningful acute changes in muscle physiology (alpha = 0.05, power = 0.80). MRS studies monitoring triglyceride (TG) content during CR (83, 84) observed that effect sizes were 1.09 and 0.59 in liver and muscle, respectively. Using the small effect size of muscle changes (alpha = 0.05, power = 0.80), the required sample size to detect organ-specific differences in MRS measurements is 28. To account for a potential drop-out rate of 15%, the sample size was set to 32.

Clinical routine laboratory examinations and other blood sample analyses will be performed on 100 samples. This number of participants was chosen to reach sufficient power for detecting differences in gut metagenome analysis. Large inter-individual differences are usually observed in the composition of fecal microbiomes. Small differences in alpha diversity (effect size 0.55) between two groups of 50 individuals can be detected with an 80% statistical power (85). Missing values are commonly encountered in fecal microbiome evaluations (85). Accounting for missing data and sufficient numbers to quantify bacteria abundance at the species level for at least 50 individuals, we estimated that 100 subjects are needed for this part of the study (power = 0.80). This is based on a fecal metagenome analysis, which included missing values for more than 50% of 612 bacterial species out of 724 species detected (86).

Data collection and management

All research data will be documented on case report forms and the study diary. A pseudonymised identification number will be allocated to each participant. Study data will be transcribed from the source documents and stored electronically in a pseudonymised, password-secured database at Buchinger Wilhelmi Development und Holding GmbH. Documents allowing the identification of subjects (e.g., signed consent forms) will be stored securely and separately from the study data. Only representatives of the principal investigator, who are obliged to maintain confidentiality, have access to these data. The follow-up data will be collected using an online questionnaire that meets general data protection regulation standards. Data will always be treated confidentially and data protection regulations will be complied with. The source data will be kept for at least 10 years after the termination of the study.

Statistical analysis

The statistical analysis of the data will be performed using R software for statistical computing. Given the longitudinal design of our study with multiple repeated measurements, a mixed model for repeated measures will be used for quantitative variables. The *p*-values from linear-mixed models in the longitudinal data analysis will be adjusted with a *post-hoc* Tukey test. Missing data will be handled with complete case analyses for the dependent variables or imputed using the

median for auxiliary variables with less than 20% missing values. Subgroups will be generated using demographic data (e.g., sex) or unsupervised clustering methods. The statistical models will be adjusted for potential confounders such as age, gender, or baseline body weights. A parsimonious approach will be used. If a covariate does not influence the variance of the model, it will be excluded from the model. We will use multivariate statistics for metabolomics or metagenomics, including unsupervised (e.g., principal component analysis) and supervised statistical methods (orthogonal partial least squares discriminant analysis, sparse partial least square discriminant analysis). In the case of multivariate analyses (e.g., microbiome data), the false discovery rate corrected p-value (q-value of 5%).

Ethics and dissemination

The ethics commission of the federal state of Baden-Württemberg approved the study protocol on 26th July 2021. It was registered in an official clinical trial register on 2nd September 2021 (ClinicalTrials.gov Identifier: NCT05031598). The ethics commission will approve all protocol amendments and update the clinical trial register. The study will be conducted in accordance with the Declaration of Helsinki and the guidelines of the International Conference of Harmonization of Good Clinical Practice Guidelines and German law.

Microbiota data will be made public in a data depository. Results will be published in peer-reviewed journals and presented at international conferences and on social media.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Board of the State Medical Chamber of Baden-Württemberg. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FG: project administration, methodology, data collection, and writing—original draft preparation. MV and PC: conceptualization, funding, methodology, data collection, data analysis, and writing—review and editing. RM and SJH: methodology, writing—review and editing. MR, CvS, SJM, and FM: methodology. FWT: conceptualization, funding acquisition, supervision, and writing—review and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

Authors FG, RM, and FWT are employees of the Buchinger Wilhelmi Development and Holding GmbH, Überlingen. Author CvS was employed by Omegametrix.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.951000/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 24 November 2022
DOI 10.3389/fnut.2022.936220

OPEN ACCESS

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

RECEIVED 05 May 2022
ACCEPTED 31 October 2022
PUBLISHED 24 November 2022

CITATION
Wu Q, Li J, Zhu J, Sun X, He D, Li J, Cheng Z, Zhang X, Xu Y, Chen Q, Zhu Y and Lai M (2022) Gamma-glutamyl-leucine levels are causally associated with elevated cardio-metabolic risks. *Front. Nutr.* 9:936220. doi: 10.3389/fnut.2022.936220

Gamma-glutamyl-leucine levels are causally associated with elevated cardio-metabolic risks

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Objective: Gamma-glutamyl dipeptides are bioactive peptides involved in inflammation, oxidative stress, and glucose regulation. Gamma-glutamyl-leucine (Gamma-Glu-Leu) has been extensively reported to be associated with the risk of cardio-metabolic diseases, such as obesity, metabolic syndrome, and type 2 diabetes. However, the causality remains to be uncovered. The aim of this study was to explore the causal-effect relationships between Gamma-Glu-Leu and metabolic risk.

Materials and methods: In this study, 1,289 subjects were included from a cross-sectional survey on metabolic syndrome (MetS) in eastern China. Serum Gamma-Glu-Leu levels were measured by untargeted metabolomics. Using linear regressions, a two-stage genome-wide association study (GWAS) for Gamma-Glu-Leu was conducted to seek its instrumental single nucleotide polymorphisms (SNPs). One-sample Mendelian randomization (MR) analyses were performed to evaluate the causality between Gamma-Glu-Leu and the metabolic risk.

Results: Four SNPs are associated with serum Gamma-Glu-Leu levels, including rs12476238, rs56146133, rs2479714, and rs12229654. Out of them, rs12476238 exhibits the strongest association (Beta = −0.38, S.E. = 0.07 in discovery stage, Beta = −0.29, S.E. = 0.14 in validation stage, combined P-value = 1.04×10^{-8}). Each of the four SNPs has a nominal association with at least one metabolic risk factor. Both rs12229654 and rs56146133 are associated with body mass index, waist circumference (WC), the ratio of

WC to hip circumference, blood pressure, and triglyceride ($5 \times 10^{-5} < P < 0.05$). rs56146133 also has nominal associations with fasting insulin, glucose, and insulin resistance index ($5 \times 10^{-5} < P < 0.05$). Using the four SNPs serving as the instrumental SNPs of Gamma-Glu-Leu, the MR analyses revealed that higher Gamma-Glu-Leu levels are causally associated with elevated risks of multiple cardio-metabolic factors except for high-density lipoprotein cholesterol and low-density lipoprotein cholesterol ($P > 0.05$).

Conclusion: Four SNPs (rs12476238, rs56146133, rs2479714, and rs12229654) may regulate the levels of serum Gamma-Glu-Leu. Higher Gamma-Glu-Leu levels are causally linked to cardio-metabolic risks. Future prospective studies on Gamma-Glu-Leu are required to explain its role in metabolic disorders.

KEYWORDS

Gamma-glutamyl-leucine, metabolic risk factors, GWAS, metabolic syndrome, Mendelian randomization

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, responsible for 32% of global deaths (1). The increasing burden of cardio-metabolic risk is closely associated with CVD (2). Metabolic syndrome (MetS) is a composite of a series of cardio-metabolic risk factors, including central obesity, hypertension, dyslipidemia, and hyperglycemia (3). Exploring the pathophysiology of MetS and its related metabolic disorders could provide novel insights to prevent the progression of CVD.

Gamma-glutamyl dipeptides are a family of bioactive peptides containing gamma-glutamyl residues and amino acids that can be derived from dietary consumption (including cheese, soy sauce, edible beans, and so on) or produced from Gamma-glutamyl-cysteine synthetase (γ-GCS) and Gamma-glutamyl transferase (γ-GGT) metabolism in humans and microorganisms (4–6). Emerging evidence has demonstrated that Gamma-glutamyl dipeptides are involved in diverse bioactivities, including inflammatory activities, oxidative stress, and glucose metabolism *via* activating calcium-sensing receptors (CasR) in different organs (7–10). As a G-protein-coupled receptor, CaSR can modulate a large variety of cellular processes that are associated with cardiovascular health, such as modulation of insulin secretion, release of nitric oxide, upregulation of apoptosis and cell proliferation, and activation of the NLRP3 inflammasome (11). In epidemiological studies, disturbed Gamma-glutamyl dipeptide levels have also been implicated in multiple diseases, including obesity, MetS, type 2 diabetes, non-alcoholic fatty liver diseases, and CVDs (12–16). Our previous targeted metabolomics study also revealed that one of the Gamma-glutamyl dipeptides, Gamma-glutamyl-leucine (Gamma-Glu-Leu) levels, were significantly higher in patients with MetS (17). Inconsistently, Zheng et al. reported multiple Gamma-glutamyl dipeptides including Gamma-Glu-Leu to be associated with lower alcohol consumption (18). By using different Gamma-glutamyl dipeptides to establish a metabolite score, they found this score negatively associated with inflammation biomarkers and the incidence of CVDs (18). Given that most prior studies were based on cross-sectional designs, it still could not be decided whether the associations between Gamma-glutamyl dipeptides and the CVD-related metabolic factors were contributed by confounding factors, reverse causality, or real causal effectors. Much effort is required to determine the causal relationships between Gamma-glutamyl dipeptides and the metabolic risk.

Using genetic variants as instrumental variables (IVs), the Mendelian randomization (MR) approach has been widely applied to infer the causal-effect relationships (19, 20). Since genotype is assorted randomly at conception and the randomization process is emulated within observational studies, it is less likely to be affected by potential confounding factors or reverse causations (21, 22). As an intermediate phenotype between genetics and clinical disease endpoints, the metabolite phenotype has been found to have a high heritability (23). Genome-wide association studies for metabolites (mGWAS) have identified a hundred gene loci regulating metabolite levels (met-QTL) (24–26). On the one hand, these identified gene loci improved our understanding of the role of metabolites in disease etiologies; on the other hand, by using these identified genetic variants to proxy levels of metabolites, several previous studies

have found that plasma metabolite levels, such as branched-chain amino acids and 2-hydroxybutyric acid, were causally associated with obesity and type 2 diabetes (21, 27).

Therefore, we hypothesize that the levels of Gamma-Glu-Leu are associated with single-nucleotide polymorphisms (SNPs) across the whole genome and that genetically determined Gamma-Glu-Leu has a causal effect on the cardio-metabolic risks. In this study, we aimed to identify the genetic variants associated with circulating levels of Gamma-Glu-Leu and to determine the causal relationships between the genetically proxied levels of Gamma-Glu-Leu and the cardio-metabolic factors using a MR approach.

Materials and methods

Study population

Subjects were recruited from our previous cross-sectional survey on MetS in Hangzhou, Zhejiang of China, which was a questionnaire-based epidemiological investigation conducted in 2010 and consisted of 862 patients with MetS and 880 healthy controls. The detailed information for the study population has been previously described (28, 29). Fasting blood samples for each subject were collected and then frozen at −80°C immediately. All subjects undertook a whole-genome SNP genotyping and were further divided into discovery (n = 1,157) and validation (n = 240) subsets to undertake an untargeted metabolomics analysis. After quality control of genotyping and metabolomics data, 1,062 subjects and 227 subjects with good-quality data on Gamma-Glu-Leu measurement and SNP genotyping were included as the discovery and replication samples, respectively. The study overview is shown in **Figure 1A**.

Epidemiological investigation and clinical measurements

Using a standardized protocol, a questionnaire-based interview was conducted for each subject by trained investigators. Covariates of demographic characteristics, including age, sex, history of CVDs, type 2 diabetes, hypertension, cancer, liver diseases, kidney diseases, and drug use, were investigated.

Values of body weight, height, waist circumference (WC), hip circumference (HC), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were measured by well-trained assistants using a standardized protocol. Body mass index (BMI) was calculated as the body weight in kilograms divided by the square of the height in meters. WC was determined at the midpoint between the lowest coastal ridge and the upper border of the iliac crest. Blood pressure was measured in a sitting position using a mercury sphygmomanometer. The values of SBP and DBP were reported as the means of three repeat measurements at 30 s intervals.

Serum uric acid (UA), total triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting insulin were measured using biochemical auto-analyzers. Fasting plasma glucose (FPG) was analyzed by the glucose oxidase method with a Beckman glucose analyzer. The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as fasting insulin (μU/L) × fasting glucose (nmol/L)/22.5.

Gamma-glutamyl-leucine measurement

Serum Gamma-Glu-Leu measurement data was retrieved from our previous untargeted metabolomics study, which was conducted using the Agilent 1290 Infinity coupled with 6545 Q/TOF-MS system (Agilent Technologies, Santa Clara, CA, USA) under the positive and negative ion modes. The chromatographic separation was performed with the Waters BEH C8 analytical column (100 mm × 2.1 mm, 1.7 μm). The mobile phase consisted of acetylene solution (B) and water (A) (both contain 0.1% fore lime acid) in the positive ion mode or methanol solution (B) and water (A) (both contain 10 mmol/L ammonium acetate) in the negative ion mode. Detailed experimental conditions, data acquirement, data preprocessing, and data quality control have been previously described (17, 20). In total, 1,793 ion features were detected in the 240 subjects and 2,238 ion features were detected in the 1,157 subjects. Metabolites were identified according to the public metabolomics databases (The Human Metabolome Database and METLIN) and confirmed using available in-house reference compounds (17).

Genotyping, imputation, and quality control

Single nucleotide polymorphism genotyping was performed using Illumina Human-OmniExpress 760 k chips (Illumina, San Diego, CA, USA) (28). The staff who performed the DNA analysis were not aware of the clinical status of the subjects. The initial genetic quality control was conducted by removing sex discrepancies, ethnic outliers, probable relatives, and those with a call rate < 0.95 and excessive genome-wide heterozygosity. SNPs with minor allele frequency < 0.05, Hardy-Weinberg equilibrium $P < 1 \times 10^{-4}$, call rate < 0.95, and not in autosomal chromosomes were excluded. The University of Michigan imputation server was used to complete the imputation (30). Before imputation, all alleles were aligned to the forward strand

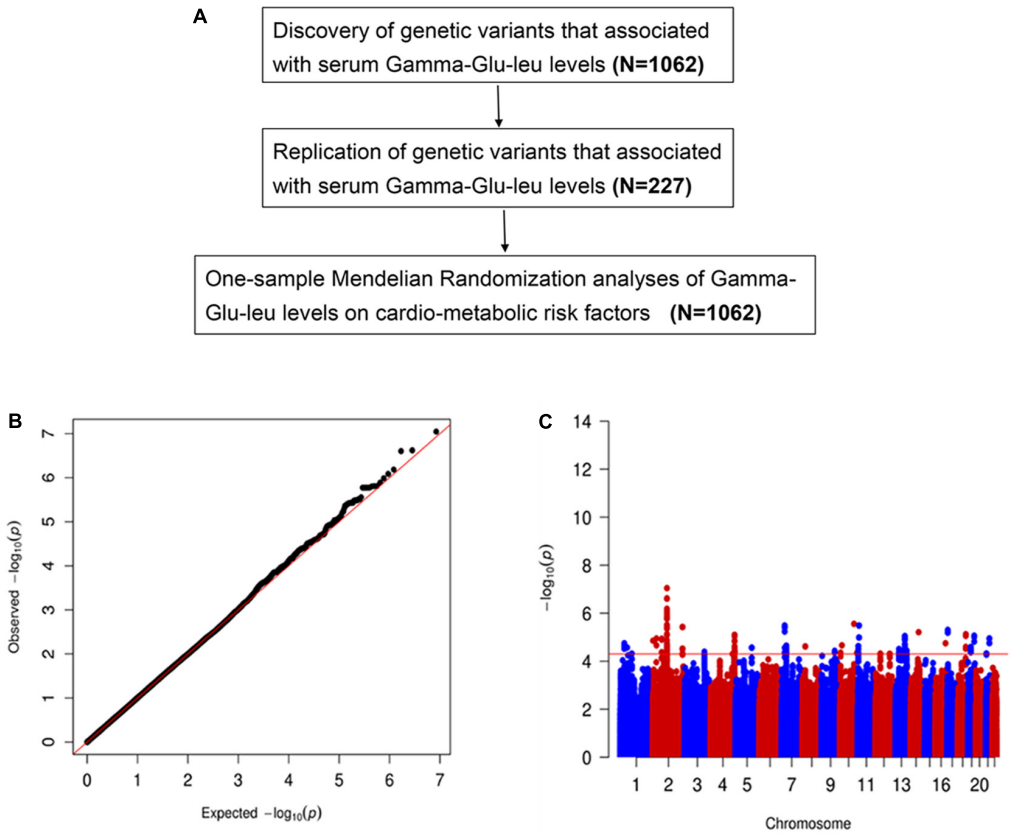

FIGURE 1
(A) The study overview. The cardio-metabolic risk factors include body mass index, waist circumference, waist circumference to hip circumference ratio, total triglyceride, total cholesterol, low-density lipoprotein cholesterol, low-density lipoprotein cholesterol, systolic blood pressure, diastolic blood pressure, fasting plasma glucose, insulin resistance index, and insulin and uric acid. **(B)** The quantile-quantile plot for genomic associations of Gamma-glutamyl-leucine (Gamma-Glu-Leu) (N = 1,062). **(C)** The Manhattan plot for genomic associations of Gamma-Glu-Leu (N = 1,062).

of build 37 and converted to VCF files. The genotype phasing was conducted using EAGLE with the 1000 Genomes Project Phase 3 Version 5 of EAS as the reference panel. Following imputation, SNPs with poor imputation quality ($r^2 < 0.3$) were filtered. Consequently, 40,001,312 SNPs remained.

Statistical analysis

The normality of variables was visually checked using a Q-Q plot. Continuous variables were reported as mean (standard deviation) or median (interquartile range) and were compared using the Student's t-test or Mann–Whitney U test. Categorical variables were reported as numbers (percentages) and were compared using the Chi-square test.

Genome-wide association study analyses were conducted using linear regressions under an additive genetic model implemented in PLINK2. Before analyses, for Gamma-Glu-Leu quantification data, inversed-normalized residuals adjusted for age, sex, and the first two genetic principal components were calculated using the R package "GenABEL." The significance threshold for GWAS was set at $P < 5 \times 10^{-8}$. The suggestive threshold for GWAS was set at $P < 5 \times 10^{-5}$. A clump procedure implemented in PLINK was run for all suggestive GWAS signals (1 Mb, $R^2 < 0.5$). The clumped SNPs were then selected for the replication stage. SNPs with $P < 0.05$ and consistent effect directions were considered successfully replicated. A meta-analysis was performed using the inverse-variance model using the METAL software. The Q-Q and Manhattan plots were drawn using the R package "qqmen." The regional plots for associations between SNPs and Gamma-Glu-Leu were drawn using Locuszoom based on the Asian population of the 1000 Genome Project. SNPs were annotated with available website databases, including PhenoScanner, the GWAS catalog, GTEx, SNiPA, and HaploReg. The detailed information is shown in **Supplementary Table 1**.

One-sample MR analyses were used to evaluate the causal relationships between Gamma-Glu-Leu and the cardio-metabolic risk factors, including the main components of the

metabolic syndrome: obesity-related traits (BMI, WC, and W/H), lipid traits (TG, TC, LDL-C, and HDL-C), blood pressure (SBP and DBP), and glycemic traits (FPG, HOMA-IR, and insulin). Given that hyperuricemia has been previously suggested to be involved in the MetS, and may be an independent predictor of MetS (31), the UA was also included as a cardio-metabolic risk factor in this study. SNPs that were independently and consistently associated with Gamma-Glu-Leu were used to construct an additive weighted gene risk score (wGRS) as the IV of Gamma-Glu-Leu. The formula is as follows: β means the effect size of the association between the SNP and Gamma-Glu-Leu; *N* means the number of risk alleles; i means the number of instrumental SNPs.

$$\mathbf{w}GRS = \frac{\sum_{1}^{\mathbf{i}} \beta\mathbf{i} \times \mathbf{N}}{\mathbf{i} \times \sum_{1}^{\mathbf{i}} \beta\mathbf{i}}$$

To verify the consumption of MR, the F statistics were calculated to evaluate the strength of the IV. The detailed MR analysis process has been provided in **Supplementary Figure 1**. First, a linear regression was performed to assess the association between wGRS and Gamma-Glu-Leu (βZX); then, the associations of the wGRS with the metabolic risk factors were examined (βZY); and finally, observational estimates of the relationships between Gamma-Glu-Leu and the metabolic risk factors were calculated (βXY). The causal estimates were assessed using the Wald-type estimator. All regression models were adjusted for age and sex. In addition, to evaluate horizontal pleiotropy effects on the results, we calculated the relationships between genetically predicted Gamma-Glu-Leu levels and potential confounding factors (age and gender). By querying the Phenoscanner V3 and GWAS catalog, we also assessed if the identified Gamma-Glu-Leu-associated SNPs were associated with any secondary phenotypes. The statistical significance was set at $P < 0.05$. A two-tailed test was used for all statistical analyses in this study. All statistical analyses were performed using the R version 4.0.3 software.

Results

Subject characteristics

A total of 1,062 subjects and 227 subjects were included in the discovery stage and replication stage, respectively. The average age in the discovery stage and validation stage were 57.4 ± 0.3 and 62.4 ± 0.8 years, respectively. The proportions of male and female were 49.9% and 61.2%, respectively. Compared with subjects in the discovery stage, subjects in the validation stage were more likely to be male and older and had a lower BMI and higher WC, and W/H ratio ($P < 0.05$). There was no significant difference in SBP, DBP, TG, TC, UA, HDL-C, LDL-C, insulin, fasting glucose, and HOMA-IR (**Table 1**).

Two-stage genome-wide association study analyses for Gamma-glutamyl-leucine

In the discovery stage, GWAS analyses showed that the genomic inflation factor for Gamma-Glu-Leu was 1.004, indicating no evidence of population stratification. The Q-Q and Manhattan plots were shown in **Figures 1B,C**. No significant GWAS signal was found to be associated with Gamma-Glu-Leu ($P < 5 \times 10^{-8}$, $N = 1{,}062$). A total of 36 suggestive SNPs were identified to be associated with Gamma-Glu-Leu ($P < 5 \times 10^{-5}$, **Supplementary Table 2**). The association between rs12476238 on chromosome 12 and Gamma-Glu-Leu had the lowest *P*-value ($P = 8.99 \times 10^{-8}$). In the replication stage, four SNPs were successfully replicated to be associated with Gamma-Glu-Leu (**Table 2**), including rs12476238, rs56146133, rs2479714, and rs12229654. **Figure 2** shows that the T allele of rs12229654, the T allele of rs12476238, the G allele of rs2479714, and the G allele of rs56146133 were associated with higher serum Gamma-Glu-Leu levels. The regional plots for the four Gamma-Glu-Leu-associated SNPs are shown in **Figure 3**, and rs56146133, rs12476238, rs12229654, and rs2479714 can be mapped to the genes of P2RY1, SULTIC2P, MYL2, and FAM155A, respectively.

After searching the available SNP annotation databases, rs12229654 was reported to be associated with Gamma-glutamyl transpeptidase (GGT), alcohol consumption behavior (drinker/non-drinker status), quantity of drinks, HDL-C levels, BMI, glycemic traits, and 1 h glucose tolerance levels, specifically in East Asian populations ($P < 5 \times 10^{-8}$). Out of them, rs12229654 showed the most significant association with Gamma glutamyl-transpeptidase levels (the effect size for the risk allele G: Beta = 0.019, S.E. = 0.0007, $P = 9.00 \times 10^{-58}$). rs12476238 was reported to be associated with the gene expression of GGC2 and DNA methylation of cg02082929 in the whole blood ($P < 5 \times 10^{-8}$). In HaploReg, this SNP showed associations with histone modification and DNase from multiple tissues, as well as a motif (BDP1) change. rs56146133 was found to be associated with the gene expression of MBNL1 and the DNA methylation of cg16754766 and cg14921522 in the whole blood ($P < 5 \times 10^{-8}$). rs2479714 was reported to have associations with two CpG markers cg13810695 and cg05124117 in the whole blood ($P < 5 \times 10^{-8}$) (**Supplementary Tables 3, 4**).

Table 3 presents the associations of the four SNPs with cardio-metabolic risk factors in this study. All four SNPs had marginal associations with at least one metabolic trait ($5 \times 10^{-5} < P < 0.05$). Notably, rs56146133 was associated with all the listed metabolic traits except for HDL-C, LDL-C, TC, and UA, and rs12229654 was associated with BMI, WC, W/H ratio, SBP, DBP, TG, and UA ($5 \times 10^{-5} < P < 0.05$). rs12476238 was associated with BMI and DBP ($5 \times 10^{-5} < P < 0.05$). The effect directions for all associations between the four SNPs and metabolic risk factors were consistent with their associations with Gamma-Glu-Leu levels.

TABLE 1 Characteristics of subjects in genome-wide association study (GWAS) analyses.

	Discovery stage (N = 1,062)	Replication stage (N = 227)	P
Men, N (%)	530 (49.9)	139 (61.2)	< 0.01
Age (year)†	57.4 (0.3)	62.4 (0.8)	< 0.01
BMI (kg/m^2)†	24.46 (0.11)	23.86 (0.21)	0.01
WC (cm)†	82.64 (0.32)	85.9 (0.71)	< 0.01
W/H ratio†	0.87 (0.002)	0.9 (0.004)	< 0.01
SBP (mmHg)†	140.6 (0.71)	141.48 (1.68)	0.63
DBP (mmHg)†	83.56 (0.40)	82.8 (0.90)	0.44
TG (mmol/L)*	1.66 (1.31)	1.61 (1.15)	0.34
LDL-C (mmol/L)†	2.16 (0.02)	2.14 (0.05)	0.71
TC (mmol/L)†	4.74 (0.03)	4.69 (0.07)	0.46
Uric acid (umol/L)*	5.75 (0.01)	5.76 (0.02)	0.47
HDL-C (mmol/L)†	1.5 (0.01)	1.51 (0.02)	0.80
Insulin (mU/L)*	3.9 (3.4)	3.6 (3.2)	0.12
Glucose (mmol/L)*	5.14 (1.12)	5.16 (1.1)	0.25
HOMA-IR*	0.92 (0.96)	0.86 (0.98)	0.33

*Data was presented as median (interquartile range).
†Data was presented as mean (standard deviation).
BMI, body mass index; WC, waist circumference; W/H ratio, waist circumference to hip circumference ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance.

TABLE 2 Four single nucleotide polymorphism (SNPs) that associated with Gamma-Glu-Leu in the two-stage genome-wide association study (GWAS) analyses.

SNP	CHR	POS	REF	ALT	A1	Gene hit	MAF	Discovery stage			Validation stage			P-meta
								BETA	SE	P	BETA	SE	P	
rs12476238	2	108940336	T	C	T	SULT1C2P1	0.11	−0.38	0.07	8.99E-08	−0.29	0.14	3.77E-02	1.04E-08
rs56146133	3	152513774	A	G	G	P2RY1	0.22	0.21	0.05	4.01E-05	0.29	0.11	1.09E-02	1.62E-06
rs2479714	13	107715021	G	A	G	FAM155A	0.75	−0.22	0.05	3.61E-05	−0.25	0.11	2.95E-02	3.12E-06
rs12229654	12	111414461	T	G	G	LINC01405	0.25	−0.21	0.05	4.85E-05	−0.28	0.10	7.33E-03	1.49E-06

SNP, single nucleotide polymorphism; CHR, chromosome; POS, position (hg19); REF, reference allele; ALT, alternative allele; MAF, minor allele frequency; A1, minor allele; SE, standard error.

Causal associations of Gamma-glutamyl-leucine with cardio-metabolic risk factors

Genetically predicted serum Gamma-Glu-Leu levels were not significantly associated with age and gender ($P > 0.05$, Supplementary Table 5). In Phenoscanner and the GWAS catalog, we also did not find that the identified four SNPs had associations with possible confounding factors (Supplementary Table 3). Therefore, all of them were used to construct a GRS of Gamma-Glu-Leu, which was then used as its IV (Beta = 0.25, S.E. = 0.03, $P = 1.26 \times 10^{-14}$). The F statistic for this IV was 46.39, suggesting that it was sufficient for the MR analyses. After adjustment for age and sex, observational associations showed that higher levels of Gamma-Glu-Leu were significantly associated with all listed metabolic risk factors except for HDL-C ($P < 0.05$). For the causal estimates, similarly, higher genetically determined levels of Gamma-Glu-Leu were positively associated with all listed metabolic risk factors except for HDL-C and LDL-C (Figure 4 and Supplementary Table 6, $P < 0.05$). Among these outcomes, higher Gamma-Glu-Leu displayed the most significant causal relationship with high HOMA-IR [Beta: 0.62 (95% CI: 0.32–0.91), $P = 3.6 \times 10^{-5}$].

Discussion

In this study, we first reported that four SNPs were associated with serum Gamma-Glu-Leu levels in the East Asian population, including rs12476238, rs56146133, rs2479714, and rs12229654. Furthermore, each of these SNPs was nominally associated with at least one cardio-metabolic risk factor. MR analyses revealed that higher serum levels of Gamma-Glu-Leu were causally linked to the risk of cardio-metabolic

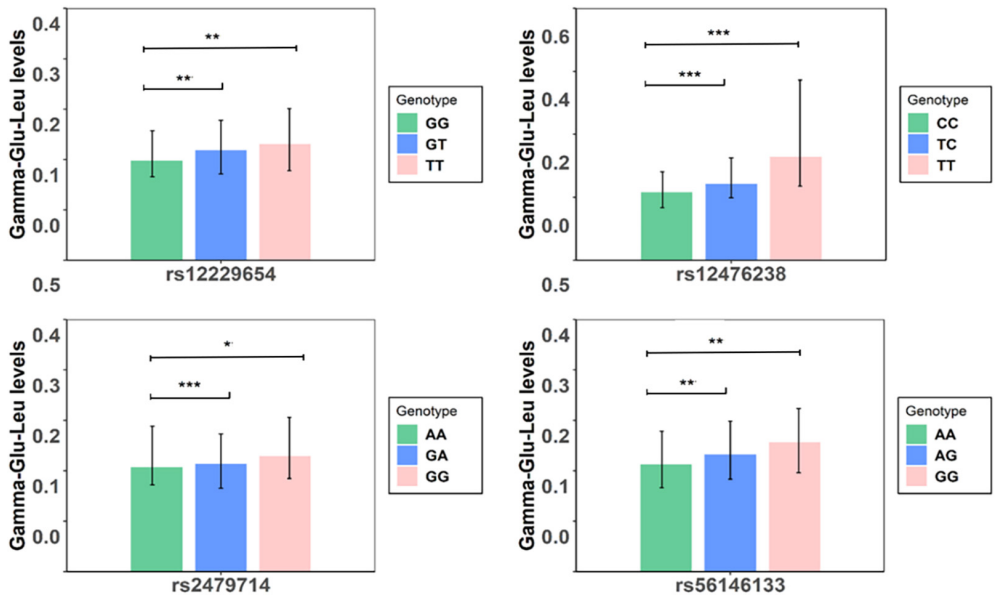

FIGURE 2
Serum levels of Gamma-glutamyl-leucine (Gamma-Glu-Leu) in subjects with different genotypes (N = 1,062). Symbol *denotes that serum levels of Gamma-Glu-Leu were significantly different between groups. ***P-value < 0.001;**P-value < 0.01; *P-value < 0.05.

factors. Overall, our study provides the causal evidence between Gamma-Glu-Leu levels and the metabolic risks. Further mechanistic research is needed to explore how this metabolite affects metabolic abnormality.

rs12229654 is located in 12q24.11 and is within a lncRNA gene (LINC01405). Its upstream gene is called MYL2, encoding a regulatory light chain associated with the cardiac myosin β (or slow) heavy chain. Its downstream gene is called CUX2, encoding a protein that contains three CUT domains and a homeodomain. Both domains are DNA-binding motifs. Previous GWAS studies have reported that rs12229654 was associated with GGT, BMI, HDL-C, glycemic traits, alcohol consumption behavior and quantity of drinks, and the risk of hypertension and T2D in the East Asian population (32–36). Consistently, our study found that rs12229654 was associated with Gamma-Glu-Leu (a metabolic product of GGT), BMI, WC, W/H ratio, TG, TC, and blood pressure. GGT is a liver enzyme involved in GSH metabolism, and the liver is the main alcohol-detoxification organ. The associations among rs12229654, GGT, as well as its related metabolite (Gamma-Glu-Leu), alcohol consumption behavior and amount of drinks suggested that the observed associations of rs12229654 with serum Gamma-Glu-Leu and cardio-metabolic risk factors may be partly explained by drinking behavior and its followed abnormal liver function (12, 37). Of note, allele G of rs12229654 only showed up in the East Asian population (MAF = 0.15 in the 1000 Genome Project) but not in other ethnic groups. Since most annotation databases on SNP regulatory elements were based on the European population, little annotated information on this SNP was reported. Future functional studies are required to uncover the mechanism of rs12229654 in metabolic disorders.

rs12476238 is located in 2q12.3, its mapped gene is a pseudogene called sulfotransferase Family 1C Member 2 (SULT1C2). The Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) previously reported nominal associations between rs12476238 and insulin and HOMA-IR (38). Similarly, a nominal relationship between rs12476238 and HOMA-IR was also reported in this study, indicating that rs12476238 may have an impact on glucose balance. According to the annotation database, rs12476238 is a *trans*-eQTL that can regulate the gene expression of its downstream gene GCC2 (39). GCC2 encodes a membrane protein localized to the *trans*-Golgi network and is related to the vesicular transport between the endosomes and the Golgi. rs12476238 was also associated with CpG marker cg02082929, which is close to the exon of GCC2 (40). In HaploReg, rs12476238 showed regulatory effects on histone modification and DNase in multiple tissues, suggesting that GCC2 may be the target gene of rs12476238. Notably, the frequency of allele T of this SNP was 0.87 in the Asian population of the 1000 genome projects, which was significantly higher than in other populations (EUR: 0.15; AFR: 0.08). Extensive studies are required to verify whether GCC2 links the association between rs12476238 and Gamma-Glu-Leu.

rs56146133 is located in 3q25.2 and can be mapped to the gene for purinergic receptor P2Y1. The product of this gene belongs to the family of G-protein-coupled receptors. Gene Ontology annotations on this gene include G-protein-coupled receptor activity and signaling receptor activity. rs56146133 was

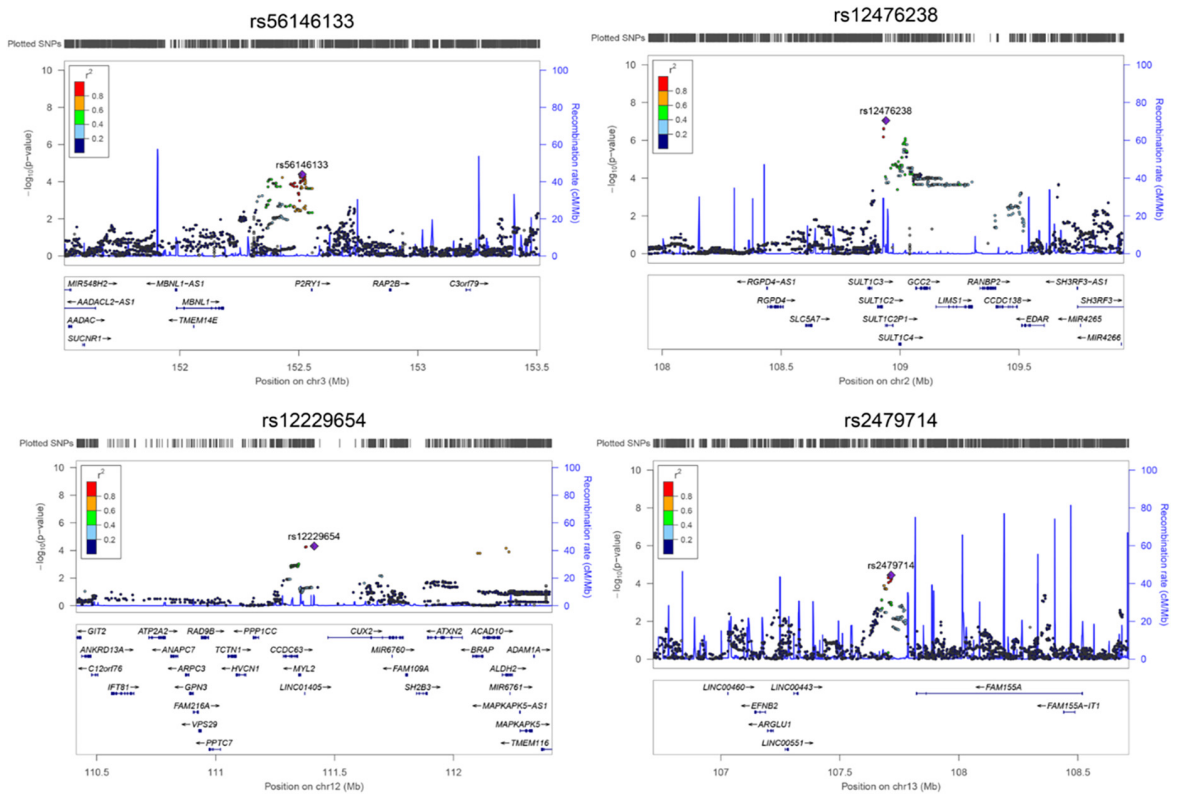

FIGURE 3
The regional plot for associations between four single nucleotide polymorphisms (SNPs) and Gamma-glutamyl-leucine (Gamma-Glu-Leu) levels (N = 1,062). Single nucleotide polymorphism position was based on hg19. Regional plot was drawn using Locuszoom based on the Asian population of the 1000 Genome Project.

associated with the gene expression of MBNL1, which is located ∼330 kb downstream of this SNP. MBNL1 encodes a member of the muscle-blind protein family. This protein is a C3H-type zinc finger protein and modulates alternative splicing of pre-mRNAs. Gloria et al. have reported that MBNL1 activates insulin receptor exon 11 inclusion by enhancing U2AF65 binding and splicing of the upstream intron (41). Alternative splicing regulates developmentally and tissue-specific gene expression programs, disruption of which has been implicated in numerous diseases. In this study, we observed that rs56146133 was associated with Gamma-Glu-Leu and multiple metabolic risk factors, indicating that this gene locus may have a regulatory role on metabolic risk.

rs2479714 is located at 13q33.3 and can be aligned to its nearby gene, FAM155A. This gene has been predicted to be involved in calcium ion import across the plasma membrane. No studies have reported the association of rs2479714 with any traits or diseases, but one gene variant, rs1509091, within 13q33.3 has been identified as having a suggestive relationship with serum pyroglutamine measurement ($P = 3 \times 10^{-6}$) (42). Pyroglutamine, also known as 5-oxoproline, is an intermediate metabolite in GSH metabolism. The Gamma-glutamyl amino acid that is released into the cytosol can be further catabolized into 5-oxoproline and amino acids by Gamma-glutamyl cyclotransferase (GCT) (43, 44), which suggested that the gene locus of 13q33.3 may be associated with the GSH cycle.

Using identified four SNPs as IV of Gamma-Glu-Leu, we found higher serum Gamma-Glu-Leu levels causally linked to multiple cardio-metabolic risks, which was consistent with previous metabolomics research (17, 45). Gamma-Glu-Leu is partly derived from GSH degradation by GGT, whose elevation may be due to an increased turnover of GSH and an increased GGT activity. It has been demonstrated that an elevated GGT activity and a depletion of GSH are associated with the risk of new onset of MetS and type 2 diabetes (46–49). Additionally, Gamma-Glu-Leu exhibited a negative association with levels of selenoprotein P (50), which is also an important antioxidant substance synthesized in the liver and has a critical role in maintaining the balance of glucose and lipid metabolism (51). Little is known about the pathological function of Gamma-Glu-Leu. Previous experimental studies reported the beneficial effects of Gamma-Glu-Leu and Gamma-glutamyl valine including anti-inflammation and hypoglycemic action; however, most of them were conducted *in vitro* cell models

TABLE 3 Associations of Gamma-glutamyl-leucine (Gamma-Glu-Leu)-associated single nucleotide polymorphism (SNPs) with the cardio-metabolic risk factors (N = 1,062).

Traits	rs12229654			rs56146133			rs12476238			rs2479714		
	Beta	SE	*P*	Beta	SE	*P*	Beta	SE	*P*	Beta	SE	*P*
BMI	−0.58	0.18	**0.001**	0.37	0.19	**0.049**	0.55	0.25	**0.030**	−0.22	0.19	0.252
WC	−1.75	0.51	**0.001**	1.49	0.53	**0.005**	1.30	0.72	0.073	−1.17	0.53	**0.027**
W/H	−0.01	0.03	**0.003**	0.01	0.003	**0.007**	0.01	0.004	0.14	−0.01	0.003	0.075
SBP	−3.20	1.10	**0.004**	3.34	1.16	**0.004**	2.08	1.55	0.181	−2.23	1.15	0.054
DBP	−1.62	0.64	**0.012**	2.21	0.67	**0.001**	1.96	0.89	**0.028**	−0.73	0.66	0.274
TC	−0.10	0.05	**0.03**	0.02	0.47	0.65	0.02	0.06	0.75	−0.07	0.05	0.127
LDL-C	−0.06	0.04	0.104	−0.01	0.04	0.82	−0.01	0.05	0.83	−0.05	0.04	0.144
TG	−0.03	0.01	**0.018**	0.03	0.01	**0.011**	0.03	0.02	0.097	−0.005	0.01	0.720
UA	−0.03	0.01	**0.018**	0.01	0.01	0.247	0.02	0.02	0.251	−0.02	0.01	0.136
HDL-C	0.01	0.02	0.776	−0.02	0.02	0.300	0.03	0.03	0.320	−0.004	0.02	0.845
Insulin	−0.20	0.17	0.243	0.35	0.17	**0.044**	0.42	0.23	0.069	−0.28	0.17	0.100
FPG	−0.10	0.07	0.162	0.16	0.07	**0.024**	0.12	0.09	0.223	−0.06	0.07	0.408
HOMA-IR	−0.07	0.04	0.049	0.12	0.04	**0.001**	0.12	0.05	**0.014**	−0.06	0.04	0.090

Values of TG were log transformed. Values of UA, FPG, HOMA-IR, and insulin were natural logarithm transformed. The beta was adjusted for age, sex and the first two genetic components. BMI, body mass index; WC, waist circumference; W/H ratio, waist circumference to hip circumference ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; SE, standard error. Values in bold denote $P < 0.05$.

(9, 52, 53). Future interventional studies in human and animal models are required to longitudinally assess the metabolic effects of Gamma-Glu-Leu, and further reveal the molecular mechanisms of its involvement in the pathophysiology of metabolic disturbances.

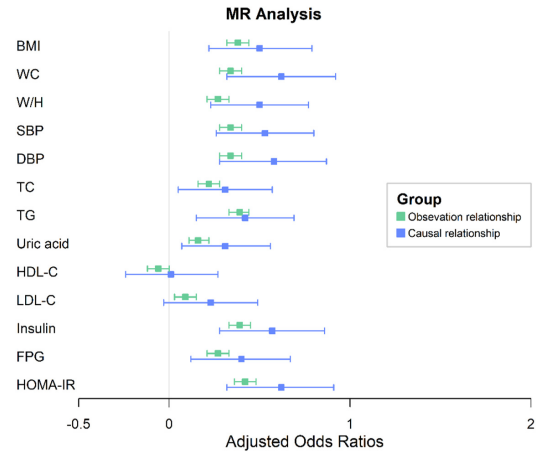

FIGURE 4
One-sample MR analysis between Gamma-glutamyl-leucine (Gamma-Glu-Leu) and cardio-metabolic risk factors. Colors in green denote the observational relationships. Colors in blue denote the causal relationships. The error bar signifies the beta and 95% CIs. Beta was adjusted for age and sex; BMI, body mass index; WC, waist circumference; W/H ratio, waist circumference to hip circumference ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; MR, Mendelian Randomization; CI, confidence interval.

Our study has some strengths. First, using two-stage GWAS analyses, we first reported the genetic associations of Gamma-Glu-Leu and identified four SNPs to be associated with serum levels of Gamma-Glu-Leu, which provides mechanistic insights into the involvement of Gamma-Glu-Leu in the metabolic disorders. In addition, using the MR approach, we revealed that higher Gamma-Glu-Leu may causally contribute to elevated cardio-metabolic risks.

Several other limitations should also be considered. First, all subjects of this study were derived from East China, thus the findings of this study cannot be generalized to other populations. Second, a few significant SNPs associated with Gamma-Glu-Leu were identified, which may be due to our limited sample size, although this study was the largest GWAS for this metabolite in East China populations. Thirdly, information on lifestyle exposure was not collected in the epidemiological investigation, such as drinking behavior, so its effect on the genetic associations of Gamma-Glu-Leu cannot be analyzed. Fourth, as a well-established cardio-metabolic risk factor, levels of C-reactive protein were not measured in this study, which prevented us from exploring the relationship between Gamma-Glu-Leu and systemic inflammation. Fifth, the instrumental SNPs used to construct the IV did not obtain the GWAS significance threshold, which might cause a weak instrumental bias. However, the F statistics of the IV were larger than 10, indicating that the IV was enough for the MR analyses. Additionally, the gene pleiotropy in the MR analyses cannot be completely avoided.

Conclusion

Four SNPs (rs12476238, rs56146133, rs2479714, and rs12229654) may regulate the levels of serum Gamma-Glu-Leu. Higher Gamma-Glu-Leu levels are causally linked to the cardio-metabolic risk. Future interventional studies on Gamma-Glu-Leu are required to explain its role in metabolic disorders.

Data availability statement

The data presented in this study are deposited in the figshare repository, accession number: https://doi.org/10.6084/m9.figshare.20103644.v1.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee at the School of Medicine, Zhejiang University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ML, YZ, and QC: conceptualization. QW, XS, DH, ZC, and JuL: data curation. JiL and JZ: formal analysis. YZ and YX: funding acquisition. QW, DH, ZC, and JuL: investigation. QW, JuL, and XS: methodology. ML and YZ: project administration. YZ: resources and supervision. QW and JZ: software. QW: validation, visualization, and writing – original draft. YZ and XZ: writing – review and editing. All authors approved the final edited version of this manuscript.

Funding

This study was supported by grants from the National Key Research and Development Program of China (2017YFC0907004) and Basic Public Research Program from Zhejiang Province (GF21H260034).

Acknowledgments

We would like to thank all participants and investigators that took part in this 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.936220/full#supplementary-material

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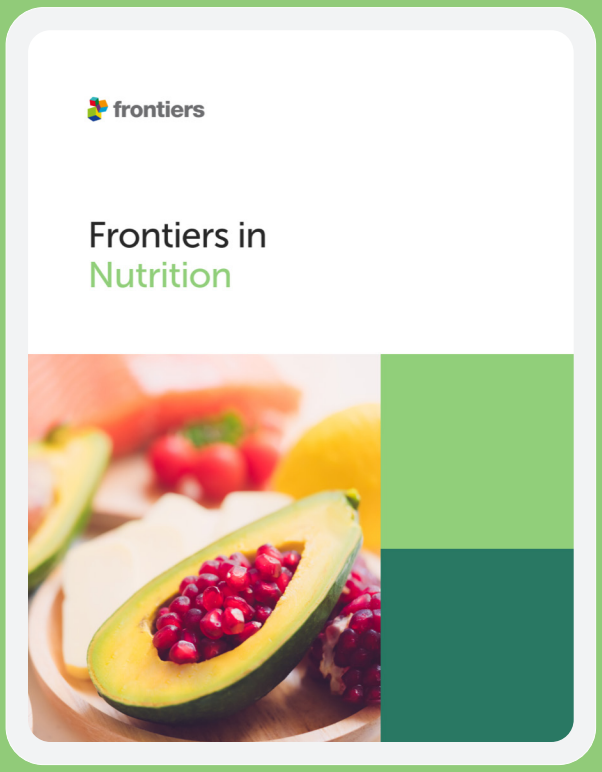

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