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Biomarkers: Precision nutrition in chronic diseases

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Biomarkers: Precision nutrition in chronic diseases

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Editorial: Biomarkers: precision nutrition in chronic diseases

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Editorial on the Research Topic
Biomarkers: precision nutrition in chronic diseases

Chronic diseases, such as cardiovascular diseases, diabetes, cancer, and arthritis, constitute significant causes of morbidity and mortality on a global scale, with their prevalence steadily rising across all age groups, genders, and ethnicities (1). Growing evidence indicates that precision nutrition plays a crucial role in the prevention and management of chronic diseases, garnering recognition as a key area of focus for health research in the next decade (2, 3). Nonetheless, one of the primary challenges in precision nutrition lies in the accurate and reliable assessment of foods and nutrients, particularly with regards to complex foods and macromolecules. Additionally, there is a need to determine how these foods and nutrients impact the health and disease status of individuals.

Promisingly, robust evidence strongly supports the use of biomarkers as an intermediary tool that effectively establishes a connection between precision nutrition and chronic diseases (4). This connection facilitates the objective assessment of food consumption and provides precise determinations of the biological effects of complex foods and ingredients (5, 6). Despite these advancements, our current understanding of how precision nutrition regulates biomarkers to prevent chronic diseases with individual variations is still in its infancy. The molecular mechanisms that underlie the involvement of key biomarkers in chronic diseases remain inadequately elucidated, necessitating comprehensive and extensive research efforts to bridge this knowledge gap. Therefore, the objective of this Research Topic is to gather the latest research that uncovers the role of key biomarkers in chronic diseases and explores how precision nutrition can modulate this process in diverse populations. By investigating the interaction between precision nutrition, biomarker discovery, and chronic diseases, we can gain valuable insights into the implementation of precision nutrition approaches for the effective prevention and management of chronic diseases.

For this Research Topic, a total of 72 manuscripts were received, out of which 25 have been published. Such a high number of submissions indicates that the topic is currently a prominent research focus and a significant area of interest. Presented below is a brief overview of the 25 published articles.

Several novel potential biomarkers for various chronic diseases have been identified in this Research Topic. Among them, a single substance serves as the biomarker, such as

25-hydroxyvitamin D for coronary heart disease (Zhang H. et al.), retinol for non-alcoholic fatty liver (Niu et al.), fluorescent advanced glycation end products for type 2 diabetes (Liu R. et al.), and branched-chain amino acids for moyamoya disease (Zeng et al.). Additionally, the ratio of different substances has been proposed as the biomarker. Hyun et al. and Xia et al. found that the creatine to cystatin C (Cr/CysC) ratio and the albumin to globulin ratio can serve as non-invasive biomarkers for the prognosis of chronic kidney disease and urological cancers, respectively. Notably, Gao et al. have found that the Cr/CysC ratio could also be a potential biomarker for osteoporosis. Furthermore, Cai et al. identified the geriatric nutritional risk index as a potential marker for stroke in elderly hypertensive patients, and Ávila et al. found the level of phosphorus combined with albumin as a potential marker for all-cause mortality and cardiovascular mortality. Intriguingly, Zhang Y. et al. demonstrated that differentially expressed genes related to ferroptosis could be employed as new biomarkers for identifying ischemic stroke and guiding therapeutic interventions. These discovered biomarkers provide an important theoretical foundation for the prevention and management of related chronic diseases, as well as contribute to the understanding of the pathogenesis underlying various chronic diseases.

This Research Topic encompasses some studies focusing on the application of precision nutrition to prevent chronic diseases based on biomarkers, which are typical physiological indicators of such conditions. For instance, studies by Tao et al., Yang et al., Li et al., and Fang et al. demonstrate that dietary supplementation of functional factors, namely anthocyanin, glutamine, Vitamin K, and fatty acids, can respectively alleviate heart failure, high salt-induced hypertension, vascular calcification, and bone mineral loss. However, it is important to exercise caution regarding the dosage of dietary functional factors as excessive fatty acid intake can contribute to metabolic diseases. Other articles highlight the positive effects of complex foods on various chronic diseases. For example, Huo et al. demonstrate that dietary fruit consumption improves functional constipation, Shahdadian et al. endorse a plant-based diet for managing metabolic syndrome, Hevilla et al. propose specific oral nutritional supplements (ONS) to address inflammation/oxidation, and Zamani et al. suggest saffron for mitigating cardiovascular diseases. Notably, Hevilla et al. also observe a synergistic effect of probiotic supplementation in conjunction with specific ONS on inflammation/oxidation. Additionally, Huang et al. demonstrate that combined training, involving resistance training along with high-intensity interval training or moderate-intensity continuous training, is beneficial for non-alcoholic fatty liver disease treatment. In addition to these dietary strategies for preventing or treating chronic diseases, Liu T.-h. et al. mention that dietary patterns also contribute to the onset of such conditions, such as a high-salt diet exacerbating the intestinal aging process. These dietary functional factors and complex foods present effective preventive measures for managing chronic diseases. However, the specific mechanisms responsible for the regulation of these biomarkers have not been thoroughly explored, highlighting the need for further research in this area.

During the process of mining biomarkers, it is crucial to consider the individual differences among research subjects. Jáni et al. demonstrate that some factors such as birth length, puberty onset, and visceral fat levels can collectively influence the biological aging process, accounting for 21% of the observed variation. Furthermore, the findings reviewed by Wuni et al. suggest that additional factors, such as individual socio-economic status and architectural environment, also influence the same process. Consequently, it becomes imperative to enhance the reliability of biomarker mining using cutting-edge techniques such as bioinformatics technology. Cole et al. employed genetic heritability as a tool to evaluate the accuracy of dietary questionnaire variables in their research, thereby bolstering the credibility of biomarker mining in dietary studies. Additionally, Barbe et al. effectively circumvented the impact of individual variability on nutritional intervention in immune and metabolic health by utilizing metabolomics-based clustering methods. Similarly, Xu et al. employed a bidirectional Mendelian randomization study to establish hypothetical causal relationships between circulating vitamin D and estimated bone mass, plasma triglycerides, and total cholesterol. This approach helped avoid inconsistencies in results caused by the presence of confounding factors. Despite the advances made by these approaches in bolstering the credibility of biomarker mining, the process remains susceptible to numerous unpredictable confounders. Therefore, further advancements are required to enhance the causal verification of biomarkers through animal experiments and human clinical studies.

In conclusion, this Research Topic has contributed to a better understanding of additional biomarkers associated with chronic diseases and the measures of precision nutrition interventions. Furthermore, the topic addresses factors that may interfere with biomarker identification and presents effective techniques for circumventing these challenges. However, the causal verification of biomarkers and the molecular mechanisms underlying their precise nutritional regulation still require further clarification, which will be the central focus of the articles included in Volume II of this Research Topic.

Author contributions

ZZ prepared the first draft. Y-LL and SS critically reviewed and edited it. All authors approved the submitted version.

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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.

Publisher's note

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Predictive value of 25-hydroxyvitamin D level in patients with coronary artery disease: A meta-analysis

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Background: A consensus has not been made about the predictive value of blood vitamin D level in patients with coronary artery disease (CAD). This meta-analysis aimed to assess the association between blood 25-hydroxyvitamin D level and adverse outcomes in patients with CAD.

Methods: Two independent authors searched the articles indexed in PubMed and Embase databases until June 28, 2022. Cohort studies or *post-hoc* analysis randomized trials evaluating the value of 25-hydroxyvitamin D level in predicting cardiovascular or all-cause mortality, and major adverse cardiovascular events ([MACEs] including death, non-fatal myocardial infarction, heart failure, revascularization, stroke, etc.) were included.

Results: The literature search identified 13 eligible studies for our analysis, including 17,892 patients with CAD. Meta-analysis showed that the pooled adjusted risk ratio (RR) was 1.60 (95% confidence intervals [CI] 1.35–1.89) for all-cause mortality, 1.48 (95% CI 1.28–1.71) for cardiovascular mortality, and 1.33 (95% CI 1.18–1.49) for MACEs. Leave-out one study sensitivity analysis suggested that the predictive values of blood 25-hydroxyvitamin D level were reliable.

Conclusions: Low blood 25-hydroxyvitamin D level is possibly an independent predictor of cardiovascular or all-cause mortality and MACEs in patients with CAD. Baseline 25-hydroxyvitamin D level may provide useful information in CAD patients.

KEYWORDS

25-hydroxyvitamin D, coronary artery disease, major adverse cardiovascular events, mortality, meta-analysis

Introduction

Coronary artery disease (CAD) is the most common type of heart disease worldwide, which can be manifested as stable ischemic heart disease or acute coronary syndrome (ACS). Despite the improvement in medical therapy and surgical revascularization, CAD remains a major determinant of morbidity and premature

death (1). A more aggressive secondary prevention can be implemented by employing early risk stratification for cardiovascular events and death in patients with CAD.

Biomarkers play an important role in risk stratification and management of CAD (2, 3). Vitamin D is a hormone precursor that maintains calcium homeostasis. The blood level of 25-hydroxyvitamin D is identified as the best estimation of vitamin D state (4). Increasing attention has been focused on the effect of vitamin D on the management of cardiovascular disease (5). Vitamin D deficiency or insufficiency is prevalent in patients with CAD (6–8). Low blood 25-hydroxyvitamin D level is emerging as a predictive biomarker for patients with CAD (9–13). However, inconsistent findings (14–17) have been recorded on the predictive value of Vitamin D deficiency in these patients.

No previous meta-analysis has systematically assessed the predictive association of Vitamin D deficiency with adverse outcomes in patients with CAD. Therefore, the current meta-analysis aimed to evaluate the predictive value of blood 25-hydroxyvitamin D level of patients with CAD in terms of cardiovascular death, all-cause mortality, and cardiovascular events.

Methods

Search strategy

The current meta-analysis was carried out under the Preferred Reporting Items for Systematic Reviews and Meta-analysis guideline (18). Two independent authors identified the eligible studies indexed in PubMed and Embase databases using the following combination of items: ("vitamin D" OR "25-hydroxyvitamin D") AND ("coronary artery disease" OR "coronary heart disease" OR "ischemic heart disease" OR "ischaemic heart disease" OR "acute coronary syndrome" OR "myocardial infarction" OR "angina") AND ("death" OR "mortality" OR "cardiovascular event") AND ("follow-up" OR "follow up" (Supplementary Text S1 in Supplementary material). The final updated search was conducted on June 28, 2022. References of pertinent articles were manually scanned to identify potentially eligible studies. To minimize publication bias, we also reviewed the ClinicalTrials.gov and full-text database of Chinese Excellent Doctoral and Master's Dissertations to identify any gray and unpublished literature.

Study selection

The inclusion criteria are as follows: (1) population: patients were with CAD; (2) exposure: blood 25-hydroxyvitamin D level at baseline; (3) comparison: patients with the bottom vs. reference top 25-hydroxyvitamin D level; (4) outcome measures: cardiovascular or all-cause mortality, and major adverse cardiovascular events ([MACEs] including death, non-fatal myocardial infarction, heart failure, revascularization, stroke, etc.); (5) reported multivariable adjusted risk estimate for the above mentioned outcomes; and (6) study design: retrospective or prospective cohort studies or *post-hoc* analysis randomized trials. When multiple articles were obtained from the same population, we selected the publication with the longest follow-up. The exclusion criteria are as follows: (1) studies reported the in-hospital outcomes; (2) studies provided risk summary by continuous 25-hydroxyvitamin D level; and (3) cross-sectional study or meeting abstract.

Data extraction and quality assessment

The following data was abstracted by two authors independently: last name of the first author, publication year, origin of study, study design, subtype of CAD, number of patients, gender distribution, age of patients at enrollment, length of follow-up, cutoff value of vitamin D deficiency, definition of MACEs, endpoints, fully adjusted risk summary, and confounders included in the fully adjusted models. Two authors independently assessed the study quality according to the Newcastle-Ottawa Scale (NOS) for cohort studies (maximum score of 9 points) (19). Studies with NOS point $\geq$ 7 indicated high methodological quality. Any discrepancies were settled by discussing with a third author (Y Fan) to reach consensus.

Statistical analysis

All data were analyzed using STATA 12.0 (STATA Corp LP, College Station, TX, USA). To evaluate the association between blood 25-hydroxyvitamin D level and adverse outcomes, we poled the most fully adjusted risk ratios (RR) and 95% confidence intervals (CI) with the bottom vs. the reference top category of 25-hydroxyvitamin D level. Heterogeneity between studies was determined using the Cochran's Q statistic ($p < 0.10$ was considered significant) and the I^2 statistic ($I^2 \geq 50\%$ was considered significant). A random effect model was used for data analysis in the presence of statistically significant heterogeneity. A fixed-effect model was used in the absence of significant heterogeneity. To test the credibility of the pooling results, we conducted a leave-out one study sensitivity analysis to recalculate the risk estimates. Subgroup analyses were conducted to investigate the effect of the types of CAD, sample sizes, publication time, and length of follow-up. Begg's test, Egger's test, and funnel plot were used to investigate the publication bias. The certainty of evidence was summarized *via* the GRADE analysis.

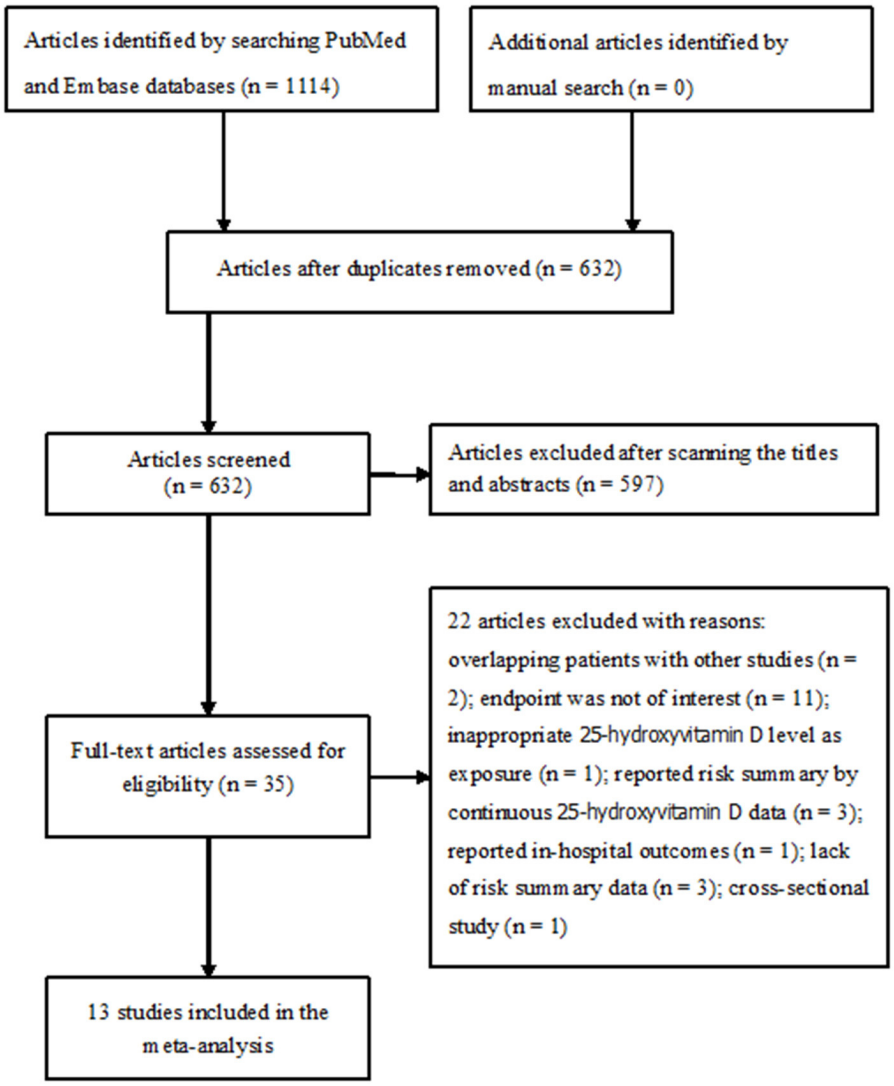

FIGURE 1
Flow chart of the study selection process.

Results

Search results and study characteristics

A total of 1,114 records were identified from initial electronic database search. After excluding duplicates, 632 records were left. After reading the titles and abstracts, 597 obviously unrelated records were excluded. Thirty-five articles were retrieved for full-text assessment, and 13 studies (9–17, 20–23) satisfied the inclusion criteria (Figure 1).

The main features of the included studies are presented in Table 1. These included studies were published between 2010 and 2021. All articles adopted the prospective designs. Four studies (10, 11, 17, 21) included patients with ACS, one study (22) enrolled patients with post-acute myocardial infarction (AMI), one study (13) included stable angina patients, and others did not report the specific type of CAD. Sample sizes ranged from 252 to 4,114, with a total of 17,892 patients with CAD. The length of follow-up varied from 12 months to 11.9 years. According to the NOS criteria, all studies were deemed as high methodological quality (Supplementary Table S1).

All-cause mortality

Ten studies (9–14, 16, 17, 20, 23) investigated the value of 25-hydroxyvitamin D level in predicting all-cause mortality.

TABLE 1 Main characteristic of the included studies.

Author/ year	Region	Patients (% male)	Age (years)	Cutoff value of 25(OH) D level	Definition of MACEs	Outcome measures HR/RR (95% CI)	Follow-up	Adjusted variables
Grandi et al. (14)	Germany	Stable CAD 1,125 (84.4)	30–70	<15 *vs.* >30 ng/mL	Non-fatal MI, ischemic stroke, or CV death	Total death 0.94 (0.39–2.26); MACEs 0.83 (0.37–1.86)	8.1 years	Age, sex, season, smoking, hypertension, DM, BMI, TG, LDL, HDL, TC, number of affected vessels, previous MI, creatinine clearance; treatment, CRP
Lerchbaum et al. (9)	Austria	CAD 2,069 (100)	54–74	<11.2 *vs.* ≥11.2 ng/mL	—	Total death; 1.77 (1·47–2.13); CV death; 1.24 (0.96–1.60)	7.7 years	Age, BMI, active smoking, physical activity, DM, CRP, prevalent CAD, serum calcium, parathyroid hormone.
Ng et al. (10)	UK	AMI 1,259 (72)	65.7 ± 12.8	<7.3 *vs.* >20 ng/mL	Death, HF admission, recurrent MI	Total death; 1.25 (0.75–2.08); MACEs; 1.61 (1.15–2.27)	1.5 years	Age, sex, previous MI, hypertension, DM, Killip class, eGFR, NTproBNP, smoking, electrocardiogram ST change
Siasos et al. (15)	Greece	CAD 252 (91)	62 ± 11	<30 *vs.* ≥30 ng/mL	CV death, non-fatal MI, or stroke, admission for cardiovascular causes	MACEs; 7.24 (0.99–53.5)	1.25 years	Age, sex, kidney function, dyslipidemia, hypertension, DM, smoking, obesity, severity of CAD
Welles et al. (16)	USA	CAD 946 (81)	65.4±11	<30 *vs.* ≥20 ng/mL	CV death, HF, MI, stroke	Total death; 1.18 (0.92–1.52); CV death; 1.13 (0.76–1.70); MACEs; 1.11 (0.85–1.44)	8.0 years	Age, sex, race/ethnicity, season of blood draw, college graduation, tobacco use, multivitamin use, physical activity, DM, hypertension, depression, BMI, SBP, DBP, hemoglobin A1c, TG, HDL, CRP, phosphorus, parathyroid hormone, fibroblast growth factor 23
De Metrio et al. (11)	Italy	ACS 814 (72)	67±12	< 9.0 *vs.* >22 ng/mL	Death, arrhythmias, cardiogenic shock, AKI, major bleeding, APE	Total death; 2.51 (1.35–4.65); MACEs; 1.85 (1.25–2.75)	1.0 year	Age, BMI, DM, LVEF, creatinine, HDL, TC, TG
Naesgaard et al. (17)	Norway	ACS 871 (61)	69.6±14.4	Quartiles 1 *vs.* Quartiles 4	—	Total death; 1.27 (0.92–1.75); CV death; 1.20 (0.58–2.50)	7.0 years	Age, sex, smoking, hypertension, BMI, index diagnosis, DM, chronic HF, previous CAD, hypercholesterolemia, use of statins, troponin-T, eGFR, hsCRP, BNP, β-blockers

(Continued)

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TABLE 1 Continued

Author/ year	Region	Patients (% male)	Age (years)	Cutoff value of 25(OH) D level	Definition of MACEs	Outcome measures HR/RR (95% CI)	Follow-up	Adjusted variables
Gerling et al. (12)	Canada	CAD 2,975 (60)	63.6±12	≤ 40.2 *vs.* ≥ 91.8 nmol/L	—	Total death; 1.84 (1.36–2.50)	5.8 years	Age, sex, BMI, smoking, renal disease, hypertension, hyperlipidemia, type 2 DM, family history of heart disease, prior MI, congestive HF
Yu et al. (20)	China	CAD 1,387 (65.1)	40–85	≤2.11 *vs.* ≥4.88 ng/mL	—	Total death; 1.36 (0.88–2.12); CV death; 1.49 (0.87–2.56)	6.7 years	Age, sex, BMI, smoking, DM, SBP, TC, HDL, extent of CAD, acute CAD, coronary revascularization, use of statins, ACEI/ARB, β-blockers, season of blood-drawing, physical activity, eGFR, calcium, parathyroid hormone, and CRP
Degerud et al. (13)	Norway	Stable angina 4,114 (71.9)	61.8±10.4	≤13.6 *vs.* > 13.6–32.1 ng/mL	—	Total death; 1.94 (1.66–2.27); CV death; 1.87 (1.49–2.36)	11.9 years	Age, sex, study site, smoking, BMI, SBP, eGFR
Beska et al. (21)	UK	NSTE-ACS 294 (61.9)	80.5±4.8	<9.45 *vs.* ≥9.45 ng/mL	Death, ACS, stroke, revascularization, major bleeding	MACEs; 1.20 (0.72–2.0)	1.0 year	Age, sex, time of blood collection, hypertension, previous MI, congestive HF, Charlson index, Rockwood Frailty Score, hemoglobin, hs-CRP, vitamin D supplementation
Aleksova et al. (22)	Italy	Post-MI 1,081 (70.9)	66.7±11.5	≤20 *vs.* >20 ng/mL	Death, angina/MI, HF	MACEs; 1.3 (1.04–1.64)	2.2 years	Age, sex, season, multivessel disease, previous coronary events/revascularization, CRP, eGFR, LVEF, ACEI/ARB, β-blockers
Verdoia et al. (23)	Italy	CAD 705 (77.6)	67.3±10.8	<12.7 *vs.* ≥21.6 ng/mL	Death, MI, TVR	Total death; 3.6 (1.43–8.9); CV death; 4.28 (0.57–32); MACEs; 1.32 (1.07–1.63)	2.7 years	Age, sex, DM, CKD

HR, hazard ratio; RR, risk ratio; CI, confidence intervals; HF, heart failure; 25(OH) D, 25-hydroxyvitamin D; BMI, body mass index; DM, diabetes mellitus; LVEF, left ventricular ejection fraction; SBP, systolic blood pressure; DBP, diastolic blood pressure; BNP, B-type natriuretic peptide; NT-proBNP, N-terminal prohormone brain natriuretic peptide; LDL, low density lipoprotein cholesterol; HDL, high-density lipoprotein; TC, total cholesterol; TG, triglycerides; MI, myocardial infarction; AF, atrial fibrillation; eGFR, estimated glomerular filtration rate; CKD, chronic kidney disease; CAD, coronary artery disease; ACS, acute coronary syndrome; TVR, target vessel revascularization; APE, acute pulmonary edema; AKI, acute kidney injury; CRP, C-reactive protein; hs-CRP, high sensitivity C-reactive protein; ACEI, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers.

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Figure 2 provides a pooling risk summary of the association between 25-hydroxyvitamin D level and all-cause mortality. Under a random effect model meta-analysis, the pooled adjusted RR of all-cause mortality was 1.60 (95% CI 1.35–1.89) for the bottom vs. the reference top category of 25-hydroxyvitamin D level, having significant heterogeneity (I^2 =60.1%; p = 0.007). Leave-out one study sensitivity analysis did not alter the statistical significance of the original risk estimate. In addition, the value of blood 25-hydroxyvitamin D level in predicting all-cause mortality was consistently found in each named subgroup (Table 2). Publication bias was not detected in this outcome according to the Begg's test (p = 1.000), Egger's test (p = 0.567), and symmetrical funnel plot (Supplementary Figure S1).

Cardiovascular mortality

Six studies (9, 13, 16, 17, 20, 23) evaluated the value of 25-hydroxyvitamin D level in predicting cardiovascular mortality. Figure 3 shows a pooling risk estimate of the association between 25-hydroxyvitamin D level and cardiovascular mortality. Based on fixed-effect model meta-analysis, the pooled adjusted RR of cardiovascular mortality was 1.48 (95% CI 1.28–1.71) for the bottom vs. the reference top category of 25-hydroxyvitamin D level, and no significant heterogeneity was observed between studies (I^2 = 44.0%; p = 0.112). Sensitivity analysis confirmed the robustness of the originally pooling risk estimate. The Begg's test (p = 1.000), Egger's test (p = 0.567), and symmetrical funnel plot (Supplemental Figure S2) suggested a low likelihood of publication bias.

Major adverse cardiovascular events

Seven studies (10, 11, 14–16, 21–23) evaluated the value of 25-hydroxyvitamin D level in predicting MACEs. Figure 4 provides a pooling risk estimate of the association between d-dimer level and MACEs. A fixed-effect model meta-analysis suggested that the pooled adjusted RR of MACEs was 1.33 (95% CI 1.18–1.49) for the bottom vs. the reference top category of 25-hydroxyvitamin D level, and no significant heterogeneity (I^2 = 29.9%; p = 0.189) was observed between studies. Leave-out one study sensitivity analysis did not

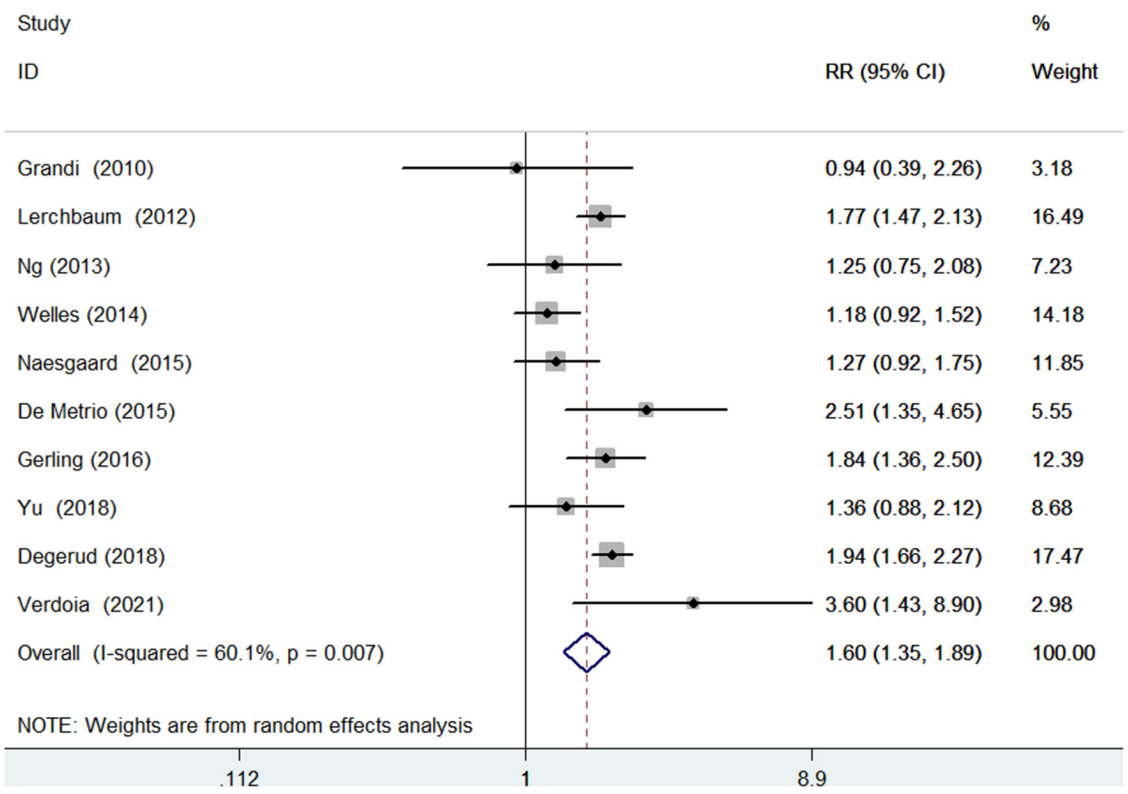

FIGURE 2
Forest plots showing pooled RR with 95% CI of all-cause mortality for the bottom vs. the reference top category of 25-hydroxyvitamin D level.

TABLE 2 Results of subgroup analysis on all-cause mortality.

Subgroup	Number of studies	Pooled RR	95% CI	Heterogeneity between studies
Types of CAD				
ACS	3	1.49	1.02–2.18	p=0.138; I^2 = 49.5%
All CAD	7	1.64	1.35–1.99	p=0.011; I^2 = 63.9%
Follow-up duration				
≥3 years	7	1.54	1.29–1.84	p=0.010; I^2 =64.4%
<3 years	3	2.08	1.12–3.86	p=0.073; I^2 =61.8%
Sample sizes				
≥1000	6	1.74	1.52–1.99	p=0.258; I^2 =23.4%
<1000	4	1.62	1.10–2.40	p=0.023; I^2 = 68.5%
Publication time				
Before 2015	6	1.43	1.14–1.81	p=0.037; I^2 =57.7%
After 2015	4	1.86	1.53–2.25	p=0.241; I^2 =28.5%

CAD, coronary artery disease; ACS, acute coronary syndrome; RR, hazard ratio; CI, confidence intervals.

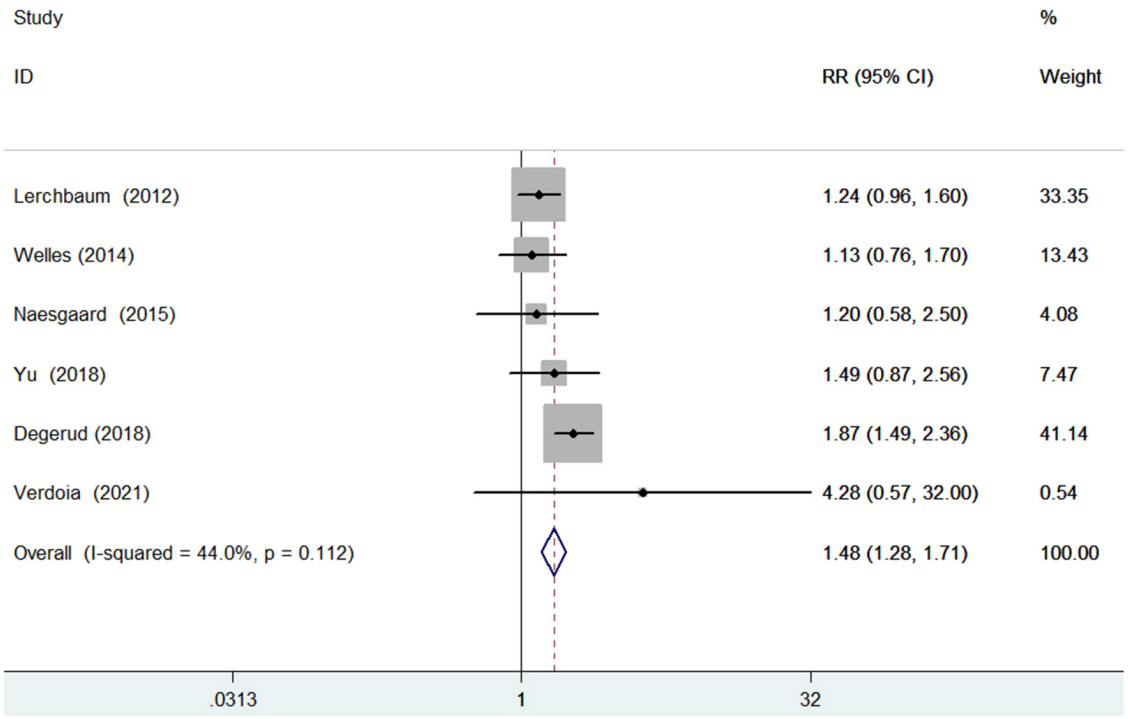

FIGURE 3
Forest plots showing pooled RR with 95% CI of cardiovascular mortality for the bottom vs. the reference top category of 25-hydroxyvitamin D level.

change the originally statistical significance of the pooling risk estimate. No evidence of publication bias was found according to the results of the Begg's test (p = 0.902), Egger's test (p = 0.428), and symmetrical funnel plot (Supplementary Figure S3).

GRADE certainty of evidence

All-cause mortality was grouped as low quality, cardiovascular mortality was classified high quality, and MACEs was classified moderate quality (Supplementary Table S2).

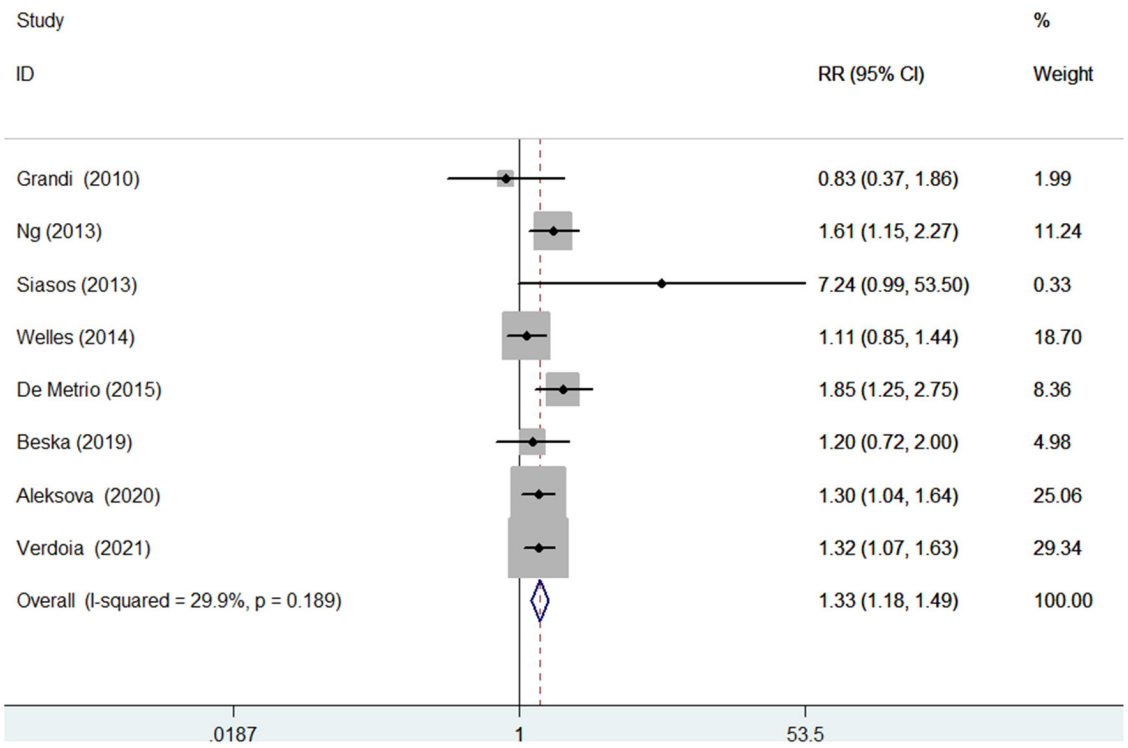

FIGURE 4
Forest plots showing pooled RR with 95% CI of major adverse cardiovascular events for the bottom vs. the reference top category of 25-hydroxyvitamin D level.

Discussion

The current meta-analysis focused on the predictive value of baseline 25-hydroxyvitamin D level in patients with CAD. This meta-analysis mainly found that 25-hydroxyvitamin D level at baseline was a significant predictor of MACEs, cardiovascular, and all-cause mortality in patients with CAD, even after adjusted multiple important confounders. Based on the comparison between the bottom and reference top 25-hydroxyvitamin D level, patients with the bottom 25-hydroxyvitamin D level conferred a 60 and 48%, higher risk of all-cause mortality and cardiovascular mortality, respectively. For the cardiovascular events, patients with the bottom category of 25-hydroxyvitamin D level had an approximately 33% higher risk of MACEs.

Several studies not meeting our criteria for inclusion also assessed the predictive value of 25-hydroxyvitamin D level in patients with CAD. The predictive role of 25-hydroxyvitamin D level was further supported *via* continuous variable analysis. In patients with stable angina pectoris, per 10 nmol/L decrease in 25-hydroxyvitamin D conferred a 9 and 10% higher risk of all-cause mortality and cardiovascular mortality, respectively (13). Based on Ludwigshafen Risk and Cardiovascular Health study, each standard deviation (SD) decrease in 25-hydroxyvitamin D level is associated with a 25% higher risk of all-cause mortality during 9.8 years follow-up in stable patients with CAD (24). Apart from the long-term outcomes, low 25-hydroxyvitamin D level is an independent predictor of cardiovascular mortality in patients with ACS (25). In patients with ST segment elevation myocardial infarction, low 25-hydroxyvitamin D level on admission is associated with high risk of no-reflow phenomenon (26).

The different types of CAD may affect the predictive value of 25-hydroxyvitamin D level. Based on subgroup analysis, the value of 25-hydroxyvitamin D level in the prediction of all-cause mortality was lower in patients with ACS (pooled RR 1.49) than in all CAD patients (pooled RR 1.64). Considering the lack of sufficient data, whether the predictive role of 25-hydroxyvitamin D level was affected by ACS subtypes was not determined. In addition, the predictive role of 25-hydroxyvitamin D level was weakened with the lengthening of follow-up in the subgroup analysis.

Several potential mechanisms may be implicated into the association of vitamin D deficiency with adverse outcomes in patients with CAD. First, low vitamin D can activate the activity of the renin-angiotensin-aldosterone system (27); Second, vitamin D deficiency may harm CAD patients by enhancing

inflammation (28, 29). Finally, low 25-hydroxyvitamin D level was closely related to the occurrence of no-reflow phenomenon after percutaneous coronary intervention (26, 30) and the severity of CAD (31).

A recent meta-analysis of four randomized clinical trials suggested that vitamin D supplementation is associated with improvements in diastolic blood pressure and parathyroid hormone in patients with CAD having vitamin D deficiency (32). However, survival and cardiovascular events were not assessed in this meta-analysis. Based on our meta-analysis, CAD patients with low blood 25-hydroxyvitamin D level should be identified as high-risk group and be closely monitored. Future randomized controlled trials are required to demonstrate whether vitamin D supplementation could improve the prognosis of patients with CAD.

Several potential limitations should be addressed in this meta-analysis. Firstly, blood 25-hydroxyvitamin D level was only detected once rather than dynamic measurement, possibly causing classification bias. Secondly, the cut-off values of lower 25-hydroxyvitamin D level, which were used for predicting adverse outcomes, varied across studies, thus making it hard for clinicians to identify patients that need supplementation of vitamin D. Thirdly, significant heterogeneity was found for all-cause mortality. The different cut-off values of low 25-hydroxyvitamin D level, types of the CAD, or length of follow-up may contribute to the existing heterogeneity. Fourthly, this meta-analysis did not analyze the predictive role of 25-hydroxyvitamin D level by continuous data analysis because of the lack of sufficient data. Fifth, when a U-shaped association of 25-hydroxyvitamin D level with worse outcomes is observed (13, 33), the selection of the bottom 25-hydroxyvitamin D level as the reference may have led to underestimation of the actual risk summary. Finally, blood level of 25-hydroxyvitamin D is strongly correlated with time spent outdoors. The lack of adjusting season or time spent outdoors may have affected the pooling risk estimate.

Conclusion

Low 25-hydroxyvitamin D level may be an independent predictor of MACEs, cardiovascular and all-cause mortality in patients with CAD. Baseline 25-hydroxyvitamin D level may provide important prognostic information in CAD patients.

Data availability statement

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

Author contributions

Study conception/design and interpretation of data: YF and XW. Literature search, data extraction, and quality assessment: HZ and PW. Statistical analysis: YJ and YS. Writing the manuscript: HZ. All the authors approved the version of the manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.984487/full#supplementary-material

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Association of circulating branched-chain amino acids with risk of moyamoya disease

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Objective: Branched-Chain Amino Acids (BCAAs) has been identified as a risk factor for circulatory disease. Nevertheless, the effects and mechanisms of BCAAs on the risk of moyamoya disease (MMD) remain unrecognized. Hence, we aimed to elucidate the association between circulating BCAAs and the risk of MMD and clinical subtypes.

Methods: We conducted a case-control study of 360 adult MMD patients and 89 matched healthy controls consecutively recruited between September 2020 and December 2021. Serum level of BCAAs was quantified by liquid chromatography-mass spectrometry. The associations between BCAAs and risk of MMD were evaluated.

Results: Increased level of serum BCAAs was observed in MMD patients ($P < 0.001$). After adjusting for traditional confounders, the elevated BCAAs level was significantly associated with the risk of MMD (Q4 vs. Q1: odds ratio, 3.10 [95% CI, 1.29–7.50]). The risk of subtypes in MMD also increased with each increment in the quartiles of BCAAs. Furthermore, BCAAs offered substantial improvement in risk reclassification and discrimination for MMD and subtypes.

Conclusion: Higher level of circulating BCAAs was associated with increased risk of MMD and clinical subtypes. This study will help to elucidate the pathogenesis of MMD, which may provide the support for facilitating the treatments and preventions.

KEYWORDS

moyamoya disease, branched-chain amino acids (BCAAs), metabolites, biomarkers, risk factors

Introduction

Moyamoya disease (MMD), characterized by progressive stenosis of distal portion of internal carotid arteries and abnormal collaterals at the base of brain, is recognized as the main cause of stroke in East Asians (1). MMD has been considered as a multifactorial disease, caused by genetic, immune, inflammation and other factors (2). Although the *RNF213* variants have been identified to be associated with angiopathy in MMD, the frequency of variants was quite low in China (2–4). Our recent study has demonstrated that traditional modifiable factors were related to the risk of MMD (5), while the well-known risk factors cannot fully account for the etiology of MMD.

Recently, progress in high-throughput multi-omics technologies has provided new insight into the pathogenesis of diseases (6). Circulating metabolites reveal the information of systemic alterations and disease mechanisms. and could act as biomarkers that accurately estimate the risk of stroke (6). Branched-Chain Amino Acids (BCAAs), consisting of leucine, isoleucine, and valine, is a compound of essential amino acids that regulates diverse functions, including cell growth, autophagy, and lipid metabolism (7). BCAAs mainly participates in biological activities by activating the mammalian target of rapamycin (mTOR) pathway. It has been shown to be associated with metabolic disorders, cardiovascular diseases and cancer (8–10). Despite few metabolomics studies have been performed in MMD patients (11, 12), the targeted outcomes and potential mechanisms of BCAAs in MMD was hitherto unrecognized.

In the current study, we enrolled a large population of MMD patients and healthy controls (HCs) and analyzed the characteristics of circulating BCAAs in MMD. We aimed to demonstrate the association of the serum BCAAs level with the risk of MMD and clinical subtypes. This work will help to identify novel biomarkers, and elucidate the pathogenesis of MMD, which may provide the support for improving the interventions and preventions.

Materials and methods

Study design and participants

In this study, we prospectively recruited adult MMD patients at the Department of Neurosurgery, Beijing Tiantan Hospital from September 2020 to December 2021. Eligible patients were age 18–60 years, unilateral and bilateral MMD diagnosed by digital subtraction angiography (DSA) following the Japanese guidelines (1). Patients were excluded if they refused to participate in the study or had inadequate Liquid chromatography-mass spectrometry (LC-MS) data of BCAAs. Finally, 360 adult patients with complete measurement of BCAAs were enrolled in the study, consisting of 114 patients of transient ischemic attack (TIA)-type MMD, 145 patients of infarction-type MMD, and 101 patients of hemorrhagic-type MMD (Figure 1). Besides, 89 age-matched HCs who underwent routine physical examination were recruited. The HCs generally had no comorbidities. The study was approved by the Ethics Committee of Beijing Tiantan Hospital. Informed consents were obtained from all participants.

Baseline data collection and laboratory assessment

Demographic data (age and sex), history of risk factors (hypertension, diabetes mellitus, hyperlipidemia, cigarette smoking, and alcohol drinking), clinical features (heart rate, blood pressure, body mass index [BMI]), clinical manifestations (TIA, infarction, and hemorrhage) were collected *via* chart views.

Fasting blood samples were collected after admission from all participants. Routine and biochemical blood tests were conducted to measure the levels of potential circulating biomarkers: white blood cell (WBC) count, lymphocyte (LY) count, neutrophil count, monocyte count, red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT) count, glucose, creatinine, uric acid, triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), apolipoprotein A_1 ($ApoA_1$), apolipoprotein B (ApoB), and homocysteine (Hcy). Hcy $\geq$ 15.0 μmol/L was considered as hyperhomocysteinemia (HHcy). Besides, peripheral inflammatory biomarkers including neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR), systemic immune-inflammation index (SII) (PLT count × neutrophil count/LY count), and monocyte-to-HDL cholesterol ratio (MHR) were calculated. Serum samples were also collected at baseline from all individuals. The serum samples were stored at −80 °C in the Central Laboratory of Beijing Tiantan Hospital. We used LC-MS techniques to quantitatively profile the serum metabolites of BCAAs. The level of BCAAs was calculated as the sum of levels of leucine, isoleucine, and valine.

Statistical analysis

All statistics analyses were performed using SPSS version 26.0 (IBM Corporation, Armonk, NY, USA) and R version 4.1.2 (R Development Core Team). Baseline characteristics were presented and compared between MMD patients and HCs. The categorical variables were presented as frequencies, and

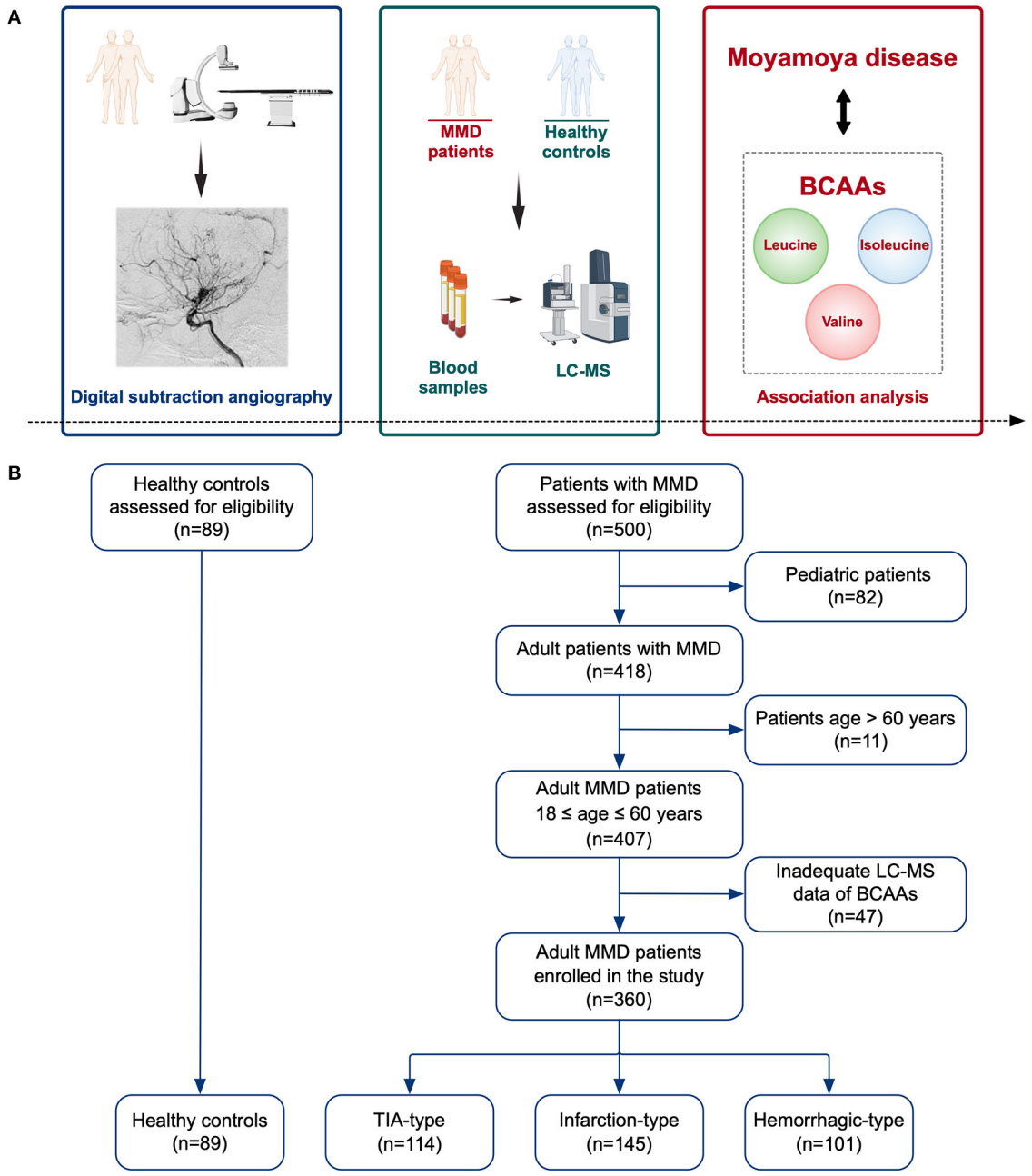

FIGURE 1
Schematic diagram of the study. **(A)** Illustration of the study methods and purpose. **(B)** Flow chart of the study participants. MMD, moyamoya disease; LC-MS, liquid chromatography-mass spectrometry; BCAAs, branched-chain amino acids; TIA, transient ischemic attack.

continuous variables were expressed as mean with standard deviation (SD) or median with interquartile range (IQR). Categorical data were compared using the χ^2 test or Fisher exact test between groups, and continuous data were compared with two-tailed Student *t*-tests or Mann-Whitney *U* tests. One-way ANOVA or Kruskal-Wallis test was used to test the trend for continuous variables across BCAAs, and the Cochran-Armitage trend χ^2 test was conducted for categorical variables. The logistic regression models were performed to identify the independent factors for MMD and its subtypes. The crude model was the unadjusted regression model of BCAAs. The model 1 adjusted for covariates including age and sex. The model 2 further adjusted for BMI, WBC count, neutrophil count, glucose, TG, TC, HDL-C, LDL-C, APO-A_1, Hcy, NLR,

TABLE 1 Baseline characteristics of HCs and MMD patients.

Variables	HCs ($n = 89$)	TIA ($n = 114$)	Infarction ($n = 145$)	Hemorrhage ($n = 101$)	*P* value
Age, y, mean ± SD	39.81 ± 11.57	40.51 ± 10.27	42.33 ± 9.96	41.78 ± 10.61	0.260
Sex, Female/Male	1.41:1	1.43:1	1.10:1	1.97:1	0.190
History of risk factors, n (%)					
Hypertension	0 (0)	35 (30.7)	67 (46.2)	29 (28.7)	< 0.001*
Diabetes mellitus	0 (0)	16 (14.0)	39 (26.9)	4 (4.0)	< 0.001*
Hyperlipidemia	0 (0)	17 (14.9)	28 (19.3)	9 (8.9)	< 0.001*
Cigarette smoking	2 (2.2)	20 (17.5)	34 (23.4)	17 (16.8)	< 0.001*
Alcohol drinking	0 (0)	14 (12.3)	20 (13.8)	8 (7.9)	0.003*
Clinical features, mean ± SD					
Heart rate, bpm	77.79 ± 9.73	78.77 ± 6.41	77.83 ± 6.72	79.30 ± 5.92	0.332
SBP, mmHg	123.64 ± 11.77	132.81 ± 12.09	134.16 ± 13.40	129.16 ± 12.25	< 0.001*
DBP, mmHg	78.46 ± 8.35	81.55 ± 9.04	83.16 ± 9.68	80.32 ± 8.96	0.001*
BMI, kg/m^2	23.96 ± 3.39	25.87 ± 4.88	25.96 ± 4.37	24.32 ± 4.11	< 0.001*
Laboratory results, median ± IQR					
WBC count, $10^9/L$	6.03 ± 1.88	6.93 ± 2.57	7.02 ± 2.39	6.43 ± 2.46	< 0.001*
LY count, $10^9/L$	1.91 ± 0.71	2.08 ± 0.79	1.97 ± 0.80	1.72 ± 0.84	< 0.001*
Neutrophil count, $10^9/L$	3.44 ± 1.62	4.24 ± 1.98	4.35 ± 1.73	3.88 ± 1.86	< 0.001*
Monocyte count, $10^9/L$	0.35 ± 0.14	0.36 ± 0.18	0.36 ± 0.16	0.34 ± 0.17	0.289
RBC count, $10^{12}/L$	4.69 ± 0.65	4.64 ± 0.72	4.68 ± 0.71	4.60 ± 0.59	0.306
HGB, g/L	144.00 ± 19.00	141.50 ± 22.00	143.00 ± 27.00	137.00 ± 22.00	0.042*
HCT, L/L	0.42 ± 0.05	0.41 ± 0.06	0.41 ± 0.08	0.41 ± 0.05	0.041*
MCV, fL	90.10 ± 5.10	90.10 ± 5.90	89.20 ± 5.30	90.00 ± 5.40	0.759
MCH, pg	30.70 ± 2.00	30.95 ± 2.00	30.70 ± 2.50	30.80 ± 2.30	0.648
MCHC, g/L	341.00 ± 15.00	342.50 ± 12.00	344.00 ± 13.00	339.00 ± 12.00	0.039*
PLT count, $10^9/L$	233.00 ± 87.00	249.50 ± 72.00	250.00 ± 80.00	244.00 ± 76.00	0.304
Fasting glucose, mmol/L	5.04 ± 0.62	5.12 ± 1.01	5.22 ± 1.42	4.91 ± 0.67	< 0.001*
Creatinine, μmol/L	57.70 ± 19.20	53.95 ± 20.05	57.80 ± 20.80	53.10 ± 21.75	0.271
Uric acid, μmol/L	310.60 ± 103.50	313.25 ± 119.60	312.00 ± 118.30	292.90 ± 113.30	0.135
TG, mmol/L	0.87 ± 0.62	1.24 ± 0.91	1.20 ± 0.75	1.13 ± 0.85	< 0.001*
TC, mmol/L	4.62 ± 0.98	4.23 ± 1.40	3.93 ± 1.26	4.35 ± 1.14	< 0.001*
HDL-C, mmol/L	1.53 ± 0.41	1.31 ± 0.42	1.25 ± 0.31	1.34 ± 0.35	< 0.001*
LDL-C, mmol/L	2.69 ± 0.87	2.36 ± 1.20	2.15 ± 1.04	2.55 ± 1.07	< 0.001*
$ApoA_1$, g/L	1.39 ± 0.28	1.32 ± 0.31	1.25 ± 0.33	1.30 ± 0.29	0.001*
ApoB, g/L	0.77 ± 0.27	0.84 ± 0.28	0.81 ± 0.28	0.82 ± 0.31	0.159
Hcy, μmol/L	10.62 ± 3.97	11.11 ± 6.87	12.29 ± 6.12	11.90 ± 4.97	0.003*
HHcy, n (%)	8 (9.0)	29 (25.4)	40 (27.6)	22 (21.8)	0.007*
NLR	1.79 ± 0.87	2.01 ± 0.87	2.15 ± 1.18	2.31 ± 1.38	0.001*
MLR	0.19 ± 0.10	0.17 ± 0.09	0.19 ± 0.10	0.20 ± 0.11	0.094
PLR	126.27 ± 76.03	120.74 ± 51.88	127.39 ± 52.94	144.74 ± 74.85	0.011*
SII, $10^9/L$	414.33 ± 289.40	508.17 ± 289.65	569.37 ± 414.91	543.60 ± 448.84	0.001*
MHR	0.23 ± 0.11	0.28 ± 0.18	0.29 ± 0.17	0.24 ± 0.16	< 0.001*

HCs, healthy controls; MMD, moyamoya disease; TIA, transient ischemic attack; SD, standard deviation; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; IQR, interquartile range; WBC, white blood cell; LY, lymphocyte; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; $ApoA_1$, apolipoprotein A_1; ApoB, apolipoprotein B; Hcy, homocysteine; HHcy, hyperhomocysteinemia; NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index; MHR, monocyte-to-HDL cholesterol ratio.
*$P < 0.05$, significant difference.

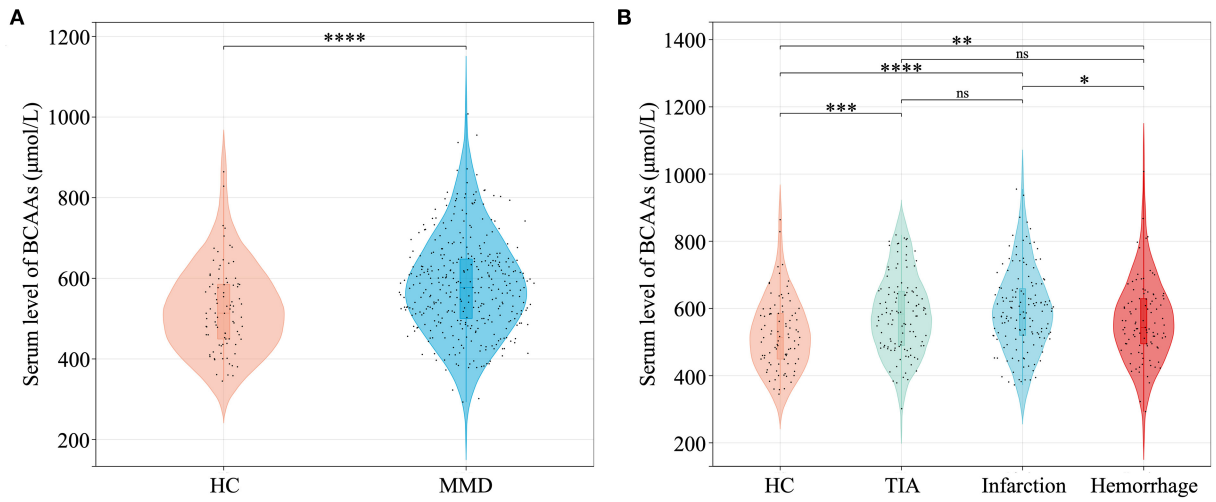

FIGURE 2
Quantitative analysis of serum BCAAs level between MMD patients and HCs. **(A)** Comparison of BCAAs level between HCs and MMD patients. **(B)** Comparison of BCAAs level between HCs and MMD subtypes. BCAAs, branched-chain amino acids; HC, healthy control; MMD, moyamoya disease; TIA, transient ischemic attack; ns, not significant. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

SII, and MHR. Furthermore, we evaluated the predictive performance of models for the risk of MMD and its subtypes by establishing receiver-operating characteristic (ROC) curves and calculated the area under the curve (AUC). Moreover, the performance of BCAAs in the basic model built based on traditional risk factors were assessed. The net reclassification index (NRI) and integrated discrimination improvement (IDI) were calculated in risk classification by adding BCAAs to the basic model. $P < 0.05$ was considered statistical significance.

Results

A total of 360 MMD patients (114 cases with TIA, 145 cases with infarction, and 101 cases with hemorrhage) and 89 matched HCs were included in the study.

Baseline characteristics and BCAAs of MMD patients and HCs

Baseline characteristics of MMD cases and HCs were shown in Table 1. History of risk factors for stroke (hypertension, diabetes mellitus, hyperlipidemia, cigarette smoking, and alcohol drinking) were more prevalent in MMD patients ($P < 0.05$ for all). In MMD patients, the levels of systolic blood pressure (SBP), diastolic blood pressure (DBP), and BMI were significantly higher than in HCs. Patients in groups of MMD subtypes had a higher level of WBC count, neutrophil count, glucose, TG, Hcy, NLR, SII, and MHR than in HC group ($P < 0.05$ for all). Levels of laboratory results including LY count, HGB, HCT, MCHC, TC, HDL-C, LDL-C, ApoA$_1$, and PLR were significantly different between groups ($P < 0.05$ for all). In addition, patients with MMD and its subtypes had a significantly higher level of BCAAs than that of HCs ($P < 0.05$ for all), while patients with hemorrhagic-type MMD had a lower level of BCAAs than that of infarction-type ($P < 0.05$) (Figure 2). The significant differences of individual BCAAs (leucine, isoleucine, and valine) between MMD patients and HCs were similar to the total BCAAs (Supplementary Figure S1).

Characteristics of MMD patients and HCs according to BCAAs quartiles

Clinical characteristics of MMD patients and HCs according to the BCAAs quartiles were shown in Table 2. Patients with higher level of BCAAs tended to be male; have risk factors of hypertension, diabetes mellitus, hyperlipidemia, cigarette smoking, and alcohol drinking; have higher levels of blood pressure, BMI, WBC count, LY count, RBC, HGB, HCT, glucose, creatinine, uric acid, TG, ApoB, and MHR ($P < 0.05$ for all). Characteristics according to the quartiles of individual BCAAs were summarized in Supplementary Table S1–S3.

Association of BCAAs with the risk of MMD and its subtypes

Figure 3 showed the associations of serum total BCAAs with the risk of MMD and its subtypes. The proportion of the

TABLE 2 Characteristics of HCs and MMD patients according to BCAAs quartiles.

Variables	Total ($N = 449$)	BCAAs quartiles†, μmol/L				
		Q1 ($n = 112$)	Q2 ($n = 112$)	Q3 ($n = 112$)	Q4 ($n = 113$)	*P* for Trend
Age, y, mean ± SD	41.24 ± 10.53	40.18 ± 10.85	41.79 ± 10.61	41.38 ± 10.13	41.63 ± 10.60	0.378
Sex, male (%)	187 (41.6)	16 (14.3)	37 (33.0)	61 (54.5)	73 (64.6)	< 0.001*
History of risk factors, n (%)						
Hypertension	131 (29.2)	22 (19.6)	26 (23.2)	40 (35.7)	43 (38.1)	< 0.001*
Diabetes mellitus	59 (13.1)	4 (3.6)	12 (10.7)	10 (8.9)	33 (29.2)	< 0.001*
Hyperlipidemia	54 (12.0)	9 (8.0)	7 (6.3)	10 (8.9)	28 (24.8)	< 0.001*
Cigarette smoking	73 (16.3)	5 (4.5)	10 (8.9)	20 (17.9)	38 (33.6)	< 0.001*
Alcohol drinking	42 (9.4)	1 (0.9)	6 (5.4)	14 (12.5)	21 (18.6)	< 0.001*
Clinical features, mean ± SD						
Heart rate, bpm	78.39 ± 7.19	77.93 ± 6.58	77.21 ± 7.14	79.9 ± 7.92	78.52 ± 6.87	0.139
SBP, mmHg	130.61 ± 13.07	127.77 ± 11.84	129.36 ± 12.10	133.16 ± 14.13	132.12 ± 13.51	0.002*
DBP, mmHg	81.18 ± 9.23	80.43 ± 9.01	80.06 ± 8.80	82.06 ± 10.52	82.16 ± 8.41	0.065
BMI, kg/m^2	25.17 ± 4.35	23.40 ± 3.72	24.84 ± 4.30	25.96 ± 4.53	26.47 ± 4.21	< 0.001*
Laboratory results, median ± IQR						
WBC count, 10^9/L	6.63 ± 2.27	6.20 ± 2.10	6.51 ± 2.67	6.86 ± 2.53	6.94 ± 2.10	< 0.001*
LY count, 10^9/L	1.91 ± 0.82	1.80 ± 0.75	1.82 ± 0.80	1.95 ± 0.80	2.19 ± 0.74	< 0.001*
Neutrophil count, 10^9/L	4.07 ± 1.85	3.63 ± 1.58	4.01 ± 1.86	4.29 ± 2.09	4.25 ± 1.82	0.008*
Monocyte count, 10^9/L	0.35 ± 0.16	0.32 ± 0.14	0.34 ± 0.17	0.37 ± 0.13	0.35 ± 0.16	0.014*
RBC, 10^{12}/L	4.64 ± 0.67	4.41 ± 0.58	4.65 ± 0.73	4.79 ± 0.59	4.86 ± 0.63	< 0.001*
HGB, g/L	141.00 ± 24.00	134.50 ± 15.00	141.00 ± 23.00	147.00 ± 25.00	149.00 ± 22.00	< 0.001*
HCT, L/L	0.41 ± 0.07	0.39 ± 0.05	0.41 ± 0.07	0.43 ± 0.07	0.43 ± 0.06	< 0.001*
MCV, fL	90.00 ± 5.40	90.25 ± 6.20	89.75 ± 5.40	89.65 ± 5.60	89.40 ± 5.00	0.726
MCH, pg	30.80 ± 2.30	30.70 ± 2.70	30.90 ± 1.90	30.80 ± 2.20	30.70 ± 2.10	0.304
MCHC, g/L	342.00 ± 13.00	339.50 ± 12.00	343.00 ± 15.00	343.00 ± 15.00	344.00 ± 12.00	< 0.001*
PLT count, 10^9/L	246.00 ± 79.00	249.00 ± 79.00	243.50 ± 87.00	248.50 ± 68.00	238.00 ± 84.00	0.644
Fasting glucose, mmol/L	5.09 ± 0.90	4.95 ± 0.77	5.04 ± 0.80	5.12 ± 0.86	5.27 ± 1.50	< 0.001*
Creatinine, μmol/L	55.60 ± 20.55	49.65 ± 14.08	53.90 ± 19.02	59.80 ± 19.00	62.90 ± 20.70	< 0.001*
Uric acid, μmol/L	307.70 ± 115.60	262.15 ± 89.30	292.70 ± 97.80	326.75 ± 99.80	365.50 ± 124.10	< 0.001*
TG, mmol/L	1.15 ± 0.81	0.90 ± 0.53	1.05 ± 0.78	1.18 ± 0.75	1.44 ± 0.95	< 0.001*
TC, mmol/L	4.26 ± 1.21	4.34 ± 1.17	4.17 ± 1.36	4.26 ± 1.08	4.31 ± 1.40	0.764
HDL-C, mmol/L	1.34 ± 0.39	1.48 ± 0.45	1.34 ± 0.37	1.33 ± 0.33	1.22 ± 0.35	< 0.001*
LDL-C, mmol/L	2.41 ± 1.13	2.40 ± 0.99	2.41 ± 1.24	2.42 ± 0.94	2.49 ± 1.23	0.806
$ApoA_1$, g/L	1.30 ± 0.29	1.39 ± 0.31	1.33 ± 0.26	1.28 ± 0.27	1.25 ± 0.34	< 0.001*
ApoB, g/L	0.82 ± 0.27	0.76 ± 0.24	0.76 ± 0.29	0.86 ± 0.24	0.86 ± 0.31	0.002*
Hcy, μmol/L	11.43 ± 5.16	10.63 ± 4.54	10.66 ± 4.68	11.85 ± 4.83	12.78 ± 6.21	< 0.001*
HHcy, n (%)	99 (22.0)	20 (17.9)	18 (16.1)	25 (22.3)	36 (31.9)	0.006*
NLR	2.06 ± 1.15	2.01 ± 1.19	2.06 ± 1.18	2.14 ± 1.33	2.01 ± 1.04	0.652
MLR	0.19 ± 0.10	0.19 ± 0.10	0.19 ± 0.09	0.20 ± 0.10	0.18 ± 0.10	0.397
PLR	127.39 ± 58.01	132.14 ± 68.32	137.51 ± 69.92	127.29 ± 54.66	114.72 ± 55.28	0.001*
SII, 10^9/L	505.35 ± 379.14	493.37 ± 439.04	526.63 ± 381.10	577.35 ± 333.42	476.38 ± 330.73	0.851
MHR	0.26 ± 0.16	0.21 ± 0.11	0.25 ± 0.14	0.29 ± 0.16	0.30 ± 0.18	< 0.001*

HCs, healthy controls; MMD, moyamoya disease; BCAAs, branched-chain amino acids; SD, standard deviation; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; IQR, interquartile range; WBC, white blood cell; LY, lymphocyte; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; $ApoA_1$, apolipoprotein A_1; ApoB, apolipoprotein B; Hcy, homocysteine; HHcy, hyperhomocysteinemia; NLR, neutrophil-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index; MHR, monocyte-to-HDL cholesterol ratio.

†Serum levels of BCAAs in quartiles: Q1, < 488.9 μmol/L; Q2, 488.9-564.0 μmol/L; Q3, 564.0-639.7 μmol/L; and Q4, ≥ 639.7 μmol/L.

*P<0.05, significant difference.

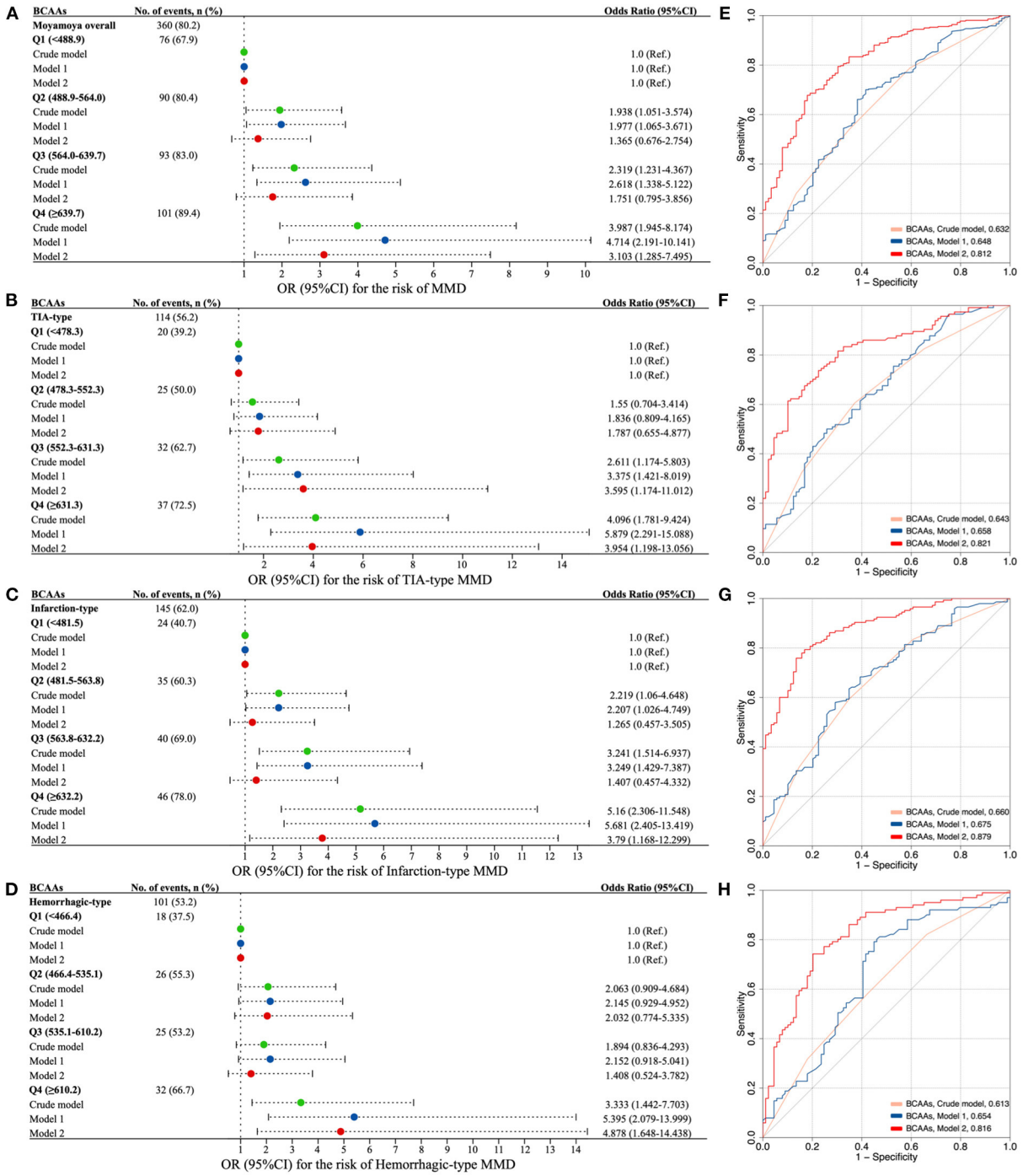

FIGURE 3
The association of circulating BCAAs level with the risk of MMD and clinical subtypes. **(A–D)** Forest plots for the association of BCAAs with MMD **(A)** and subtypes [**(B)** TIA-type; **(C)** Infarction-type; **(D)** Hemorrhagic-type]. E-H. ROC curves with AUC of different models for the risk of MMD **(E)** and subtypes [**(F)**, TIA-type; **(G)** Infarction-type; **(H)** Hemorrhagic-type]. Model 1, adjusted for age and sex. Model 2, further adjusted for BMI, WBC count, neutrophil count, glucose, TG, TC, HDL-C, LDL-C, APO-A_1, Hcy, NLR, SII, and MHR. BCAAs, branched-chain amino acids; OR, odds ratio; CI, confidence interval; MMD, moyamoya disease; TIA, transient ischemic attack.

presence of MMD in the quartiles of BCAAs increased from 1st to 4th quartiles. After adjusting for age and sex, subjects in the second to last quartiles (Q2–Q4) of BCAAs were associated with a higher risk of MMD than those in the first quartile

TABLE 3 Performance of models with BCAAs to predict the risk of MMD and its subtypes.

Variables†	NRI, continuous		IDI	
	Estimate (95%CI), %	*P* value	Estimate (95%CI), %	*P* value
Moyamoya overall				
Basic model	Ref.		Ref.	
Basic model + BCAAs quartiles	35.4 (13.2–57.6)	0.002*	1.7 (0.3–3.2)	0.022*
Basic model + BCAAs continuous	35.2 (12.4–58.0)	0.002*	2.1 (0.5–3.7)	0.011*
TIA-type				
Basic model	Ref.		Ref.	
Basic model + BCAAs quartiles	38.7 (11.5–65.9)	0.005*	2.6 (0.3–4.9)	0.027*
Basic model + BCAAs continuous	41.5 (14.5–68.6)	0.003*	2.4 (0.2–4.7)	0.036*
Infarction-type				
Basic model	Ref.		Ref.	
Basic model + BCAAs quartiles	37.6 (12.9–62.4)	0.003*	2.0 (0.1–3.8)	0.038*
Basic model + BCAAs continuous	24.1 (−1.9–50.1)	0.069	2.2 (0.3–4.1)	0.023*
Hemorrhagic-type				
Basic model	Ref.		Ref.	
Basic model + BCAAs quartiles	48.2 (20.7–75.7)	< 0.001*	4.6 (1.7–7.5)	0.002*
Basic model + BCAAs continuous	40.6 (12.8–68.5)	0.004*	2.7 (0.6–4.8)	0.013*

BCAAs, branched-chain amino acids; MMD, moyamoya disease; NRI, net reclassification index; IDI, integrated discrimination improvement; CI, confidence interval; TIA, transient ischemic attack.

†Basic model included age, sex, BMI, WBC count, Neutrophil count, glucose, TG, TC, HDL-C, LDL-C, $ApoA_1$, Hcy, NLR, SII, and MHR.

*P<0.05, significant difference.

(Q1). After additionally adjusting for covariates of BMI, WBC count, neutrophil count, glucose, TG, TC, HDL-C, LDL-C, APO-A_1, Hcy, NLR, SII, and MHR, cases in Q4 of BCAAs were significantly associated with a higher risk of MMD than those in Q1 (odds ratio [OR] 3.10, 95% confidence interval [CI] 1.29-7.50, $P = 0.012$). The ROC curves with AUC of models for the occurrence of MMD were constructed in Figure 3. In contrast to the Crude model and Model 1 (AUC: 0.632, 0.648, respectively), the Model 2 yielded to a prominent improvement in the predictive value (AUC: 0.812).

Consistently, the risk of three subtypes of MMD increased with each increment in the quartiles of BCAAs (Figure 3). Q3 and Q4 of BCAAs were strongly associated with the occurrence TIA-type MMD compared with Q1 in Model 2 (OR 3.60, 95% CI 1.17-11.01, $P = 0.025$; OR 3.95, 95% CI 1.20–13.06, P = 0.024, respectively). Q4 of BCAAs was significantly associated with the risk of infarction-type and hemorrhagic-type MMD compared with Q1 in Model 2 (OR 3.79, 95% CI 1.17–12.30, $P = 0.027$; OR 4.88, 95% CI 1.65–14.44, $P = 0.004$, respectively). In contrast to the Crude model and Model 1, the Model 2 consistently showed prominent improvements in the predictive values of subtypes of TIA, infarction, and hemorrhagic MMD (AUC: 0.821, 0.879, 0.816, respectively) (Figure 3).

Besides, the risk of MMD and its subtypes increased with each increment in the quartiles of individual BCAA (Supplementary Figure S2). Similarly, Q4 of leucine, isoleucine, and valine were markedly associated with the risk of MMD and its subtypes compared with Q1 in Model 2, respectively. The ROC curves with AUC of individual BCAAs models for the presence of MMD and subtypes were constructed in Supplementary Figure S3. Analogously, the predictive values of MMD and its subtypes of Model 2 were all noticeably enhanced, compared with the Crude model and Model 1.

Improvement in the prediction models for the risk of MMD and its subtypes

We compared the performance of different models for predicting the risk of MMD and its subtypes (Table 3). The addition of BCAAs to the basic model moderately improved the performance verified by NRI. The NRI of BCAAs in quartiles for the presence of MMD was 35.4% (95%CI 13.2–57.6%). A similar performance for predicting the risk of MMD was validated for BCAAs and the basic model. The IDI of BCAAs in quartiles for the occurrence of MMD was 1.7% (95%CI 0.3–3.2%). In addition, the predictive performance of BCAAs in MMD subtypes evaluated by NRI and IDI was consistent. Significant improvements were also observed after the addition of BCAAs to the basic model by NRI and IDI.

Discussion

In this large case-control study of 449 participants, we firstly investigated the association of serum metabolite of BCAAs with the risk of MMD and clinical subtypes. We identified that the serum level of BCAAs was significantly higher in MMD patients than in HCs. The elevated level of BCAAs was strongly associated with increased risks of MMD and subtypes. Collectively, our findings outlined the crucial relevance of increasing serum BCAAs with the risk of MMD.

Amino acids are important nutrients for humans. As the precursors of proteins, amino acids participate in various life activities and metabolism (13). BCAAs is an essential amino acid that regulates cell growth, autophagy, neurotransmitter synthesis, carbohydrate and lipid metabolism (14). The excessive intake of amino acids and metabolic disorders of BCAAs would result in the accumulation of serum BCAAs, which have been verified in animal experiments and clinical studies (15, 16). Therefore, the increment of BCAAs is often considered as the evidence of metabolic disorders. Current studies have confirmed that BCAAs is associated with diabetes, obesity, insulin resistance and other diseases (7). The metabolites of BCAA pathway accumulate as the risk factor for insulin resistance, and the association was identified in type 2 diabetes and cardiovascular disease (7, 17). Previous studies have shown that abnormal metabolism of BCAAs was associated with a variety of cardio-cerebrovascular diseases, including coronary heart disease, heart failure, and carotid artery stenosis (18–20). Few metabolomics studies have been conducted in patients with MMD (11, 12). The studies consistently demonstrated that the level of serum valine in MMD patients was significantly lower than that in HCs, while the result of isoleucine was quite the opposite. It seems possible that the inverse result of valine is due to the different technology of mass spectrometry used for the serum metabolome. In general, our findings indicated that the altered levels of BCAAs could be linked to MMD.

As nutrient signaling molecules, BCAAs mainly transduce the mTOR pathway (8). BCAAs, especially leucine, participate in many biological activities by activating mTOR. One study showed that isoleucine in mitochondria was involved in the vascular oxidative stress, leading to the endothelial dysfunction (21). MMD is a multifactorial disease, affected by genetic and environmental factors (22). Various risk factors can cause an elevation of free radicals, continuingly producing excessive reactive oxygen species (ROS) that result in cellular damage (23). Jung et al. found that the oxidative stress level of endothelial colony-forming cells (ECFCs) in patients with MMD was significantly higher than in HCs (24). The angiogenesis capacity of endothelial cells in MMD patients was increased by administrating the ROS scavengers. Endothelial cells are prone to have oxidative stress response and generate various biologically active substances by exposed to the microenvironment in plasma, causing a functional impairment in endothelial cells through various pathways. Therefore, we hypothesized that high concentration of BCAAs could induce the activation of mTOR, resulting in oxidative stress, mitochondrial dysfunction, and apoptosis, which may be one of the mechanisms involved in the pathogenesis of MMD.

BCAAs may generate a chronic inflammatory response by increasing the expression of pro-inflammatory cytokines (e.g., TNF-α and IL-6), leading to changes in endothelial and smooth muscle cell phenotypes, and thereby producing the pathological conditions. Some studies have identified that isoleucine was positively correlated with IL-6, endotoxin and oxLDL, (25) while leucine was positively related to TNF-α and HOMA-IR (26). The supplementation of BCAAs in blood monocytes stimulated redox through NADPH oxidase and the generation of ROS throughout the mitochondria activation of NF-κB, resulting in the release of pro-inflammatory factors (27). Recent studies have found that the expression levels of periphery inflammatory factors in MMD were higher than those in HCs, including TNF-α, IL1-β, IL-6, etc. (28, 29). The microenvironment formed by abnormally secreted inflammatory factors may promote the proliferation and angiogenesis of cells in affected vessels (30). The occurrence of chronic inflammation may generate the vascular damage and the formation of micro-vessels, leading to the hemorrhage and infarction (31). In conclusion, BCAAs may have an impact on endothelial and smooth muscle cells through oxidative stress and inflammatory responses, and thereby develop the phenotype of moyamoya vasculopathy.

Our study showed that several factors were associated with the quartiles of BCAAs. There were growing trends of diabetes and BMI along with the level of BCAAs. It has been verified that BCAAs was related to the metabolic disorders, including diabetes and obesity (7). Although diabetes and obesity are comorbidities in patients with MMD (2), BCAAs is still the independent risk factor after adjustment for glucose and BMI in the multivariate regression models. In addition, we confirmed that the TG level were positively correlated with BCAAs. BCAAs exert an influence on the lipid metabolism (32). It is logically consistent with our previous case-control study which has shown that the dyslipidemia was linked to MMD (5). We also detected that the elevated levels of hematologic indicators (RBC, HGB, HCT) were in parallel with the increment of BCAAs. Recent study has shown that BCAAs was related to the iron metabolism, and the circulating BCAAs was decreased in patients with anemia (33). Therefore, we concluded that the disorder of BCAAs may serve as a bridge connecting multiple metabolic abnormalities and diseases.

In this study, total and individual BCAAs were all analyzed. The differences among three individual BCAAs tended to be similar. Although BCAAs is a compound of three

substances, each BCAA may have distinct effects. Isoleucine and valine, while not leucine, mediated the metabolic health (34). It is only isoleucine, not leucine and valine, that improved the brain perfusion (35). In addition, we found that circulating BCAAs in MMD patients is higher than that of HCs. The consequence of significant difference is the initial trigger of onset or the result of second strike cannot be clarified based on the current case-control study. Hence, it is vital to demonstrate the function of each BCAA metabolite in MMD.

Several limitations should be considered in this study. First, this is a single-center study with relatively small sample size. Although the potential bias was inevitable, this is the largest study to investigate the association of serum BCAAs and MMD. Second, the study was conducted in a Chinese population with adult MMD, the findings may not be generalized to the overall populations of MMD. Third, the information of patient-level diets was not included in the study. The pattern of diets may have an impact on the outcomes. Fourth, the results of serum metabolites are affected by many factors. Although the confounders have been adjusted in the regression models, we can only demonstrate the association of serum BCAAs with the risk of MMD. Further *in vitro* or *in vivo* experiments, and larger prospective cohort studies with follow-up outcomes are warranted to reveal the effect and mechanism of BCAAs on the pathogenesis of MMD.

Conclusions

Our study indicated that higher circulating BCAAs level was associated with increased risk of MMD and clinical subtypes. This work will help to elucidate the pathogenesis of MMD, which may provide the support for facilitating the interventions and preventions.

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 Beijing Tiantan Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study concept and design: CZ and PG. Collection and assembly of data: CZ, PG, CL, XYu, and YZhai. Data analysis and interpretation: WL, QH, JL, XL, and JW. Manuscript writing: CZ. Manuscript revision: XYe, QZ, RW, and YZhang. Provision of study materials: JZ and DZ. Final approval of manuscript: all authors.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.994286/full#supplementary-material

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Serum creatinine to cystatin C ratio and clinical outcomes in adults with non-dialysis chronic kidney disease

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Background: Studies have suggested that the serum creatinine/cystatin C (Cr/CysC) ratio is a surrogate marker for muscle wasting is associated with adverse outcomes in several disease conditions. To clarify the utility of the Cr/CysC ratio as a prognostic marker in chronic kidney disease (CKD) we evaluated the association between the Cr/CysC ratio clinical outcomes in patients with non-dialysis CKD.

Methods: This prospective observational cohort study included 1,966 participants of the KoreaN cohort study Outcomes in patients With CKD (KNOW-CKD). We evaluated associated factors with the serum Cr/CysC ratio and association between the serum Cr/CysC ratio and composite outcomes of all-cause death and cardiovascular events (CVEs).

Results: The mean age was 54 ± 12 (SD) years and 61% were men. The mean serum Cr/CysC ratio was 10.97 ± 1.94 in men and 9.10 ± 1.77 in women. The Cr/CysC ratio correlated positively with urinary creatinine excretion, a marker of muscle mass. In the fully adjusted Cox proportional hazard model, the Cr/CysC ratio was associated with the occurrence of adverse outcomes through a median follow-up of 5.9 years [hazard ratio (HR) = 0.92, 95% confidence interval (CI) = 0.85–0.99 for the composite outcomes, HR = 0.87, 95% CI, 0.78 – 0.97 for all-cause death, and HR = 0.93; 95% CI, 0.84–1.04 for CVEs]. In subgroup analyses, there were interactions of the Cr/CysC ratio with age and sex for risk of the clinical outcomes, but not eGFR group.

Conclusion: A higher Cr/CysC ratio is associated with a lower risk of the composite outcomes, especially all-cause mortality, even after adjusting for eGFR. These suggest that the Cr/CysC ratio is a useful prognostic marker in CKD.

KEYWORDS

creatinine/cystatin C ratio, death, cardiovascular events, chronic kidney disease, muscle wasting

Introduction

Chronic kidney disease (CKD) is a prevalent condition worldwide that contributes an important risk of morbidity and mortality (1). CKD is also an important risk factor for protein-energy wasting (2). Uremia in CKD leads to accelerated muscle protein breakdown combined with low dietary energy and protein intakes (3, 4). Muscle wasting induces mobility limitations, loss of independence, and vulnerability to disease complications. Muscle wasting is an important determinant of adverse outcomes in CKD. Several methods are available to measure muscle mass, including imaging techniques, anthropometric parameters, and biochemical markers. Imaging studies such as dual-energy X-ray absorptiometry (DXA) are a standard method for assessing muscle mass (5). In adults with CKD, DXA is a standard method to measure body composition despite being influenced by volume status (6). However, a wide range of methods can be used to assess muscle mass. Availability, cost, and ease of use can determine whether techniques are better suited to clinical practice or research.

Serum creatinine and cystatin C are well-established markers of kidney function (7). Creatinine is generated in proportion to muscle mass, but cystatin C is not affected by muscle mass (8). Loss of muscle mass during the wasting process of CKD is accompanied by a decline in serum creatinine, but not in cystatin C. We postulated the serum Cr/CysC ratio is a surrogate marker for muscle wasting in CKD. Previous studies have reported that the Cr/CysC ratio was associated with muscle mass in patients who were critically ill (9), older adults (10), or had several chronic diseases. Moreover, the Cr/CysC ratio was associated with clinical outcomes in patients in the intensive care unit (9) and those receiving continuous kidney replace therapy (11). A cross-sectional study of patients with non-dialysis CKD reported that Cr/CysC was independently associated with skeletal muscle mass and strength (12). They suggested that Cr/CysC could be a surrogate marker for detecting muscle wasting in CKD. However, the implication of Cr/CysC in the clinical outcomes of CKD are uncertain.

To clarify the utility of the serum Cr/CysC ratio as a prognostic markers in CKD, we analyzed a prospective cohort dataset from the KoreaN cohort study for Outcome in patients With CKD (KNOW-CKD). In this study, we evaluated factors associated with the serum Cr/CysC ratio and the association between the serum Cr/CysC ratio and composite outcomes of all-cause death and cardiovascular events (CVEs) in adults with non-dialysis CKD.

Materials and methods

Study participants and design

The KNOW-CKD (ClinicalTrials.gov identifier NCT01630486) is a nationwide prospective cohort study investigating the clinical outcomes of Koreans with non-dialysis dependent CKD (13). Between 2011 and 2016, a total of 2,238 adults age 20–75 years with non-dialysis CKD stage G1-G6 were enrolled from nine tertiary care hospital. Subjects were excluded if they had a history of malignancy, advanced heart failure, a single kidney, liver cirrhosis, chronic lung disease or other factors according to the study protocol. We analyzed 1,966 participants from this cohort who underwent extensive laboratory tests, completed a health questionnaire, and for whom follow-up data were available (**Supplementary Figure 1**). This study protocol was approved by the Institutional Review Board of the participating centers. Informed consents were obtained from all participants.

Data collection and measurements

Baseline demographic characteristics, medical history, and lifestyle factors were collected by self-report and a review of the medical records. A history of hypertension was defined as a self-reported history of hypertension or current use of antihypertension medication. Diabetes mellitus was defined as a fasting serum glucose level $\geq$ 126 mg/dL, a history of diabetes, or current use of antidiabetic medication. Urinary albumin excretion was determined using the spot urine albumin-to-creatinine ratio (ACR).

Main exposure of interest

Sample for serum creatinine and cystatin C were collected at baseline after overnight fasting. Serum creatinine was measured using an isotope dilution mass spectrometry-calibrated method and cystatin C was measured using immunonephelometry with calibration against a reference at a central lab. The estimated glomerular filtration rate (eGFR) was calculated by using the CKD Epidemiology Collaboration (CKD-EPI) creatinine equation (14). The serum Cr/CysC ratio (creatinine in mg/L to cystatin C in mg/L) was calculated from the values measured concomitantly at baseline. Because the serum Cr/CysC ratio was differed by sex, we categorized the participants into male and female quartiles according to Cr/CysC ratio.

Study outcomes

Patients were followed up until their last visit, initiation of renal replacement therapy, or death before March 31, 2021. All-cause death or CVE during follow up was the primary outcomes. CVE was defined as the occurrence of a fatal or non-fatal CVE during follow up including any coronary artery events (unstable angina, myocardial infarction, coronary intervention, or coronary surgery), hospitalization for heart failure, ischemic or hemorrhagic stroke, or symptomatic arrhythmia. The composite outcome of all-cause death and CVEs was assessed.

Statistical analysis

We initially considered our primary predictor variable, Cr/CysC ratio, as a continuous variable. The Cr/CysC ratio in males was greater than that in females (**Figure 1**). We thus divided the study population into sex-specific quartiles for descriptive purposes in the analyses. We described the baseline characteristics across group using the mean ± standard deviation or median and interquartile range for continuous variables and number with percent for categorical variables.

For cross-sectional analyses at baseline, we used a linear regression model of the Cr/CysC ratio controlled for demographic, comorbid, and laboratory factors. We initially considered unadjusted models and then adjusted for age, sex, eGFR, and ACR. In the full adjusted model, we also adjusted for comorbidity and laboratory data.

Cumulative event probabilities were estimated using a Kaplan-Meier analysis and log-rank tests. Cox proportional hazard models were developed to determine the association between Cr/CysC ratio and composite outcomes, all-cause death, and CVEs. The Cr/CysC ratio was evaluated as a continuous variable and a categorical variable of quartiles. The data were expressed as hazard ratio (HR) with 95% confidence interval (CI). Model 1 considered baseline age, sex, eGFR, and the natural log of ACR. Model 2 added systolic blood pressure, body mass index, C-reactive protein, serum albumin, and a history of diabetes or cardiovascular disease. Moreover, to assess their effects on our findings, we tested for associations between the Cr/CysC ratio and outcomes stratified by age, sex, and eGFR group. The Cox model for all-cause death and CVEs was used in cubic spline analyses, with each curve having four equally distributed knots, placed at the 5th, 35th, 65th, 95th percentiles of the Cr/CysC ratio. The cubic spline model used a Cr/CysC ratio of 7.0 as reference value. All analyses were performed using Stata version 17 (STATA Corp.).

Results

Baseline characteristics and patient outcomes

The baseline characteristics of the study participants are presented according to the sex-specific quartiles of Cr/CysC ratio (**Table 1**). The average age was 54 ± 12 years, 1,190 males (61%), and eGFR was 54 ± 12 mL/min/1.73 m^2. The distribution of Cr/CysC ratio by age and sex is show in **Figure 1**. The average serum Cr/CysC ratio of males was 10.97 ± 1.94, and that of females was 9.10 ± 1.77. Compared with participants in quartile 1, those in the higher quartiles were younger, and more likely to be non-smokers, and have no history of diabetes or cardiovascular diseases. They were also likely to have higher 24-h urine creatinine and serum albumin values.

A total of 258 composite outcomes occurred: 130 all-cause of deaths and 163 CVEs occurred during a median follow-up of 5.9 years. The incidence rates of composite outcomes were 34.2, 24.5, 21.1, and 14.4/1,000 person-years according to quartiles, respectively. The trends in the rate of the composite outcomes, all-cause death, and CVEs were all statistically significant ($P < 0.01$) (**Table 2**).

The serum creatinine/cystatin C ratio and clinical parameters at baseline

The Cr/CysC ratio had a week negative correlation with the natural log of C-reactive protein and a weak positive correlation with serum albumin. The Cr/CysC ratio had a significant positive correlation with 24-h urine creatinine ($r = 0.376$, $P < 0.001$) (**Supplementary Figure 2**).

In multivariable linear regression analysis, the Cr/CysC ratio had a negative association with age, female sex, eGFR, cardiovascular disease, current smoking, and the natural logs of ACR and CRP. Cr/CysC ratio had a positive association with serum albumin and 24-h urine creatinine (coefficient of determinant, $R^2 = 0.391$) (**Supplementary Table 1**).

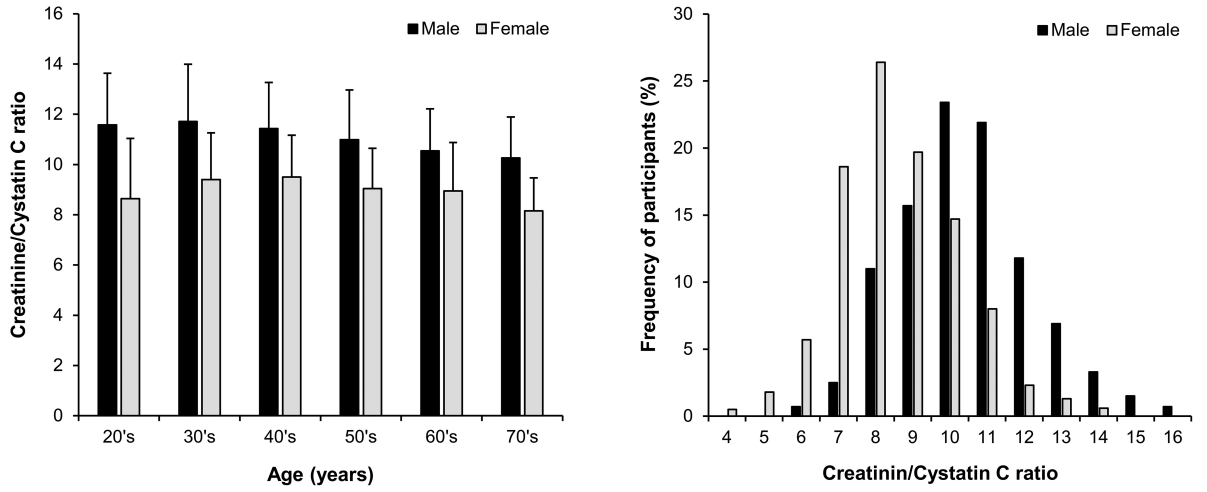

FIGURE 1
Distribution of the serum creatinine/cystatin C ratio (mean ± SD) by age and sex in 1,966 participants with non-dialysis chronic kidney disease.

The serum creatinine/cystatin C ratio and the composite outcomes, all-cause death, and cardiovascular events

The Kaplan-Meier curves revealed that the cumulative probabilities of the composite outcomes, all-cause death, and CVEs were significantly lower among patients in quartile 1 (Q1) of baseline Cr/CysC ratio compared with other quartiles (log-rank $P < 0.01$) (**Supplementary Figure 3**).

The association of Cr/CysC ratio with composite outcomes, all-cause death, and CVEs were evaluated using multivariable Cox proportional hazards regression analyses (**Table 3**). The HRs of the Cr/CysC ratio as a continuous variable for composite outcome, all-cause death, and CVEs were 0.92 (95% CI, 0.85 − 0.99, $P = 0.05$), 0.87 (95% CI, 0.78 − 0.97, $P = 0.02$), and 0.93 (95% CI, 0.84 − 1.04, $P = 0.2$), respectively, in model 2. The HRs for Q4, the quartile with the highest Cr/CysC ratio, for the composite outcome, all-cause death, and CVEs were 0.69 (95% CI, 0.45 − 0.99, $P = 0.05$), 0.54 (95% CI, 0.30 − 0.97, $P = 0.04$), and 0.72 (95% CI, 0.43 − 1.21, $P = 0.2$), respectively, in model 2.

The associations between the Cr/CysC ratio and the composite outcome, all-cause death, and CVEs were showed using a cubic spline analysis. The risks for the composite outcome, all-cause death, and CVEs were lower with greater Cr/CysC ratio. The risk for all-cause death became progressively lower as the Cr/CysC ratio increased (**Figure 2**).

Subgroup analysis

We further examined the effect of modification of the Cr/CysC ratio on risk of the composite outcome, all-cause death, and CVEs in several subgroups (**Figure 3**). The association between the Cr/CysC ratio and the composite outcome, all-cause death, and CVEs was consistent across eGFR subgroups (< 45 vs. ≥ 45 mL/min/1.73 m^2). The relationship between the Cr/CysC ratio quartile and composite outcomes was attenuated in younger adults (age < 50 years) (P for interaction = 0.05). The relationship between the Cr/CysC ratio quartile and composite outcomes was attenuated in males (P for interaction = 0.02). We found significant interactions among subgroups by age and sex.

Discussion

In this study, we found that the serum Cr/CysC ratio was associated with the risk of a composite outcome of all-cause death and CVEs in adults with non-dialysis CKD, regardless of kidney function. The serum Cr/CysC ratio had a significantly positive correlation with urinary creatinine excretion, a muscle mass marker (15). A higher Cr/CysC ratio was strongly associated with a lower risk of all-cause death and that association was independent of demographics, comorbidities, and clinical factors at baseline. Among the subgroups of patients, the association between the Cr/CysC ratio and the composite outcomes was consistent across eGFR subgroups. These findings suggest that the Cr/CysC ratio could be a prognostic marker of clinical outcomes in CKD.

Serum creatinine and cystatin C are widely used endogenous glomerular filtration markers (7). Creatinine is an end product of muscle catabolism. Its main non-GFR determinants include muscle mass and protein intake, and it varies substantially by age, sex, and chronic illness (16, 17). Cystatin C is low-molecular weight protein enzymes produced by nucleated cells that is involved in the inflammatory cascade. Its serum concentration

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TABLE 1 Baseline characteristics according to serum Cr/CysC ratio quartiles in 1,966 adults with chronic kidney disease.

Variables	Total (*n* = 1,966)	Quartile of creatinine/Cystatin C ratio				*P* for trend
		Q1 (*n* = 492)	Q2 (*n* = 491)	Q3 (*n* = 492)	Q4 (*n* = 491)	
Male range	6.45–24.14	6.45–9.69	9.69–10.81	10.81–11.97	11.98–24.14	
Female range	4.62–23.49	4.62–7.90	7.90–8.91	8.91–10.11	10.14–23.49	
Age, years	53.6 ± 12.3	56.7 ± 12.4	55.7 ± 11.4	52.5 ± 11.9	49.4 ± 12.06	< 0.001
Male sex	1,190 (61%)	298 (61%)	297 (61%)	298 (61%)	297 (61%)	0.9
BMI, kg/m^2	24.57 ± 3.89	24.70 ± 3.82	24.60 ± 3.23	24.57 ± 3.20	24.43 ± 3.27	0.2
Creatinine, mg/dL	1.81 ± 1.15	1.44 ± 0.69	1.63 ± 0.83	1.78 ± 0.91	2.37 ± 1.69	< 0.001
Cystatin C, mg/L	1.75 ± 0.92	1.77 ± 0.80	1.70 ± 0.83	1.68 ± 0.85	1.85 ± 1.14	0.2
eGFR, mL/min/1.73m^2	53.6 ± 31.0	61.0 ± 30.8	54.8 ± 29.7	52.5 ± 30.4	46.0 ± 31.4	< 0.001
UACR, mg/g	348 [78–1053]	462 [131–1444]	306 [64–1049]	312 [71–940]	331 [66–972]	0.07
24-h U creatinine, mg/day	1,177 ± 412	1,087 ± 383	1,154 ± 374	1,188 ± 409	1,273 ± 455	< 0.001
Systolic BP, mmHg	128 ± 16	129 ± 17	128 ± 14	127 ± 16	128 ± 17	0.07
Diastolic BP, mmHg	77 ± 11	77 ± 12	77 ± 11	77 ± 11	77 ± 11	0.2
C-reactive protein, mg/L	0.6 [0.2–1.7]	0.8 [0.3–2.2]	0.7 [0.3–1.8]	0.6 [0.2–1.5]	0.5 [0.2–1.3]	<0.001
Albumin, g/dL	4.19 ± 0.42	4.08 ± 0.48	4.23 ± 0.40	4.21 ± 0.38	4.23 ± 0.41	<0.001
Hemoglobin, g/dL	12.8 ± 2.0	12.7 ± 1.9	13.0 ± 1.9	13.0 ± 1.9	12.7 ± 2.2	0.7
Diabetes	665 (34%)	191 (39%)	181 (37%)	148 (30%)	145 (30%)	<0.001
Hypertension	1,889 (96%)	478 (97%)	471 (96%)	474 (96%)	499 (95%)	0.3
Cardiovascular disease	309 (16%)	91 (19%)	102 (21%)	69 (14%)	47 (10%)	<0.001
Current smoker	294 (15%)	99 (20%)	68 (14%)	77 (16%)	50 (10%)	<0.001

Continuous variables expressed as mean ± standard deviation or median [interquartile range]; categorical variables, as number (percentage). BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; UACR, urine albumin-creatinine ratio.

TABLE 2 Incidence of the composite outcomes, all-cause death, and cardiovascular events according to quartile of CysC/Cr ratio.

Outcomes	Total (*n* = 1,966)	Quartile of creatinine/Cystatin C ratio				*P* for trend
		Q1 (*n* = 492)	Q2 (*n* = 491)	Q3 (*n* = 492)	Q4 (*n* = 491)	
No. of person-years	11,033	2,601	2,781	2,887	2,764	
Composite outcomes						
No of incidence	258	89	68	61	40	
Incidence rate (1,000 person-year)	23.4	34.2	24.5	21.1	14.4	<0.001
All-cause death						
No. of incidence	130	46	33	31	20	
Incidence rate (1,000 person-year)	11.8	17.7	11.9	10.7	7.2	<0.001
Cardiovascular events						
No. of incidence	163	55	43	40	25	
Incidence rate (1,000 person-year)	14.8	21.1	15.5	13.9	9.0	0.01

can be affected by non-GFR determinants such as inflammation, cardiovascular disease, obesity, and smoking (18, 19).

Previous studies reported a positive correlation between the Cr/CysC ratio and muscle mass in patients with intensive care unit (9), older adults (10), type 2 diabetes (20), chronic lung disease (21), or cancer (22). Kashani et al. (9) defined the Cr/CysC ratio as a sarcopenic index that correlated significantly with muscle mass measured abdominal CT scan. As a sarcopenic index, it predicted mortality in 226 patients receiving intensive care. Moreover, a recent study reported that the Cr/CysC ratio correlated with muscle quality (myosteatosis) and physical performance in older adults, independent of muscle mass (23). The Cr/CysC ratio was associated with major adverse CVEs in patients with obstructive coronary artery disease. In patients receiving intensive care and continuous kidney replacement therapy, a higher Cr/CysC ratio was associated with longer survival (11). In a cross-sectional study of 272 patients with CKD, the Cr/CysC ratio correlated with skeletal muscle mass and hand grip strength, and appeared to be a surrogate marker for muscle wasting (12). In our study, serum Cr/CysC was

TABLE 3 Hazard ratio for death and cardiovascular events based on the Cr/CysC ratio in 1,966 adults with chronic kidney disease.

	Crude		Model 1		Model 2	
	Hazard ratio (95% CI)	***P***	**Hazard ratio (95% CI)**	***P***	**Hazard ratio (95% CI)**	***P***
Composite outcomes						
Continuous (per 1 unit Cr/CysC)	0.93 (0.87–0.99)	0.02	0.87 (0.80–0.95)	0.01	0.92 (0.85–0.99)	0.05
Categorical						
Q1	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q2	0.67 (0.48–0.92)	0.01	0.75 (0.53–1.01)	0.08	0.82 (0.59–1.14)	0.2
Q3	0.53 (0.38–0.74)	<0.001	0.62 (0.44–0.87)	0.01	0.71 (0.50–1.01)	0.06
Q4	0.39 (0.27–0.53)	<0.001	0.55 (0.37–0.82)	0.01	0.69 (0.45–0.99)	0.05
All-cause death						
Continuous (per 1 unit Cr/CysC)	0.94 (0.86–1.02)	0.1	0.83 (0.74–0.93)	0.01	0.87 (0.78–0.97)	0.02
Categorical						
Q1	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q2	0.68 (0.42–1.02)	0.06	0.66 (0.42–1.04)	0.08	0.78 (0.49–1.23)	0.3
Q3	0.58 (0.37–0.91)	0.02	0.57 (0.35–0.91)	0.02	0.68 (0.42–1.10)	0.1
Q4	0.39 (0.23–0.66)	<0.001	0.42 (0.24–0.74)	0.01	0.54 (0.30–0.97)	0.04
Cardiovascular events						
Continuous (per 1 unit Cr/CysC)	0.92 (0.85–1.00)	0.05	0.89 (0.80–0.99)	0.04	0.93 (0.84–1.04)	0.2
Categorical						
Q1	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q2	0.67 (0.45–0.99)	0.05	0.79 (0.52–1.19)	0.3	0.80 (0.53–1.21)	0.3
Q3	0.53 (0.35–0.81)	0.01	0.67 (0.43–1.03)	0.07	0.72 (0.46–1.12)	0.1
Q4	0.38 (0.24–0.61)	<0.001	0.60 (0.36–1.00)	0.05	0.72 (0.43–1.21)	0.2

Model 1, adjusted for age, sex, estimated glomerular filtration rate, and natural log of albuminuria. Model 2, additionally adjusted for diabetes, cardiovascular disease, body mass index, systolic blood pressure, current smoking, albumin, and the natural log of C-reactive protein. CI, confidence interval.

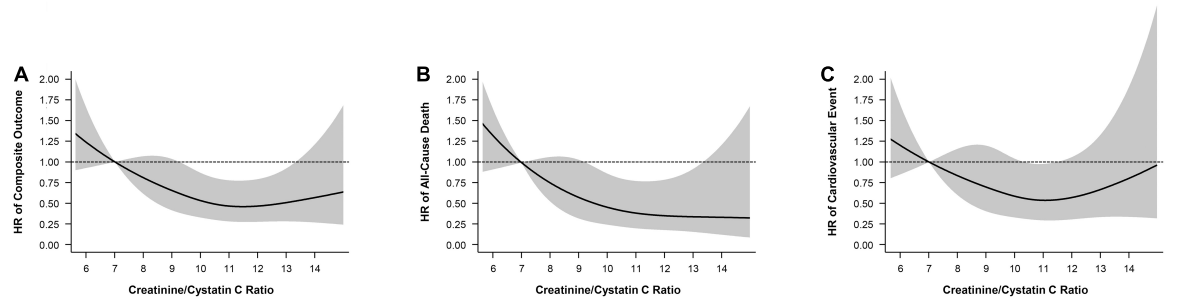

FIGURE 2
Cubic spline model shows relationship of creatinine/cystatin C ratio with **(A)** composite outcomes, **(B)** all-cause death, and **(C)** cardiovascular events. Adjustments were made for model 2 variables (age, sex, eGFR, ACR, diabetes, cardiovascular disease, body mass index, smoking, albumin, C-reactive protein).

independently and positively correlated with urine creatinine excretion. Thus, Cr/CysC could be represent as a muscle wasting marker in CKD.

For reasons similar to those put forward for creatinine and cystatin C, a larger difference between cystatin C- and creatinine-eGFR has been associated with lower frailty, injurious falls, hospitalization, CVEs, and mortality in adults with hypertension of a cohort of the Systolic Blood Pressure Intervention Trial (SPLINT) (24). Kim et al. (25) reported that a positive difference between cystatin C- and creatinine-eGFR in the KNOW-CKD cohort was associated with a higher risk of CVEs and accelerated coronary artery calcification. However, information about the association between the straight-forward serum Cr/Cys C ratio and long-term clinical outcomes from CKD is limited. In this study, we showed that a higher Cr/CysC ratio was associated with a lower risk of CVEs and all-cause death. Among the subgroups of patients, we found significant interaction

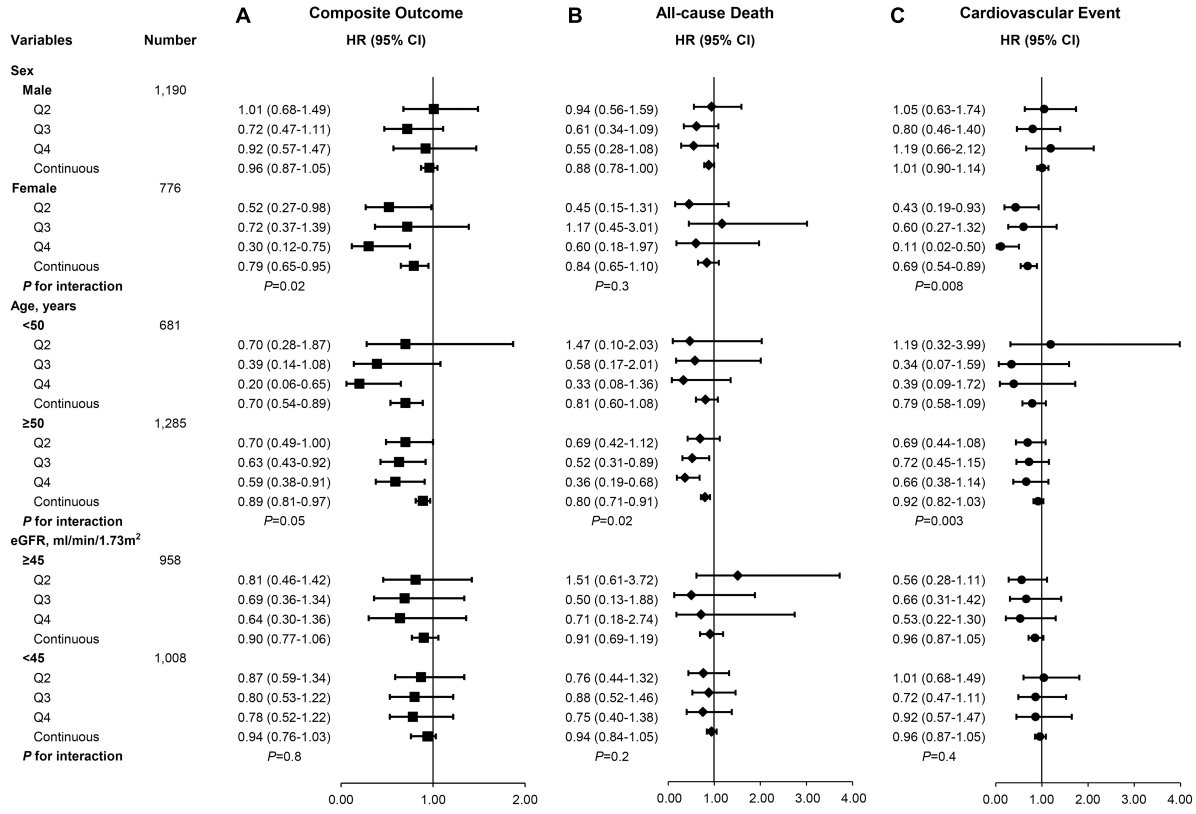

FIGURE 3
There were different risks of **(A)** composite outcomes, **(B)** all-cause death, and **(C)** cardiovascular events according to subgroups in the multivariable-adjusted Cox proportional hazards model (Model 2).

among subgroups by age and sex, but no interaction among subgroups by eGFR.

Patients with CKD have many risk factor for muscle wasting, including poor appetite, inflammation, insulin resistance, and metabolic acidosis (2, 3). Muscle wasting is thus relevant in CKD, but it goes underdiagnosed. There are several methods for assessing muscle mass with imaging, such as DXA, computed tomography or magnetic resonance imaging (4, 5). However, those tests are costly, entail radiation, and are not available in all clinical settings. Bioimpedance is an inexpensive alternative for assessing body composition, but it is greatly influenced by hydration status and limb size in CKD. Instead, we suggest the serum Cr/Cys C ratio, which is readily available and time effective for capturing sarcopenia and serve as a biomarker of adverse outcomes in CKD.

This study has several limitations. First, in the KNOW-CKD cohort, all-cause death, CVEs, and the composite outcomes occurred in only 130 (6.6%), 163 (8.3%), and 258 (13.1%) patients, respectively, which is lower than in other CKD cohorts. We previously showed that our cohort had a lower cardiovascular risk burden than other cohorts (26, 27) and our lower event rate could have decreased the statistical power of our results. Second, we adjusted for several clinical factors in multivariable analyses, but other factors might also influence serum creatinine and cystatin C levels. For example, we did not evaluate protein intake, volume status, exercise habits, medications, and thyroid function. Third, we did not have data about sarcopenia. We did not measure muscle mass using an image analysis or muscle function by grip strength or walking speed. We therefore could not directly investigate the association between the serum Cr/Cys C and sarcopenia in CKD. Forth, our study participants were all Korean patients with CKD. Sex and age modified the association between Cr/CysC ratio and clinical outcomes. A recent study reported that a higher Cr/CysC ratio was associated with lower mortality in both non-black and black race people. However, the effect was more significant among black people (28). Therefore, it might be difficult to generalize our findings to all patients with CKD. Further studies are required to extrapolate our present findings. Despite those limitations, our study has several strengths. We used comprehensive health history and laboratory data from the nationwide KNOW-CKD cohort. All blood samples were sent to a single central laboratory for accurate measurement of serum creatinine and cystatin C. We found that the serum Cr/Cys C ratio is a simple marker for clinical outcomes. The serum Cr/CysC level was associated with 24-h urine creatinine,

albumin and CRP, and might be link for muscle mass, nutrition, and inflammation in CKD.

Conclusion

In conclusion, the serum Cr/Cys C ratio is associated with the risk of all-cause of death and CVEs among adults with non-dialysis CKD. These findings suggest that the Cr/CysC ratio could be used a prognosis marker for adults with non-dialysis CKD. Further evaluations are needed for its generalized application of our results.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: KNOW-CKD data. And I did not detect any particular expressions.

Ethics statement

The studies involving human participants were reviewed and approved by Kangbuck Samsung Hospital IRB. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YH, K-BL, and YK: research idea and study design. WC, SH, and K-HO: data acquisition. HK and SP: statistical analysis. K-BL, HP, YK, and K-HO: supervision and mentorship. All authors have read and approved the final version of the manuscript.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.996674/full#supplementary-material

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Effect of fruit intake on functional constipation: A systematic review and meta-analysis of randomized and crossover studies

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Functional constipation (FC) is commonly treated with fruits whose efficacy remains unclear. We conducted a meta-analysis of fruit intervention for FC and provided evidence-based recommendations. We searched seven databases from inception to July 2022. All randomized and crossover studies on the effectiveness of fruits on FC were included. We conducted sensitivity and subgroup analysis. A total of 11 studies were included in this review. Four trials showed that kiwifruits have significantly increased stool frequency (MD = 0.26, 95% CI (0.22, 0.30), $P < 0.0001$, $I^2 = 0\%$) than palm date or orange juice in the fixed-effect meta-analysis. Three high-quality studies suggested that kiwifruits have a better effect than ficus carica paste on the symptom of the FC assessed by the Bristol stool scale in the fixed-effect meta-analysis [MD = 0.39, 95% CI (0.11, 0.66), $P < 0.05$, $I^2 = 27\%$]. Besides, five trials showed that fruits can increase the amount of *Lactobacillus acidophilus* [MD = 0.82, 95% CI (0.25, 1.39), $P < 0.05$, $I^2 = 52\%$], analyzed with the random-effect model. Subgroup meta-analysis based on the types of fruits suggested that fruits including pome fruit, citrus fruit, and berries have increased the effect of *Bifidobacterium* t more than the stone fruits in the random effect meta-analysis [MD = 0.51, 95% CI (0.23, 0.79), $P < 0.05$, $I^2 = 84\%$]. Totally, fruit intake may have potential symptom alleviation on the FC as evidence shows that they can affect stool consistency, stool frequency, and gut microbiota. Further large-scale studies are needed to gain more confident conclusions concerning the association between fruit intake and FC in the future.

KEYWORDS

functional constipation, fruits, randomized and crossover studies, meta-analysis, gut microbiota

Introduction

Constipation is a common functional bowel disorder, characterized by difficult, infrequent, or incomplete bowel movements (1). According to the Rome IV criteria, constipation is categorized into two subtypes: functional constipation (FC) and irritable bowel syndrome (IBS-C) (2). According to the Rome IV criteria in 2021, the global prevalence of FC was found to be 10.1% (3). In addition to its higher prevalence, chronic constipation comes with an economic burden for patients and health systems. Three million outpatient visits and 800,000 emergency room visits have been accounted for in the United States (4). The annual cost reached between $2,000 and $7,500 per patient in the United States in 2019 (5). Besides, the occurrence of constipation will also increase the poor quality of life, risk of colorectal cancer (6, 7), and higher rates of psychological distress (8). Therefore, it is necessary to emphasize the importance of successful prevention and management of constipation.

Until now, several common methods used to treat constipation have been applied in the clinic, including osmotic and stimulant laxatives, stool softeners, bulking agents, and pro-secretory agents. However, approximately half of the patients were dissatisfied with these treatment strategies due to the limited efficacy and side effects of drugs (9, 10). Dietary plays an important role in the treatment and management of constipation. The World Gastroenterology Association recommended increasing fiber intake either through dietary advice or supplementation (11). In the United Kingdom, professional guidelines in 2020 suggested that participants with constipation may consume fruits including prunes, cherries, and their fruit juices (12). Although epidemiological studies have also provided strong evidence that fruit could be beneficial in the FC, clinical trials showed inconsistent results. For example, a study with 1,088 participants including healthy and constipated patients suggested that some fruits, especially prunes, can soften the stool (13). But several trials in the clinic have found that some fruits, including ficus carica, palm date, and orange, did not affect the symptom alleviation of the FC, especially the stool consistency or frequency (14–16). In 2021, a recent systematic review of trials showed that various fruits, such as prunes, raisins, and apple fiber, could increase fecal weight. The present study suggests that apple, kiwifruit, fig paste, and orange may reduce gut transit time but prunes do not (17).

Therefore, this meta-analysis aims to evaluate the studies on the effect of fruits in patients with FC and to decide the fruit species that are most effective in treating participants with FC.

Materials and methods

Our meta-analysis was conducted according to the Cochrane Handbook for Systematic Reviews of Interventions (18) and was performed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (19). Two reviewers independently performed the literature search, study selection, data extraction, and quality assessment processes, such as the risk of bias and grading of evidence. Disagreements were resolved through discussion with the third author.

Literature search

We aim to identify randomized and crossover studies through the following clinical research databases from their inception until July 7, 2022: PubMed, EMBASE, Web of Science, Chinese Biomedical Database (CBM), the Cochrane Library, the China National Knowledge Infrastructure (CNKI), and the China Science and Technology Journal Database. Combinations of keywords and medical subject headings (MeSH) terms as follows: "constipation," "constipate," "gut microbiome," "gut transit," "stool frequency," "stool consistency," "bowel movement," "defaecation," and "randomized controlled trials," "crossover studies," and "sorbitol," "fruit," "juice," "fiber," "polyphenol," "extract," "kiwi," and "prune." In addition, we manually searched the references of the original article and then reviewed relevant articles to find possible relevant studies.

Selective criteria

Studies were eligible for inclusion if they met the following criteria: (1) The study was based on randomized controlled trials (RCTs) with a parallel or cross-over design in which fruit treatment was compared with placebo or no treatment; (2) The study population consisted of patients with functional constipation aged more than18 years; (3) The diagnosis of FC was clearly made by the use of internationally recognized criteria, such as Rome IV criteria; (4) The study used at least one of the following outcomes in clinical trials: stool consistency, stool frequency, Bristol stool score, *Lactobacillus acidophilus*, and *Bifidobacterium* spp. The following studies were excluded: (1) Case reports and reviews; (2) patients aged less than 18 years old; (3) patients with constipation who were induced by drugs or organic disease; (4) study protocols of ongoing trials without completed data.

Study screening

Our meta-analysis was conducted independently by two reviewers. The screening was performed by three processes according to the inclusion and exclusion criteria. In the

first stage, search results were downloaded from databases in EndNote and then duplicates were removed; and in the second stage, titles and abstracts of the articles were reviewed; in the last stage, the full text of studies where titles or abstracts that were insufficient to make decisions were obtained. The study screening diagram that suggested the detailed selection of studies is shown in **Figure 1**.

Data extraction

Two reviewers independently extracted the data from the corresponding eligible studies, including the study design, first author's name, year of publication, country of study, population (gender/age), duration of intervention, details of interventions (type, form, dosage), details of both the experimental treatment and the control and clinical outcomes (stool frequency, stool consistency, and gut microbiome) (**Table 1**).

Risk of bias and grading of the evidence

We assessed the risk of bias in studies with the Cochrane Risk of bias tool for randomized trials version 2 (ROB2) (20). This tool suggests five detailed domains for the quality assessments of individual processes Five detailed domains that were assessed by two authors are as follows: (1) The randomization process; (2) the deviation from the intended intervention; (3) the missing results; (4) the measurement of the outcome; (5) the selection of the reported results. These domains were judged with high risks, some concerns, or low risk of bias judgments. In addition, for crossover trials, if the order of intervention was not randomized, the risk of bias in the randomization process was defined as high in the Cochrane tool. The bias due to carryover effects was evaluated by the process of a washout period or a follow-up non-interventional period ($\geq$14 days) among the studies. Findings from these assessments have been summarized pictorially.

The Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) tool was used to examine the quality and strength of the evidence (21). The evidence was graded as high, moderate, low, or very low quality. Although RCTs were graded as studies with high-quality evidence in any type of study, not all of the RCTs had higher quality due to various factors in the study design. It was, therefore, necessary to downgrade evidence with criteria and these included: study limitation (as assessed by the Cochrane ROB2), inconsistency (without unexplained heterogeneity between studies, $I^2 > 50\%$ and $P < 0.10$), publication bias (significant evidence of small study effects), indirectness, and imprecision.

Two review authors evaluated the risk of bias with the Cochrane RoB2 and the outcome evidence with the GRADE tool independently, resolving any disagreements by a discussion with a third review author. We presented our assessment of the risk of bias and assessment of outcome evidence (**Tables 2**, **3**; **Supplementary Tables 1–5**).

Statistical analysis

Meta-analysis was conducted with Cochrane Collaboration's Review Manager 5.3. The mean differences (MDs) and 95% confidence intervals (CIs) of the outcome data for all constipation symptoms were used for meta-analysis, provided that these symptoms were reported in at least three studies. The results of this meta-analysis were expressed as MDs and 95% CIs, which were calculated for continuous data. The $\chi^{2\,2}$ tested heterogeneity between studies and I^2 suggested the degree of heterogeneity. The I^2 value greater than 50% was considered as significant heterogeneity. If data were without significant heterogeneity, the fixed effects model was used for pooled analysis. If the data had significant heterogeneity, random effects model was used for pooled analysis. A $p < 0.05$ was considered statistically significant when we tested the pooled data.

When the I^2 value was 50% or greater, possible reasons for heterogeneity were found according to the following methods: (1) Subgroup analysis was performed based on different outcomes, different types of intervention, and methodological quality; (2) A sensitivity analysis was conducted by repeating the analysis after sequential exclusion of one study at a time from the meta-analysis with more than 2 study comparisons to detect the stability of results. When the removal of a study changed the magnitude (by >10%), the significance, the direction of the association, or the evidence of heterogeneity, it was considered as having an influential effect.

Publication bias was evaluated by the funnel plot and Egger's test. $P < 0.05$ was considered to be statistically significant. But we cannot explore sources of heterogeneity or publication bias because less than 10 study comparisons were included in each outcome analysis (22).

Results

This meta-analysis includes a total number of 11 single RCTs with parallel or cross-over designs. According to the Cochrane RoB2, among the 11 studies, four studies (16, 23–25) in the current review has some overall concerns Of these, all studies have a bias due to deviations from intended interventions. Besides, the other seven studies (14, 15, 26–30) are assessed as having low risks. The assessments of risk of bias are reported in **Supplementary Tables 2, 3**. Besides, the GRADE system is used

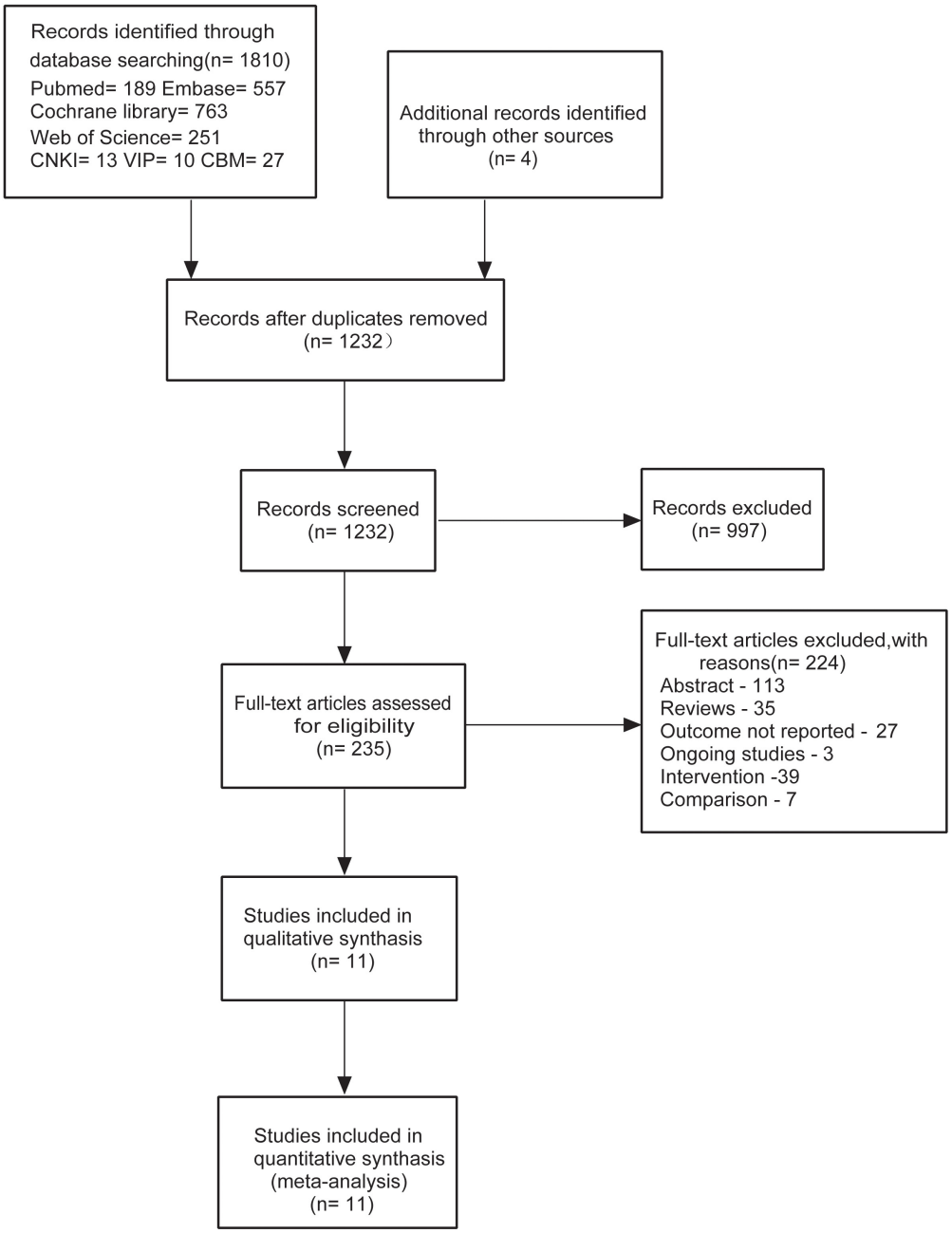

FIGURE 1
Flow diagram for the identification of relevant clinical trials examining the effect of fruits or fruit products on patients with functional constipation (FC).

to rate the certainty of evidence according to its internationally recognized standard.

Totally, fruits vs. placebo did significantly increase the stool frequency of patients with FC in the fixed-effect meta-analysis [MD = 0.26, 95% CI (0.22, 0.30), $P < 0.00001$, Figure 2]. There was no heterogeneity in this outcome of stool frequency ($I^2 = 0$). Subgroup meta-analysis by the type of fruits suggested that kiwifruits have significantly increased stool frequency [MD = 0.26, 95% CI (0.22, 0.30), $P < 0.0001$, $I^2 = 0$, Figure 3], while palm date or orange juice may not increase the stool frequency with the fixed-effect meta-analysis.

Stool consistency is one of the main methods to measure symptoms of FC. Totally, fruits or fruit products vs. placebo have greater improvement in the stool consistency of patients with FC in the fixed-effect meta-analysis [MD = −0.41, 95% CI (−0.45, −0.37), $P < 0.00001$, Figure 4]. Kiwifruits have a greater

TABLE 1 Summary of the human trials investigating the effect of fruits on functional constipation (FC).

Study	Country	Fruit product	Study design	Number	Study population	Daily dose	Comparator	Duration
Rush et al. (26)	New Zealand	Kiwifruit	Crossover RCT	48	Healthy adults	233 g	Placebo	3 weeks
Eid et al. (15)	UK	Palm date	Crossover RCT	22	Healthy adults	50 g	Maltodextrin	3 weeks
Shinohara et al. (25)	Japan	Apple	Crossover trials	8	Healthy adults	2 apples	Placebo	2 weeks
Chiu et al. (23)	China	Prune	RCT	60	Healthy adults	100 ml	Placebo drink	4 weeks
Lima et al. (16)	Brazil	Orange	Crossover trials	10	Healthy women	300 ml	Placebo	4 weeks
Mitsou et al. (24)	Greece	Banana	RCT	34	Healthy women	240 g	Placebo drink	8 weeks
Vendrame et al. (27)	Italy	Blueberry	Crossover RCT	20	Healthy male individual	250 ml	Placebo drink	6 weeks
Jamar et al. (28)	Brazil	Juçara	RCT	40	Individual with obesity	5 g	Maltodextrin	6 weeks
Baek et al. (14)	Korea	Ficus carica	RCT	80	Subject with FC	300 g	Placebo	8 weeks
Eady et al. (29)	New Zealand	Kiwifruits	Crossover RCT	32	Mildly constipated patients	3 kiwifruits	Metamucil	4 weeks
Wilkinson-Smith et al. (30)	UK	Kiwifruits	Crossover RCT	14	Healthy volunteers	300 g	Maltodextrin	3 days

TABLE 2 Risk of bias assessment of randomized controlled trials of the effect of fruit intake on FC.

References	Random sequence generation	Blinding of participants and personnel	Incomplete outcome data	Measurement of outcome	Selective reporting
Chiu et al. (23)	Low	Some concerns	Low	Low	Low
Mitsou et al. (24)	Low	Some concerns	Low	Low	Low
Jamar et al. (28)	Low	Low	Low	Low	Low
Baek et al. (14)	Low	Low	Low	Low	Low

TABLE 3 Risk of bias assessment for crossover trials of the effect of fruits intake on FC.

References	Random sequence generation[a]	Blinding of participants and personnel	Incomplete outcome data	Measurement of outcome	Selective reporting	Carryover effects
Wilkinson-Smith et al. (30)	Low	Low	Low	Low	Low	Low
Vendrame et al. (27)	Low	Low	Low	Low	Low	Low
Eid et al. (15)	Low	Low	Low	Low	Low	Low
Eady et al. (29)	Low	Low	Low	Low	Low	Low
Lima et al. (16)	Low	Some concerns	Low	Low	Low	Low
Shinohara et al. (25)	Low	Some concerns	Low	Low	Low	Low

[a]For crossover studies, studies with "Some concerns" in the random sequence generation column were those that did not specify whether the order of treatments was randomized or not.

symptom alleviation [MD = −0.41, 95% CI (−0.45, −0.37), $P < 0.0001$, Figure 5] than orange juice in the stool consistency of patients with FC by subgroup analysis.

The Bristol stool scale is used to assess the physical appearance and form of fecal samples. Totally, fruits were associated with beneficial effects on the physical appearance and form of fecal samples as evaluated by the Bristol stool scale [MD = 0.39, 95% CI (0.11, 0.66), $P < 0.05$, $I^2 = 27\%$, Figure 6]. However, a subgroup meta-analysis showed that kiwifruits have a better effect [MD = 0.67, 95% CI (0.24, 1.10), $P < 0.05$, Figure 7] than ficus carica paste on the symptom of the FC assessed by the Bristol stool scale in the fixed-effect meta-analysis.

Fruits vs. placebo have no effects on the *L. acidophilus* of patients with FC in the random-effect meta-analysis [MD = 0.49, 95% CI (−0.20, 1.19), $P > 0.05$, Figure 8]. There was high

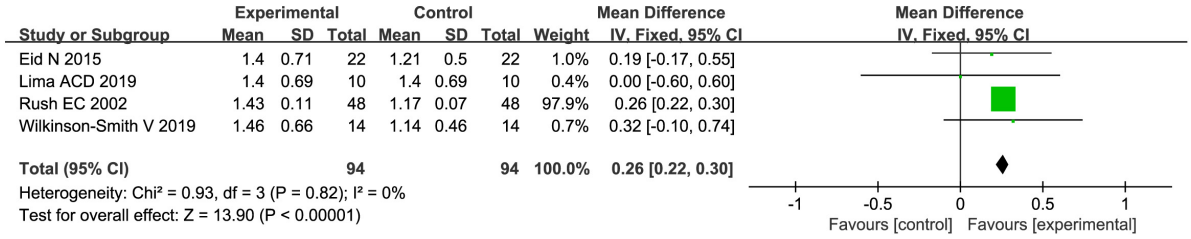

FIGURE 2
Effect of fruit intervention on stool frequency in the fixed-effect meta-analysis.

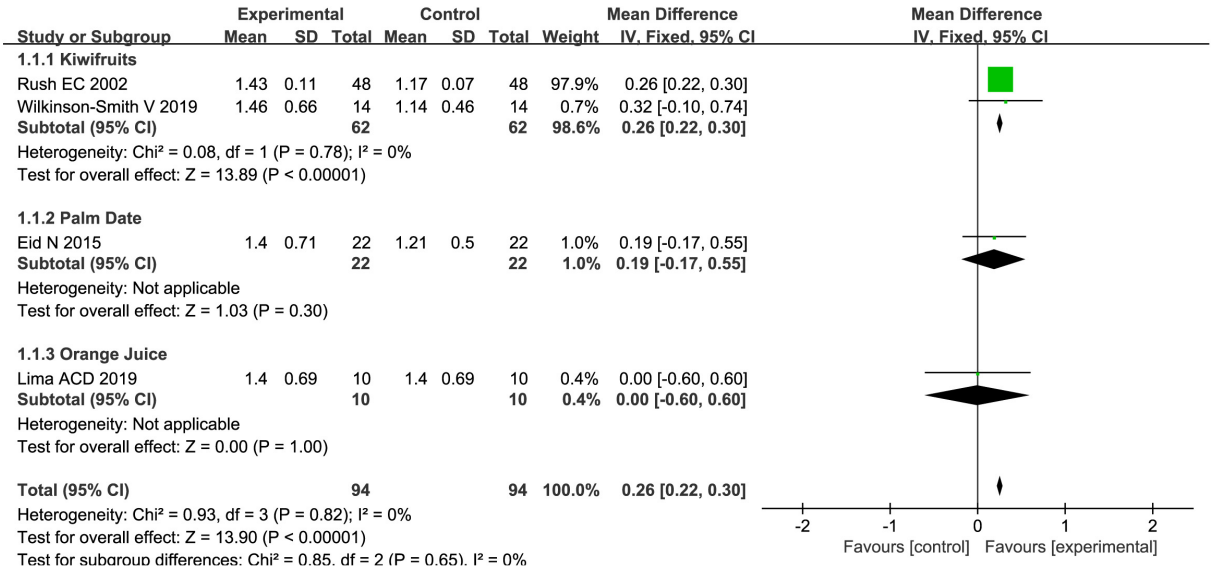

FIGURE 3
Intervention effect of different types of fruits vs. placebo for stool frequency on patients in subgroup meta-analysis.

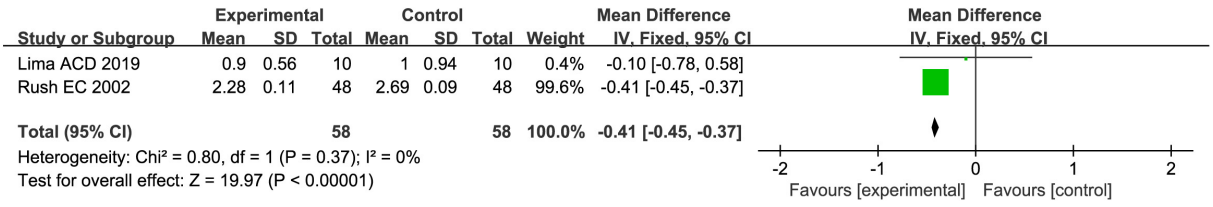

FIGURE 4
Effect of fruit intervention on stool consistency in the fixed-effect meta-analysis.

heterogeneity in *L. acidophilus* (I^2 = 84%). A sensitivity meta-analysis by removing a trial suggested that heterogeneity has been decreased to 41% (Figure 9), and an analysis with a random-effect model showed that there are significant effects on *L. acidophilus* [MD = 0.81, 95% CI (0.31, 1.31), $P < 0.05$, Figure 9]. We also conducted a subgroup meta-analysis by intervention time on patients. Fruits affect the effect of L. *acidophilus* [MD = 1.14, 95% CI (0.77, 1.50), $P < 0.05$, Figure 9] when intervention time was ≤4 weeks. Analyzed with the subgroup meta-analysis, the heterogeneity among subgroups has been reduced to 0%. The difference between estimates of the effect of fruits on *L. acidophilus* in intervention time ≤4 weeks and intervention time >4 weeks was significant (χ^2 = 5.81, $P < 0.05$ by a test of interaction; Figure 9).

Different types of fruits have various effects on the improvement of *Bifidobacterium* in patients. We performed

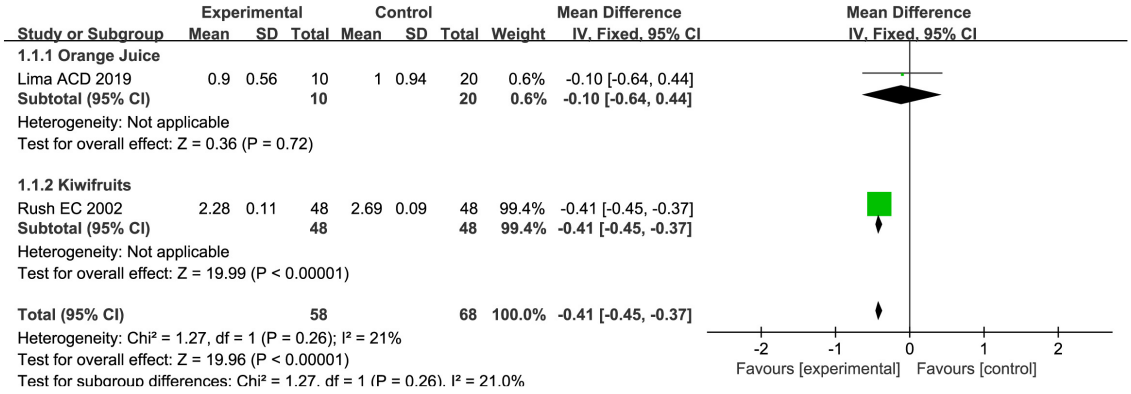

FIGURE 5
Intervention effect of different types of fruits vs. placebo for stool consistency on patients in subgroup meta-analysis.

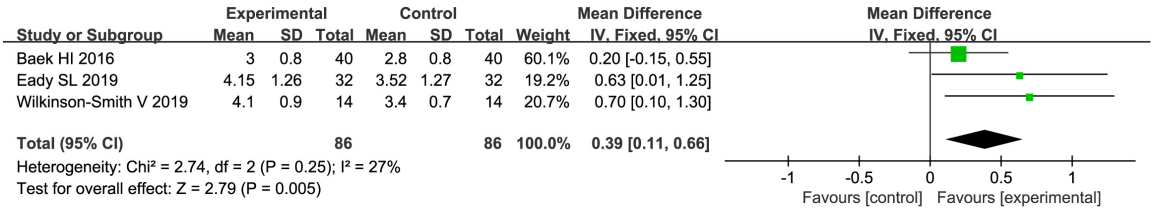

FIGURE 6
Effect of fruit intervention on the Bristol stool score in the fixed-effect meta-analysis.

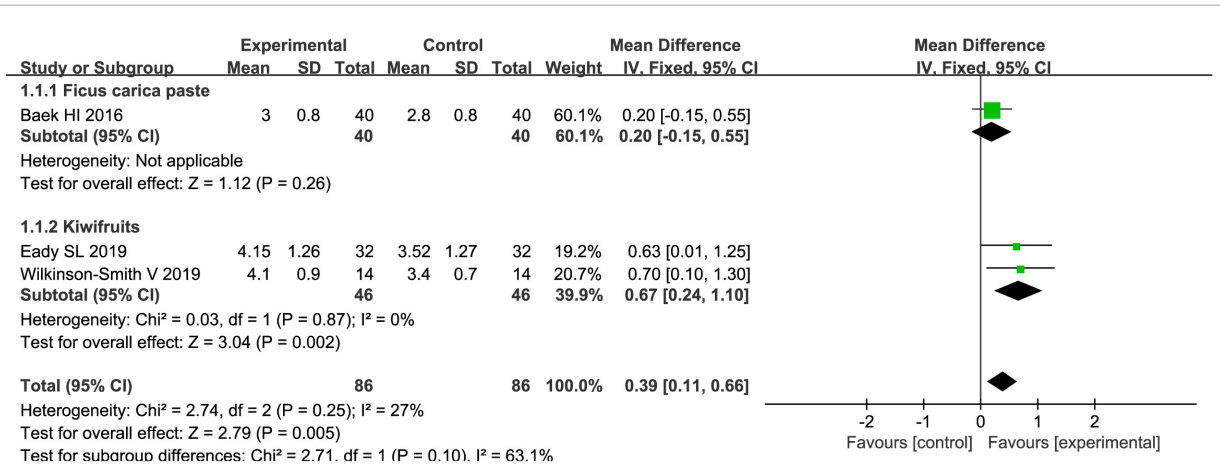

FIGURE 7
Intervention effect of different types of fruits vs. placebo for Bristol stool score on patients in subgroup meta-analysis.

a subgroup meta-analysis by the type of fruits for the *Bifidobacterium* and then suggested that fruits including pome fruits, citrus fruits, and berries have better effects on the *Bifidobacterium* than the stone fruits in the random effect meta-analysis [MD = 0.51, 95% CI (0.23, 0.79), $P < 0.05$, Figure 10]. Besides, we also conducted a subgroup meta-analysis by the intervention time where the effect of *Bifidobacterium* was increased both by ≥4 weeks and <4 weeks in the random effect meta-analysis [MD = 0.53, 95% CI (0.23, 0.82), $P < 0.05$, Figure 11].

Discussion

The present meta-analysis showed that the consumption of fruits or fruit products was significantly associated with FC in

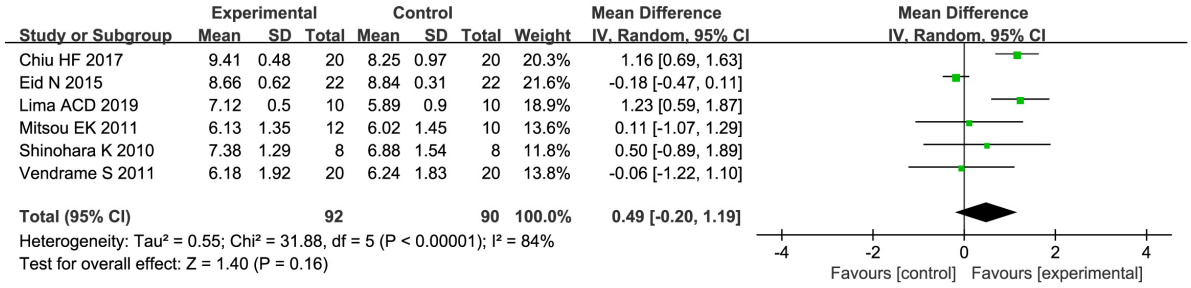

FIGURE 8
Intervention effect of different fruits vs. placebo for *Lactobacillus acidophilus* on patients.

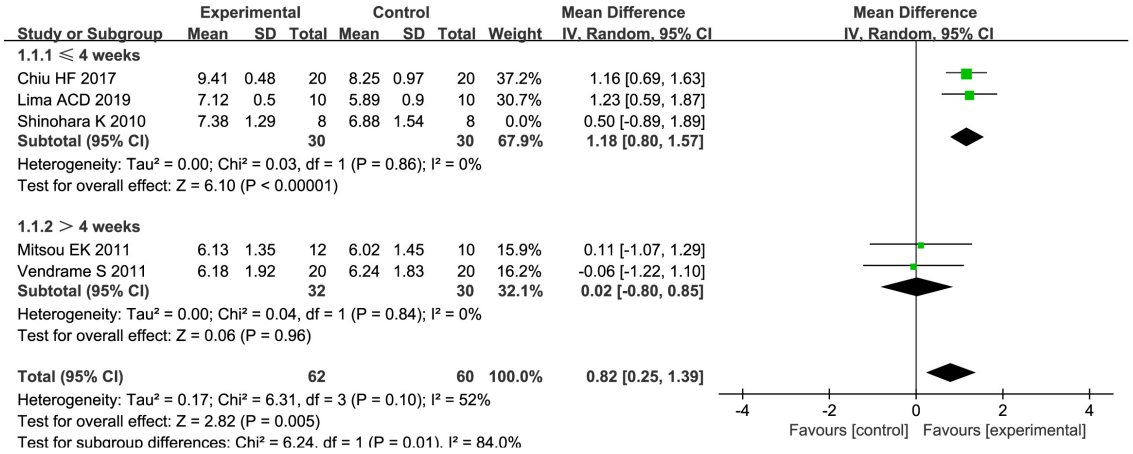

FIGURE 9
Intervention effect of different fruits vs. placebo for *L. acidophilus* on patients in subgroup meta-analysis by different intervention times.

the analysis of RCTs with parallel or cross-over design. The best of this study was the first to assess the different types of fruits in patients with FC in meta-analysis. These findings provided more support for the recommendations encouraging people to consume the most effective fruit to consume.

Fruit refers to the edible part of a plant that is, a mature ovary, consisting of seeds, covering, and any closely connected tissue. Fruit products are processed by fruits, such as frozen foods, canned food, juices, nectars, jams, and preserves. Our meta-analysis suggested that various fruits and fruit products have been shown to alter the microbiota and intestine motility in human studies, including kiwifruits (Bristol stool score, stool consistency, and bifidobacteria), blueberry (bifidobacteria), and orange (bifidobacteria). Kiwifruits are high in fiber and polyphenols and they contain vitamin C twice than orange. Pham et al. indicated that vitamin C can significantly increase microbial alpha diversity and fecal short-chain fatty acids, including butyrate and propionate, and the relative abundance of Collinsella (31). Kiwifruits have been studied for their effect on microbiota and intestine motility in *in vitro* experiments, animal studies, and human trials. Parkar et al. (32) suggested that kiwifruits produced high *Bifidobacterium* spp. compared to the control (water) in *in vitro* fermentation model (32). Various kiwifruits were investigated in animal trials, which shows that fruit components were able to increase the number of *Lactobacillus* spp. compared to the control (33, 34). In clinical trials on constipated adults, kiwifruits significantly increased bowel movement frequency in the constipated group but not in the healthy group (35, 36). Therefore, kiwifruits are more recommended to be consumed by patients with FC based on the current experiments, animal studies, and human trials.

Blueberries were popularized as a "super fruit" mainly due to abundant anthocyanin flavonoids (37). In an experiment with blueberries rich in anthocyanins on mice, gut microbiota, such as *Actinobacteria*, *Coriobacteriaceae*, and some members of *Bifidobacteriaceae*, were increased (38). A randomized crossover trial on adults suggested that the abundance of *Bifidobacterium* spp. and *L. acidophilus* increased compared to the baseline (27). To sum up, blueberry had positive effects on the microbiota composition, including *Bifidobacteria* and *L. acidophilus*. The content of fiber in oranges is higher than in other fruits, such as kiwifruits, apples, plums, and bananas. A randomized

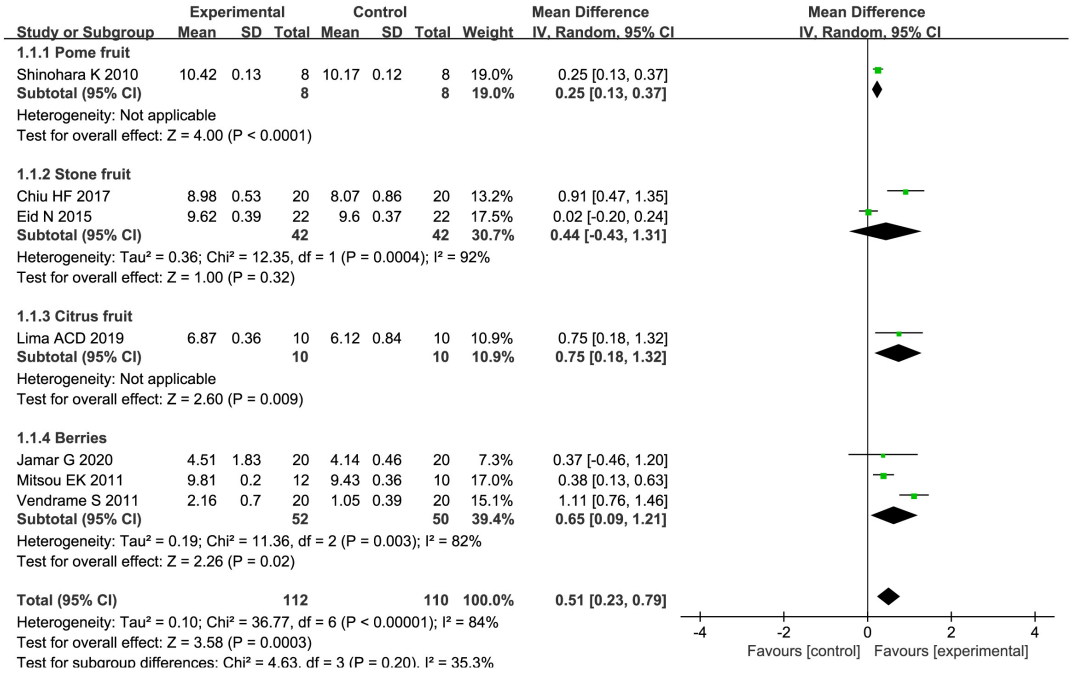

FIGURE 10
Intervention effect of different types of fruits vs. placebo for *Bifidobacterium* on patients in subgroup meta-analysis.

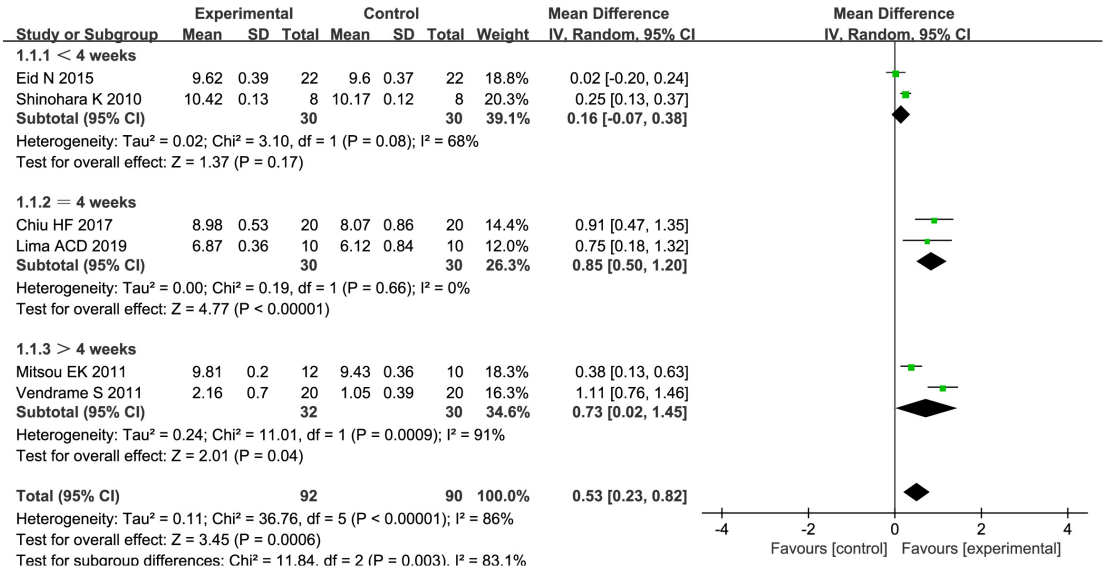

FIGURE 11
Intervention effect of the fruits vs. placebo for *Bifidobacterium* on patients in subgroup meta-analysis by types of intervention time.

crossover trial involving adults has shown that the orange juice group had a higher abundance of the Porphyromonadaceae family and Parabacteroides genus, and the Odoribacteraceae family and Butyricimonas genus than placebo (39). However, we did not equate the effect of orange juice with orange due to lack of fiber in orange juice. So, we would like to suggest that fresh orange should be performed on the human clinical trials in the future.

Ideally, long-term randomized control trials would provide the strongest level of evidence for clinical guidelines. But these studies can be challenging to perform, especially for an intervention, such as fruits and fruit products. A randomized

trial has suggested that kiwifruits may improve constipation symptoms in patients with constipation in 2021 (40), which is consistent with the results of our study that kiwifruits have significantly increased stool frequency [MD = 0.26, 95% CI (0.22, 0.30), $P < 0.0001$, $I^2 = 0$]. Although our meta-analysis suggests that kiwifruit products have a potential symptom alleviation in constipation, the key is whether the kiwifruits planted in different countries have the same symptom-improving effect. We need to be cautious in deciding whether certain types of fruits are suitable or better than others for patients with constipation. A recent systematic review published in 2021 has suggested that there is some evidence for the effects of fruits on gut motility due to gut physiology and microbiota and are helpful in constipation symptom alleviation. However, it is hard to know the effects of fruits and the specific mechanisms behind their potential (17).

Several potential mechanisms have been suggested to explain the relationship between fruit consumption and chronic constipation. Increasing evidence from epidemiological studies in humans and experimental studies in animals showed that altered microbiota has been linked to constipation, and patients with constipation have unbalanced microbiota, such as *Bacteroidetes*, *Bifidobacteria*, and *Lactobacilli*, compared to patients without constipation (41–44). Therefore, lower beneficial microbiota is one of the major causes of constipation and regulating these microbiotas could be one of the major mechanisms.

Fruits are sources of sorbitol, polyphenols, and fiber (45), which are served as a core element of the "Five a Day" fruit recommendation by World Health Organization (WHO) (46). Sorbitol is a beneficial nutrient contained in fruits. Dietary sorbitol cannot be digested and absorbed and has the ability to hold water in its molecules (47, 48). Several studies have shown that sorbitol significantly increased fecal water or fecal weight and then eased constipation (49). It is well-known that polyphenols are inhibitors in fruits for the digestion of carbohydrates. Therefore, fruit intake containing polyphenols may increase undigested carbohydrates that are ready for fermentation by gut microbiota. In addition, the results found that 90–95% of ingested polyphenols reach the colon, which can affect gut microbiota composition and can be metabolized by gut microbiota (50). Some evidence showed that polyphenols have the ability to actively regulate the gut microbiota by increasing the bacteria, such as Bifidobacterium and Lactobacillus, that are helpful for gut health (51–53). While it has been emphasized that polyphenols would be beneficial in the improvement of inflammatory bowel disease as well IBS due to their anti-inflammatory ability (53), data without enough evidence have suggested a direct effect on constipation. Dietary fiber might also contribute to improvement in constipation by different potential mechanisms. Fiber, which is the sum of carbohydrates and it cannot be digested or absorbed in the small intestine, is characterized by polymers of three or more monomeric units (54). Non-fermentable fiber can enter the lower gut intact while viscous fibers have a potential water-binding ability, which can bulk stool significantly (55). Besides, gut microbiota abundance and fecal biomass can be increased by fermentable fiber intake, and the short-chain fatty acid production may also be increased (56).

We performed subgroup analysis to identify potential sources of heterogeneity. Within the subgroup analysis, we examined intervention time as a possible source of heterogeneity; this did show significant interaction between variables ($\chi^2 = 11.84$, $P < 0.05$ by the test of interaction, **Figure 11**). After analysis by the random-effect model, it is shown that an intervention time of fewer than 4 weeks may be better for Bifidobacterium of FC among four RCTs when the fruit intervention is compared with control in adults (16, 23, 24, 27).

Heterogeneity could also be explained by the differences between the studies of the method of dietary assessment. Vendrame et al. (27) collected data *via* self-completed food frequency questionnaires (27); five studies used 3-day dietary records (14, 15, 26, 28, 29) and three studies used medical questionnaires along with dietary habits (16, 23, 24), the remaining studies did not mention the dietary assessment (25, 30). Assessing true dietary intake is inherently difficult, and the use of food frequency questionnaires has been challenged (57, 58). They are likely to cause random or systematic errors. These measurements did not estimate the real connection between diet and diseases. So, we hope that there is a need to incorporate more biological markers of fruits, such as plasma vitamin C into nutritional assessment studies in future clinical trials. Besides, the polyphenols and polyphenol-rich whole foods may have a prebiotic function, with emphasis on the bifidogenic effect, leading to increased excretion of acetate Jamar et al. (28). Although acetate has the potential ability to be a microbiota metabolite, we would like to suggest that large-scale randomized control trials are needed to gain confident conclusions concerning the association between fruit intake and microbiota metabolites in future clinical research.

The current study has some strengths. We included higher-quality studies that have a low risk of bias and high validity for each study, and there are no significant baseline differences between the control and intervention groups. Besides, this is the first study to explore the relationship between fruits and constipation by meta-analysis.

Potential limitations should be considered. First, assessment of real dietary intake like food frequency questionnaires is inherently difficult, which cannot truly estimate the true interaction between fruit and constipation. It is very necessary to emphasize a call for standardization of nutritional epidemiology. We suggest using specific biomarkers of fruit to assess the dietary intake in future clinical trials. Besides, we had to admit that publication bias is a potential concern in

the included studies because the statistical power may be limited since seven studies alone could not assess the publication bias for outcomes, such as *Bifidobacterium*.

Conclusion

Our meta-analysis of randomized and crossover studies demonstrates that intake of fruits is linked to symptom alleviation of FC. Kiwifruits have significantly increased stool frequency than palm date or orange juice in the fixed-effect analysis. Pome fruit, citrus fruit, and berries have increased the *Bifidobacterium* than the stone fruits analyzed by the random-effect model. Prune and orange can increase the number of *L. s acidophilus* compared to the banana or blueberry analyzed with the random-effect model. Further, large-scale studies are needed to gain confident conclusions concerning the association between fruit intake and FC.

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 author.

Author contributions

JH and LW: conceptualization and data curation. JL: formal analysis, investigation, and methodology. HC: supervision, validation, and visualization. QG: writing—original draft and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1018502/full#supplementary-material

SUPPLEMENTARY TABLE 1
The effect of fruits on stool frequency of patients with FC.

SUPPLEMENTARY TABLE 2
The effect of fruits on the stool of consistency patients with FC.

SUPPLEMENTARY TABLE 3
The effect of fruits on Bristol stool score of patients with FC.

SUPPLEMENTARY TABLE 4
The effect of fruits for *Lactobacillus acidophilus* in patients with FC.

SUPPLEMENTARY TABLE 5
The effect of fruits for *Bifidobacterium* in patients with FC.

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Association between the ratio of serum creatinine to cystatin C and bone mineral density in Chinese older adults patients with type 2 diabetes mellitus

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Background: The ratio of creatinine to cystatin C (Cre/CysC), a marker of muscle function and muscle mass, can be used to predict sarcopenia in different populations. Since sarcopenia is closely associated with osteoporosis, this study investigated the association between Cre/CysC and bone mineral density (BMD) in patients with type 2 diabetes mellitus (T2DM).

Method: This cross-sectional study included 391 Chinese patients with T2DM. General information, biochemical indicators, and the BMD of lumbar spine (LS), femoral neck (FN), and total hip (TH) were measured.

Results: Pearson correlation analysis showed that Cre/CysC was significantly positively correlated with the BMD of LS (r = 0.170, p = 0.001), FN (r = 0.178, $p < 0.001$), and TH (r = 0.205, $p < 0.001$). The results of stepwise linear regression suggested that Cre/CysC was the only biochemical predictor of the BMD at three sites (LS: β = 0.137, p = 0.01; FN: β = 0.097, p = 0.038; TH: β = 0.145, p = 0.002).

Conclusion: In older patients with T2DM, high Cre/CysC value is independently positively associated with BMD and hence, Cre/CysC may serve as a valuable marker of osteoporosis.

KEYWORDS

type 2 diabetes, creatinine, cystatin C, Cre/CysC, bone mineral density

Introduction

In the past few years, the incidence of diabetes has increased significantly. According to the 2021 International Diabetes Federation report, diabetes affects approximately 537 million adults aged 20–79 years worldwide, and it is predicted that 783 million people will have diabetes by 2045 (1); thus, diabetes has become an increasingly serious global health problem. Some studies have documented that the risk of osteoporosis is significantly higher in patients with type 2 diabetes mellitus (T2DM) than in those without T2DM (2). The guidelines for primary osteoporosis diagnosis mention bone mineral density (BMD) as an essential marker for the diagnosis of osteoporosis (3). A recent large-scale study assessing the determinants of clinical risk of fractures by genome-wide analysis confirmed that only BMD had a major effect on fractures, and low BMD was a pivotal cause of osteoporotic fracture (4). Therefore, in such patients, early identification of risk factors related to BMD is crucial for the early diagnosis and treatment of osteoporosis and the prevention of adverse fracture outcomes.

Sarcopenia is characterized by both decreased muscle mass and function (5). Compared with the population without diabetes, the incidence of sarcopenia in patients with T2DM increased significantly (6). Studies have shown that muscle mass and muscle function are closely related to bone health, which mainly manifested in the increased risk of falls and fractures (7–9). In the T2DM population, the presence of risk factors, such as reduced physical activity, obesity, and steroid hormone use, will further aggravate muscle and bone diseases.

Creatinine is the product of normal catabolism of muscle tissue and is proportional to muscle mass; however, as renal function affects creatinine level, it cannot be used as a potential indicator of muscle mass in clinical settings. Cystatin C, an endogenous protein, can reliably reflect the glomerular filtration rate (10). Therefore, the ratio of creatinine to cystatin C (Cre/CysC) is considered to be the ratio of muscle mass to total body mass after adjusting for renal function, which reflects muscle volume and is closely related to skeletal muscle mass volume (11). Based on this theory, previous studies have confirmed that Cre/CysC can accurately predict the incidence of sarcopenia in different populations. Osaka et al. have indicated that decreased Cre/CysC is considered an independent predictor of sarcopenia in patients with T2DM (12). Considering the close relationship between muscle mass, muscle function, and osteoporosis, Cre/CysC may be a reliable index for predicting osteoporosis. A study report on indicators of bone properties in older adults showed that Cre/CysC might be used as a biochemical marker of bone property independent of muscle mass in the population with preserved renal function (13). In another study on Japanese postmenopausal women, Cre/CysC had a positive correlation with BMD, and was suggested to be one of the surrogate marker candidates of osteoporosis (3). However, to date, the relationship between Cre/CysC and BMD in patients with T2DM has not been researched. Given these backgrounds, this study aimed to clarify the relationship between serum Cre/CysC and BMD in older patients with T2DM.

Materials and methods

Study patients

This study analyzed data collected from 391 patients at the Department of Endocrinology, Affiliated Hospital of Jining Medical College between June 2021 and April 2022. Information related to demographics, health status and function, health outcomes, including blood biomarker measurements, and BMD measurements, were collected. All patients had been diagnosed with T2DM at baseline according to the 2019 World Health Organization (WHO) standards. The exclusion criteria were as follows: patients younger than 50 years and premenopausal women; patients without anthropometric measures; those lacking blood biochemical index data of creatinine and cystatin C; those lacking BMD examination; those with the presence of malignant tumor, serious liver, kidney, heart disease, or metabolic diseases, such as those involving the pituitary, thyroid, and adrenal glands; and those receiving hemodialysis, immunosuppressive drugs, or drugs that affect bone metabolism, including vitamin D and calcium. The Human Ethics Committee of the Affiliated Hospital of Jining Medical University approved this study.

Body composition measurement

Information on the clinical characteristic, including sex, age, weight, height, body mass index (BMI), blood pressure, duration of diabetes, disease history (such as hypertension, hyperlipidemia, cerebrovascular accident, coronary artery disease, and kidney disease), and drug consumption history were obtained from the electronic medical records of the hospital. Height and weight were measured accurately to 0.1 cm and 0.1 kg, respectively. BMI was calculated as weight (kilograms)/height (meters squared). The duration of diabetes was calculated from the time when T2DM was diagnosed based on the patient's medical records to the year when blood and BMD tests were performed.

Laboratory measurements

After fasting for 8–12 h, fasting blood samples were obtained from all patients for laboratory analyses. Glucose

metabolism indices included the following: glycated hemoglobin (HbA1c), fasting blood glucose (FBG), and serum C-peptide; renal function indices: uric acid (UA), creatinine, and cystatin C; liver function indices: total protein, albumin (ALB), total bilirubin, direct bilirubin, and indirect bilirubin; serum lipid metabolism indices: total cholesterol, triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein, and very low-density lipoprotein; thyroid function indices: free triiodothyronine, free thyroxine, and thyroid-stimulating hormone. Parathyroid hormone, calcitonin, 25-hydroxy-vitamin D_3, serum calcium, serum magnesium, and serum phosphorus were also measured. In particular, creatinine (mg/dl) was determined using a sarcosine oxidase method (Diacegene, Sichuan, China), and cystatin C was measured by the particle-enhanced turbidimetric assay (Zybio, Chongqing, China). Serum creatinine (mg/L) was divided by serum cystatin C to calculate Cre/CysC (mg/L).

Bone mineral density measurement

Dual-energy X-ray absorptiometry (DEXA) was used to measure BMD (grams per square centimeter). Each patient was measured at three sites: the lumbar spine (LS), femoral neck (FN), and total hip (TH). BMD was measured according to the WHO standards as follows: normal, T-scores ≥ -1.0; osteopenia, T-scores between -1.0 and -2.5; and osteoporosis, T-scores < -2.5 (14).

Statistical analysis

All statistical analyses were performed using the SPSS software (V.26.0). Continuous data with a normal distribution are expressed as mean values ± SD, whereas non-normal distributed data are expressed as medians (quartile). An independent sample t-test or Mann–Whitney U test was used to compare the data between the two groups. Categorical data were presented in terms of frequency or percentage and analyzed with Chi-square tests. Pearson's correlation coefficients were used to examine the correlation between biochemical indices and BMD. Variables that significantly correlated with BMD were included in the multiple stepwise linear regression analysis, and multiple stepwise linear regression analysis was performed using the stepwise selection of the variables to determine the variables independently related to the BMD of the LS, FN, and TH. Receiver operating characteristic (ROC) curves were plotted with osteopenia and osteoporosis as state variable. The area under the curve (AUC) and Youden index were calculated to get cut-off points of Cre/CysC. All tests for statistical significance were two-tailed, and statistical significance was set at $p < 0.05$.

Results

Patient characteristics

The clinical and laboratory characteristics of the patients are summarized in Table 1. Among the 391 patients, 202 were men and 189 were women. The average age of patients was 61.17 ± 9.47 years. Patients had an average duration of 10 years of having T2DM. The mean HbA1c level was 8.83 ± 2.06%, which was significantly higher in women (9.07 ± 2.07) than in men (8.61 ± 2.04). The mean creatinine level was 7.03 ± 2.52 mg/L, which was significantly higher in men (7.74 ± 2.35) than in women (6.27 ± 2.38). The mean cystatin C level was 1.11 ± 0.40 mg/L, which was higher in women (1.12 ± 0.50) than in men (1.10 ± 0.27), although the difference was not statistically significant. The average Cre/CysC value was 6.40 ± 1.52, which was significantly higher in men (7.04 ± 1.46) than in women (5.72 ± 1.26). The average BMD of LS, FN, and TH were 1.07 ± 0.19, 0.89 ± 0.16, and 0.94 ± 0.16, respectively. The BMD of men at the three sites was significantly higher than that of women (Table 1).

Correlations between clinical factors and bone mineral densities

The correlations between clinical factors and BMDs of the LS, FN, and TH are shown in Table 2. Creatinine had a significantly positive correlation with LS BMD (r = 0.135, p = 0.008), although without significant correlation with FN BMD (r = 0.036, p = 0.477) and TH BMD (r = 0.058, p = 0.250). Cystatin C was significantly negatively correlated with the BMD of TH (r = −0.107, p = 0.034), but was not associated with the BMD of LS (r = −0.033, p = 0.516) and FN (r = −0.083, p = 0.102). Cre/CysC had a strong positive relationship with the BMD of LS (r = 0.170, p = 0.001), FN (r = 0.178, $p < 0.001$), and TH (r = 0.205, $p < 0.001$). The correlations between creatinine, cystatin C, Cre/CysC, and BMD are shown in Figure 1. In addition, we observed significant correlations between the age, BMI, HbA1c, UA level, HDL, serum phosphorus level, and the BMDs of LS, FN, and TH. The duration of diabetes was only related to FN BMD, whereas TG and FBG were only related to TH BMD (Table 2).

Multiple stepwise linear regression analyses of variables related to the bone mineral densities

Multiple stepwise linear regression analyses of variables related to the BMDs of LS, FN, and TH are shown in Table 3. We observed that Cre/CysC was the only biochemical predictor

TABLE 1 Characteristics of patients included in this study.

Characteristic	Total (n = 391)	Male (n = 202)	Female (n = 189)	P-value
AGE (years)	61.17 ± 7.47	60.14 ± 7.03	62.28 ± 7.79	0.005
DURATION (years)	10.00 (5.00, 17.00)	10.00 (5.00, 17.00)	10.00 (4.25, 16.00)	0.47
BMI (kg/m^2)	25.51 ± 3.89	25.47 ± 3.81	25.55 ± 3.99	0.838
HBA1C (%)	8.83 ± 2.06	8.61 ± 2.04	9.07 ± 2.07	0.029
CREATININE (mg/L)	7.03 ± 2.52	7.74 ± 2.35	6.27 ± 2.38	<0.001
CYSTATIN C (mg/L)	1.11 ± 0.40	1.10 ± 0.27	1.12 ± 0.50	0.686
CRE/CYSC	6.40 ± 1.52	7.04 ± 1.46	5.72 ± 1.26	<0.001
UA (μmol/L)	282.98 ± 93.83	302.82 ± 93.10	261.67 ± 90.10	<0.001
TP (g/L)	65.30 ± 6.30	65.63 ± 6.42	64.95 ± 6.17	0.284
ALB (g/L)	41.43 ± 4.45	42.24 ± 4.03	40.58 ± 4.72	<0.001
TBIL (μmol/L)	14.38 ± 5.33	15.42 ± 5.76	13.27 ± 4.60	<0.001
DBIL (μmol/L)	3.64 ± 1.63	3.72 ± 1.69	3.55 ± 1.57	0.288
IDBIL (μmol/L)	10.80 ± 4.37	11.69 ± 4.73	9.85 ± 3.74	<0.001
TG (mmol/L)	1.25 (0.90, 1.89)	1.28 (0.86, 2.03)	1.23 (0.90, 1.74)	0.368
TCH (mmol/L)	4.37 ± 1.22	4.22 ± 1.28	4.52 ± 1.14	0.017
HDL (mmol/L)	1.24 ± 0.29	1.20 ± 0.28	1.29 ± 0.29	0.001
LDL (mmol/L)	2.61 ± 0.85	2.50 ± 0.85	2.71 ± 0.83	0.015
VLDL (mmol/L)	0.43 (0.31, 0.57)	0.41 (0.30, 0.59)	0.44 (0.33, 0.56)	0.591
FBG (mmol/L)	7.83 ± 3.52	7.75 ± 3.72	7.91 ± 3.31	0.659
FCP (ng/mL)	1.93 ± 1.23	1.86 ± 1.15	2.00 ± 1.31	0.297
FT3 (pmol/L)	4.55 ± 1.80	4.60 ± 0.68	4.49 ± 2.51	0.554
FT4 (pmol/L)	16.97 ± 4.34	17.13 ± 2.38	16.79 ± 5.76	0.443
TSH (miu/L)	2.18 ± 1.76	1.96 ± 1.56	2.43 ± 1.93	0.01
PTH (pg/mL)	35.08 ± 16.52	34.00 ± 15.70	36.23 ± 17.34	0.205
CT (pg/mL)	5.48 (3.29, 12.03)	9.03 (4.48, 13.42)	3.71 (2.54, 5.70)	<0.001
25-(OH)D_3 (ng/mL)	17.48 ± 7.28	18.98 ± 7.66	15.83 ± 6.48	<0.001
CA (mmol/L)	2.27 ± 0.12	2.27 ± 0.12	2.26 ± 0.11	0.395
P (mmol/L)	1.24 ± 0.20	1.22 ± 0.19	1.27 ± 0.21	0.019
MG (mmol/L)	0.91 ± 0.10	0.92 ± 0.08	0.90 ± 0.12	0.091
LS BMD (G/CM^2)	1.07 ± 0.19	1.11 ± 0.17	1.02 ± 0.19	<0.001
FN BMD (G/CM^2)	0.89 ± 0.16	0.95 ± 0.15	0.83 ± 0.15	<0.001
TH BMD (G/CM^2)	0.94 ± 0.16	1.00 ± 0.15	0.89 ± 0.15	<0.001

BMI, body mass index; HbA1c, hemoglobin A1c; Cre/CysC, creatinine to cystatin C ratio; UA, uric acid; TP, total protein; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; IDBIL, indirect bilirubin; TG, triglycerides; TCH, total cholesterol; HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoprotein; FBG, fasting blood glucose; FCP, fasting C-peptide; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; PTH, parathyroid hormone; CT, calcitonin; 25-(OH)D_3, 25-hydroxy-vitamin D_3; Ca, calcium; P, phosphorus; Mg, magnesium; LS BMD, lumbar spine bone mineral density; FN BMD, femoral neck bone mineral density; TH BMD, total hip bone mineral density.

of the BMDs of LS (β = 0.137, p = 0.01), FN (β = 0.097, p = 0.038), and TH (β = 0.145, p = 0.002). In addition to Cre/CysC, BMI (LS: β = 0.216, p < 0.001; FN: β = 0.278, p < 0.001; TH: β = 0.346, p < 0.001), age (LS: β = −0.198, p < 0.001; FN: β = −0.292, p < 0.001; TH: β = −0.264, p < 0.001), and sex (LS: β = 0.146, p = 0.006; FN: β = 0.296, p < 0.001; TH: β = 0.257, p < 0.001) were also independent predictors of BMD at the three sites. Moreover, HbA1c (β = −0.094, p = 0.026) and serum phosphorus (β = 0.090, p = 0.036) were independent predictors of FN BMD. FBG (β = −0.088, p = 0.035) was a predictor of TH BMD (Table 3).

Subgroup analysis according to sex and bone mass

Considering the significant effect of sex on BMD, Pearson correlation and multivariate regression analysis of BMD were performed according to sex groups (Supplementary Tables 1, 2). The Cre/CysC has a significant correlation with BMD in men (LS: r = 0.144, p = 0.042; FN: r = 0.235, p = 0.001; TH: r = 0.284, p < 0.001), and only significantly correlated with LS BMD (r = 0.203, p = 0.005) in women; however, the positive correlation trend was observed in the BMDs of FN (r = 0.108, p = 0.138) and TH (r = 0.114, p = 0.119) in women. In multiple stepwise linear regression analysis, after adjusting for confounding factors, Cre/CysC was the independent predictor of the BMDs of FN (β = 0.157, p = 0.014) and TH (β = 0.21, p = 0.001) in men. Meanwhile, it was the independent predictor of LS BMD (β = 0.159, p = 0.02) in women.

Patients were further classified based on the T-scores as follows: normal, osteopenic, and osteoporotic groups. Of the patients, 140 men (69.3%) and 76 women (40.2%) were diagnosed as normal; 58 men (28.7%) and 81 women (42.9%) were diagnosed with osteopenia; and 4 men (2%) and 32 women (16.9%) were diagnosed with osteoporosis. Older patients,

TABLE 2 Correlations between clinical factors and BMDs.

Variable	LS BMD		FN BMD		TH BMD	
	r	*P*	*r*	*P*	*r*	*P*
Age (years)	−0.230	<0.001	−0.360	<0.001	−0.315	<0.001
Duration (years)	−0.041	0.424	−0.120	0.018	−0.099	0.051
BMI (kg/m^2)	0.244	<0.001	0.345	<0.001	0.403	<0.001
HbA1c (%)	−0.102	0.047	−0.138	0.007	−0.112	0.028
Creatinine (mg/l)	0.135	0.008	0.036	0.477	0.058	0.250
Cystatin C (mg/L)	−0.033	0.516	−0.083	0.102	−0.107	0.034
Cre/CysC	0.170	0.001	0.178	<0.001	0.205	<0.001
UA (μmol/L)	0.126	0.013	0.140	0.006	0.188	<0.001
TP (g/L)	−0.039	0.441	−0.015	0.776	−0.034	0.508
ALB (g/L)	0.013	0.794	0.051	0.312	0.062	0.225
TBIL (μmol/L)	0.035	0.499	0.085	0.095	0.082	0.109
DBIL (μmol/L)	−0.039	0.447	−0.002	0.974	−0.002	0.966
IDBIL (μmol/L)	0.049	0.335	0.089	0.080	0.087	0.086
TG (mmol/L)	0.059	0.252	0.090	0.076	0.111	0.029
TCH (mmol/L)	0.048	0.353	−0.001	0.987	−0.009	0.858
HDL (mmol/L)	−0.129	0.011	−0.169	0.001	−0.181	<0.001
LDL (mmol/L)	0.050	0.327	0.010	0.841	0.005	0.915
VLDL (mmol/L)	0.077	0.131	0.062	0.228	0.054	0.287
FBG (mmol/L)	−0.031	0.540	−0.076	0.135	−0.105	0.040
FCP (ng/mL)	0.080	0.135	0.072	0.173	0.100	0.060
FT3 (pmol/L)	−0.058	0.264	−0.018	0.731	−0.044	0.392
FT4 (pmol/L)	−0.074	0.150	−0.056	0.274	−0.093	0.068
TSH (mIU/L)	0.035	0.497	0.003	0.959	−0.002	0.975
PTH (pg/mL)	−0.032	0.543	−0.095	0.075	−0.076	0.152
CT (pg/mL)	0.071	0.253	−0.003	0.960	−0.013	0.835
25-(OH)D_3 (ng/mL)	−0.044	0.397	0.008	0.875	−0.004	0.945
Ca (mmol/L)	−0.021	0.685	−0.048	0.346	−0.031	0.544
P (mmol/L)	0.157	0.002	0.200	<0.001	0.194	<0.001
Mg (mmol/L)	−0.050	0.332	−0.059	0.249	−0.060	0.246

BMI, body mass index; HbA1c, hemoglobin A1c; Cre/CysC, creatinine to cystatin C ratio; UA, uric acid; TP, total protein; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; IDBIL, indirect bilirubin; TG, triglycerides; TCH, total cholesterol; HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoprotein; FBG, fasting blood glucose; FCP, fasting C-peptide; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; PTH, parathyroid hormone; CT, calcitonin; 25-(OH)D_3, 25-hydroxy-vitamin D_3; Ca, calcium; P, phosphorus; Mg, magnesium; LS BMD, lumbar spine bone mineral density; FN BMD, femoral neck bone mineral density; TH BMD, total hip bone mineral density.

patients with low BMI, and those with low Cre/CysC values were more likely to have osteopenia and osteoporosis. In addition, serum creatinine, UA, ALB, and serum phosphorus levels of patients with osteopenia and osteoporosis were decreased compared to patients with normal *T*-scores. However, the HDL levels of patients with osteopenia and osteoporosis were higher than that of patients with normal *T*-scores. The difference between the groups was statistically significant (Table 4).

The predictive ability of Cre/CysC about osteopenia and osteoporosis was assessed using ROC curve analysis (Figure 2). Cre/CysC showed moderate strength in predicting both osteopenia and osteoporosis with AUC = 0.671 and 0.685, respectively. The values of Cre/CysC = 6.51 (sensitivity 73.7%, specificity 56.9%) and 5.87 (sensitivity 66.7%, specificity 65.1%) were determined as cut-off points for osteopenia and osteoporosis.

Discussion

In this cross-sectional study of Chinese patients with T2DM, we found positive correlations between Cre/CysC and LS, FN, and TH BMDs. After adjusting for sex, age, BMI, and other multiple confounding factors such as serum UA and blood phosphorus, it was still an independent predictor of the above indicators. In addition, according to *T*-scores grouping analysis, the Cre/CysC of the osteoporosis group was significantly lower than that of the osteopenia and normal groups. To our

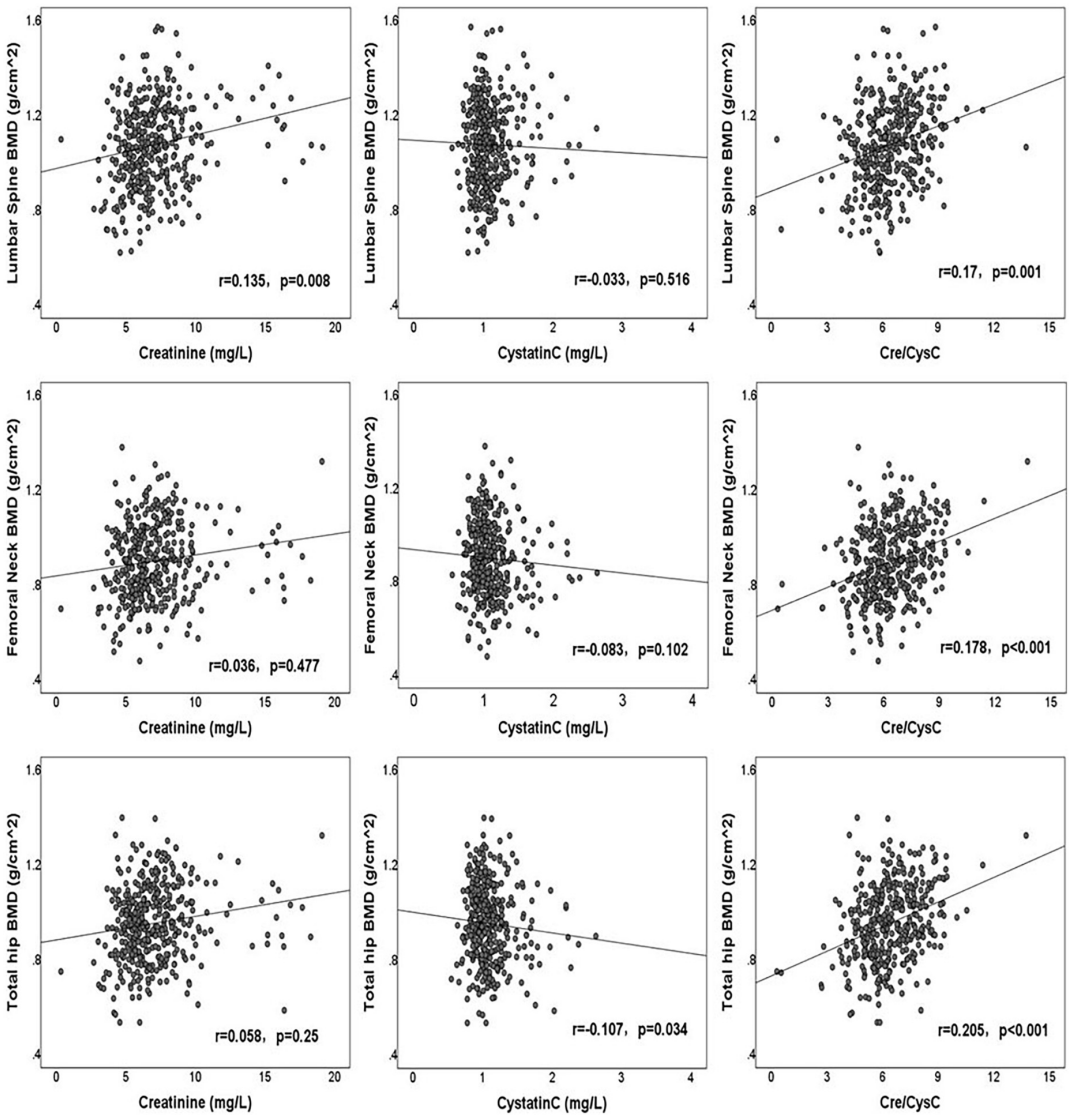

FIGURE 1
Scatter plot of creatinine, cystatin C, creatinine/cystatin C, and BMDs.

knowledge, this is the first time that the correlation between Cre/CysC and BMD has been confirmed in patients with T2DM.

Type 2 diabetes mellitus is an inflammatory chronic condition characterized by insulin resistance and impaired glucose metabolism, as well as complications of multiple organ systems (15). A cohort study reported that the incidence of sarcopenia in patients with T2DM was 15.7%, a significantly higher rate than that in healthy controls (6.9%), and the odds ratio for sarcopenia was 3.06 (16). According to the guideline of European Working Group on Sarcopenia in Older People, sarcopenia can only be diagnosed when a patient has a low muscle strength as well as low muscle mass (5). A major determinant of muscle strength is muscle myosteatosis, which could be reflected by muscle density (high muscle density means low fat infiltration) (17). Therefore, both muscle mass and muscle density are two determining factors for sarcopenia. A previous study on Korean adults with T2DM aged 50 years and older found that the total body muscle mass was an important factor related to FN BMD (2). In patients with T2DM, decreased muscle mass leads to deteriorated insulin sensitivity, aggravated diabetes (18), increased somatostatin secretion, abnormal bone metabolism, and reduced bone mass, and is associated with osteoporosis (19). Moreover, previous studies also reported that compared with the healthy control group, patients with T2DM mainly presented reduced muscle strength and performance, which was related to muscle density (20). The mechanism leading to the decrease of muscle density in patients

TABLE 3 Multiple stepwise linear regression analyses of variables related to the BMDs.

Variable	Beta (95% CI)		*P*-value
LS BMD			
Age	−0.198	(−0.007, −0.003)	<0.001
BMI	0.216	(0.006, 0.015)	<0.001
Sex	0.146	(0.015, 0.092)	0.006
Cre/CysC	0.137	(0.004, 0.030)	0.010
FN BMD			
Age	−0.292	(−0.008, −0.004)	<0.001
Sex	0.296	(0.064, 0.122)	<0.001
BMI	0.278	(0.008, 0.015)	<0.001
HbA1c	−0.094	(−0.013, −0.001)	0.026
P	0.090	(0.005, 0.138)	0.036
Cre/CysC	0.097	(0.001, 0.020)	0.038
TH BMD			
BMI	0.346	(0.011, 0.018)	<0.001
Sex	0.257	(0.053, 0.111)	<0.001
Age	−0.264	(−0.007, −0.004)	<0.001
Cre/CysC	0.145	(0.006, 0.025)	0.002
FBG	−0.088	(−0.008, 0.000)	0.035

Adopted factors: sex, age, BMI, HbA1c, Cre/CysC, uric acid, high-density lipoproteins, P for the LS BMD; sex, age, duration, BMI, HbA1c, Cre/CysC, uric acid, high-density lipoproteins, P for the FN BMD; sex, age, BMI, HbA1c, Cre/CysC, uric acid, triglycerides, high-density lipoproteins, FBG, P for the TH BMD. CI, confidence interval; BMI, body mass index; Cre/CysC, creatinine to cystatin C ratio; HbA1c, hemoglobin A1c; P, phosphorus; FBG, fasting blood glucose.

with T2DM remains unclear, but may be related to insulin resistance and decreased function and number of mitochondria (21, 22).

The circulating Cre/CysC, which can be used as a predictor of myosteatosis and muscle mass (23), was first reported in 2013 and has received widespread attention. It has been increasingly used in the screening of sarcopenia in diabetic and non-diabetic patients. Our previous studies have shown that the Cre/CysC can predict not only the muscle mass but also muscle density in patients with T2DM (24). Based on the close correlation between muscle and bone health, we further confirmed that Cre/CysC could be used as an independent predictor of BMD in the LS, FN, and TH. The positive correlation between Cre/CysC and BMD may be related to the fact that Cre/CysC is an alternative indicator of muscle density and mass; this is consistent with the findings of a small-scale study in patients with primary osteoporosis, which reported that Cre/CysC was positively correlated with LS and FN BMD (3). Furthermore, Komorita et al. confirmed that Cre/CysC was a major risk factor for brittle fractures in patients with T2DM (25). We also observed that the correlation between Cre/CysC and BMD was stronger than that between BMD and creatinine or cystatin C alone, indicating that the Cre/CysC value, rather than serum creatinine or cystatin C level, would be a more appropriate alternative indicator of bone health in patients with T2DM.

The relationship between osteoporosis and sarcopenia is reasonable in the context of the bone–muscle subunit and a common mesenchymal precursor of muscle and bone are formed from (26). The crosstalk between muscle and both has been summarized into two major aspects, including mechanical communication and biochemical communication. The mechanical communication has been investigated in detail and plays critical roles during embryonic patterning, postnatal allometric growth, and the homeostatic relationship of adult life and aging (27). Both muscle and bone could be regarded as secretory/endocrine organs. The myokines secreted by muscles may regulate the bone mineral content, such IL-15 (28), IL-8 (29) and irisin (30). On the other side, the factors released by bone including IGF-1 (26), Wnt3a (31), FGF23 (32) and osteocalcin (33), can mediate myogenesis and muscle function. Therefore, as a marker of sarcopenia, Cre/CysC can also predict BMD. The associations and possible mechanisms between Cre/CysC, sarcopenia and osteoporosis were shown in **Figure 3**.

To further verify the reliability of the conclusion, we conducted a subgroup analysis according to sex and bone mass. When stratified by sex, we found that in men, Cre/CysC was positively correlated with BMD at the three sites and was able to independently predict FN and TH BMD. In women, Cre/CysC was able to independently predict LS BMD and showed tendency for positive correlations with FN and TH BMD. There may be several reasons for the discrepancies in the association between Cre/CysC values and BMD in men and women. First, the number of women enrolled in the study was relatively lower than that of men, and the ability to detect statistical differences was weak. Second, there are significant differences in HbA1c, UA, HDL, and serum phosphorus levels between men and women, which may have weakened the association of Cre/CysC and BMD. Finally, BMI had a positive correlation with all BMDs, whereas age had a negative correlation with all BMDs. In multiple regression analysis, age and BMI as potential confounding factors may weaken the effect of Cre/CysC on BMD. Therefore, the next step is to conduct a large sample study to confirm the effect of Cre/CysC on BMD in different sex.

When stratified by *T*-scores, the Cre/CysC value of the normal bone mass group was the highest, followed by those of the osteopenic and the osteoporotic groups, wherein the difference was significant. The Cre/CysC value decreases with the decrease in bone mass, indicating that Cre/CysC is closely related to BMD. These results are consistent with previous findings that older participants with low BMD levels had increased sarcopenia incidence, decreased muscle strength, low muscle mass, and impaired physical performance (34). Although Cre/CysC showed moderate abilities in predicting osteopenia and osteoporosis in the ROC analysis, it can still help clinicians to avoid unnecessarily DEXA examination because of its low price and convenience of measurement.

In addition to Cre/CysC, this study also found that BMI was positively correlated with BMD, and was an independent

TABLE 4 Characteristics of patients in the normal, osteopenia, and osteoporosis group.

Variable	Normal (n = 216)	Osteopenia (n = 139)	Osteoporosis (n = 36)	P
SEX				
MALE	140 (69.3%)	58 (28.7%)	4 (2%)	<0.001
FEMALE	76 (40.2%)	81 (42.9%)	32 (16.9%)	
AGE (years)	58.71 ± 6.38	63.30 ± 7.07	67.69 ± 8.76	<0.001
DURATION (years)	10.50 (5.00, 16.00)	10.00 (6.00, 17.50)	10.00 (2.00, 17.50)	0.856
BMI (kg/m^2)	26.17 ± 3.73	25.01 ± 3.90	23.44 ± 3.93	<0.001
HBA1C (%)	8.50 ± 1.87	9.26 ± 2.30	9.19 ± 1.89	0.002
CREATININE (mg/L)	7.39 ± 2.67	6.68 ± 2.16	6.19 ± 2.56	0.004
CYSTATIN C (mg/L)	1.09 ± 0.27	1.12 ± 0.29	1.25 ± 0.98	0.077
CRE/CYSC	6.81 ± 1.50	6.01 ± 1.36	5.49 ± 1.40	<0.001
UA (μmol/L)	297.13 ± 97.73	273.15 ± 84.26	235.79 ± 86.53	<0.001
TP (g/L)	65.52 ± 6.41	65.18 ± 6.25	64.46 ± 5.91	0.622
ALB (g/L)	41.94 ± 4.31	41.09 ± 4.52	39.72 ± 4.59	0.011
TBIL (μmol/L)	14.73 ± 5.63	14.25 ± 5.03	12.79 ± 4.38	0.122
DBIL (μmol/L)	3.62 ± 1.65	3.64 ± 1.55	3.76 ± 1.88	0.887
IDBIL (μmol/L)	11.15 ± 4.60	10.64 ± 4.21	9.35 ± 3.17	0.063
TG (mmol/L)	1.28 (0.92, 1.94)	1.19 (0.84, 1.87)	1.17 (0.74, 1.64)	0.363
TCH (mmol/L)	4.28 ± 1.19	4.54 ± 1.30	4.24 ± 1.03	0.123
HDL (mmol/L)	1.21 ± 0.25	1.28 ± 0.29	1.31 ± 0.42	0.017
LDL (mmol/L)	2.56 ± 0.82	2.71 ± 0.87	2.48 ± 0.91	0.153
VLDL (mmol/L)	0.42 (0.32, 0.58)	0.44 (0.31, 0.57)	0.40 (0.30, 0.48)	0.587
FBG (mmol/L)	7.57 ± 3.34	8.33 ± 3.82	7.43 ± 3.22	0.108
FCP (ng/mL)	1.94 ± 1.13	2.00 ± 1.37	1.57 ± 1.17	0.212
FT3 (pmol/L)	4.55 ± 0.74	4.62 ± 2.89	4.22 ± 0.71	0.522
FT4 (pmol/L)	16.79 ± 2.58	17.33 ± 6.39	16.70 ± 2.67	0.490
TSH (miu/L)	2.19 ± 1.74	2.24 ± 1.90	1.95 ± 1.25	0.704
PTH (pg/mL)	34.36 ± 16.37	35.00 ± 15.17	39.65 ± 21.53	0.235
CT (pg/mL)	6.53 (3.75, 12.53)	5.38 (2.86, 11.44)	3.38 (2.16, 5.80)	0.150
25-(OH)D_3 (ng/mL)	17.65 ± 6.70	17.38 ± 8.32	16.87 ± 6.56	0.826
CA (mmol/L)	2.27 ± 0.12	2.27 ± 0.11	2.26 ± 0.11	0.770
P (mmol/L)	1.26 ± 0.19	1.24 ± 0.22	1.16 ± 0.19	0.020
MG (mmol/L)	0.91 ± 0.08	0.91 ± 0.08	0.94 ± 0.20	0.167
LS BMD (G/CM^2)	1.17 ± 0.14	0.98 ± 0.14	0.77 ± 0.08	<0.001
FN BMD (G/CM^2)	0.99 ± 0.12	0.79 ± 0.08	0.68 ± 0.11	<0.001
TH BMD (G/CM^2)	1.05 ± 0.12	0.84 ± 0.09	0.72 ± 0.12	<0.001

BMI, body mass index; HbA1c, hemoglobin A1c; Cre/CysC, creatinine to cystatin C ratio; UA, uric acid; TP, total protein; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; IDBIL, indirect bilirubin; TG, triglycerides; TCH, total cholesterol; HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoprotein; FBG, fasting blood glucose; FCP, fasting C-peptide; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; PTH, parathyroid hormone; CT, calcitonin; 25-(OH)D_3, 25-hydroxy-vitamin D_3; Ca, calcium; P, phosphorus; Mg, magnesium; LS BMD, lumbar spine bone mineral density; FN BMD, femoral neck bone mineral density; TH BMD, total hip bone mineral density.

predictor for LS, FN, and TH BMDs in patients with T2DM, which is in accordance with previous studies showing that increased BMD in patients with T2DM was caused by increased BMI (35, 36). Patients with T2DM are more prone to obesity and dyslipidemia, and increased BMI may lead to increased bone strain in daily activities. We also noticed a negative correlation between age and BMD, which is reasonable because BMD gradually decreases with increasing age. A significantly higher BMD was observed in men than in women based on the impact of sex differences on BMD. There was a significant positive correlation between serum phosphorus and TH BMD. Previous studies have shown that relatively high blood phosphorus levels in the normal range may be beneficial to BMD (37). Phosphate is important for osteoblast differentiation and extracellular matrix mineralization, with its level directly affecting bone metabolism (38). HbA1c is used as an indicator of diabetes control, which had a significant negative correlation with FN BMD, indicating that patients with poor control of diabetes have lower BMD, higher risk of osteoporosis, and fracture. A cohort study in Taiwan observed similar results as ours, which showed that patients with T2DM and higher HbA1c had a higher risk of fracture (39). FBG is also a common indicator of blood glucose

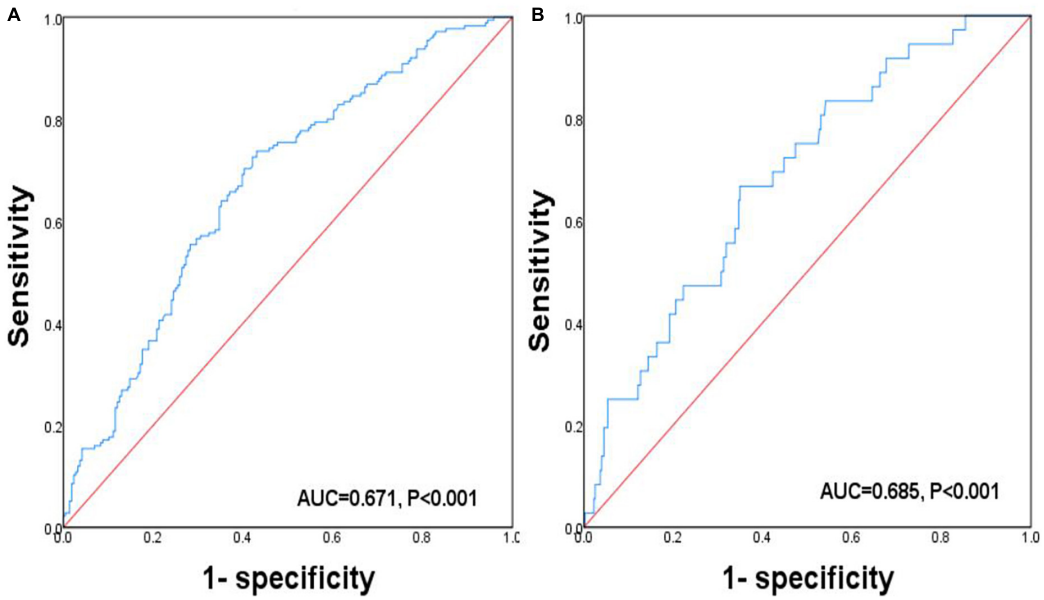

FIGURE 2
Receiver operating characteristic curve analysis related to the impact of Cre/CysC on the BMDs. **(A)** ROC curve analysis related to the impact of Cre/CysC on the diagnosis of osteopenia. **(B)** ROC curve analysis related to the impact of Cre/CysC on the diagnosis of osteoporosis.

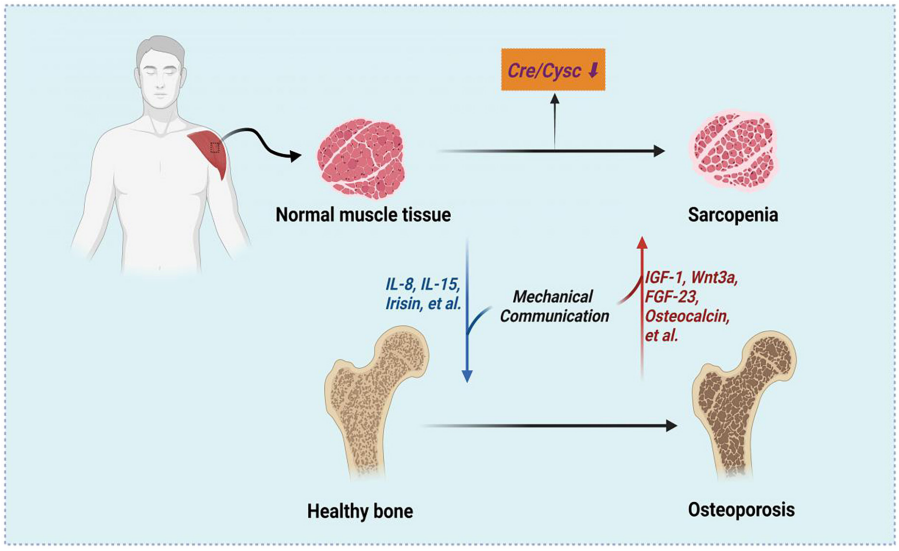

FIGURE 3
The associations between Cre/CysC, sarcopenia and osteoporosis. Created with BioRender.com.

control, and a significant negative correlation was observed with the TH BMD.

In order to eliminate or lessen the interference of sex hormones, our study only included the patients older than 50 and all the women were postmenopausal. In fact, for all women (including those of reproductive age), blood levels of estradiol and testosterone were significant determinants of BMD (40). A decline in estradiol level has been recognized as the most critical hormonal regulator of the menopause-associated decrease in BMD (41). A recent genome-wide study provided further support of the effects of estradiol on BMD in maintaining skeletal health in postmenopausal women (42). The bone-sparing effect of estrogen is antiresorption by inhibition of osteoclast activity, and testosterone, like estrogen, appears to stimulate bone turnover, acting directly or indirectly *via* conversion into estradiol in human osteoblasts to

increase androgen receptor expression and stimulate bone cell proliferation and mineralization (43, 44).

This study had a few limitations. First, due to the cross-sectional design, the causal relationship between Cre/CysC and BMDs could not be determined. Therefore, prospective studies are required for further verification. Second, blood biochemical indicators were only measured once at baseline, which may have caused measurement errors. Third, we did not evaluate the muscle mass and function (e.g., handgrip strength and gait speed) and could not further confirm that the ability of Cre/CysC to predict BMD was achieved through the muscle. Finally, this is a single-center study and participants of this study were mainly Chinese Han adults, therefore it is not clear whether our conclusion can be generalized to other ethnic groups.

Conclusion

In conclusion, the present study firstly demonstrated that the Cre/CysC may be a valuable predictor of BMD in Chinese older adults patients with T2DM. It may help clinicians to avoid unnecessarily DEXA examination and important clinical significance because of its low price and have convenience of measurement.

Data availability statement

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

Ethics statement

This study was approved by the Ethics Committee of the Affiliated Hospital of Jining Medical University (2020-BS-008).

Author contributions

TG and MZ conceived and designed this study. TG collected the data and drafted a manuscript. TG and FL analyzed the data. YH, MJ, and GL contributed to the collection of materials and the revision of the article. FL, QY, BB, and MZ made critical changes to the writing of the article. All authors have access to the database, contributed to this article, and approved its publication.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1035853/full#supplementary-material

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Serum albumin to globulin ratio prior to treatment as a potential non-invasive prognostic indicator for urological cancers

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Background: Numerous clinical studies have reported an association between the pretreatment albumin to globulin ratio (AGR) and survival outcomes of urological cancers. However, these conclusions remain controversial. Therefore, we performed a meta-analysis to explore the prognostic value of the AGR in urinary system tumors.

Methods: We retrieved eligible studies published up to June 2022 through a comprehensive search of multiple databases. Pooled hazard ratios (HRs) with 95% confidence intervals (CI) for overall survival (OS), cancer-specific survival (CSS), recurrence-free survival (RFS), progression-free survival (PFS), and biochemical recurrence-free survival (BRFS) were used to evaluated the predictive effect of the AGR before treatment in urinary system tumors. Heterogeneity test, random-effects models, fixed-effects models and sensitivity tests were used for analyses.

Results: A total of 21 studies with 18,269 patients were enrolled in our meta-analysis. We found that patients with urinary system cancer with low AGR prior to treatment had poor OS [HR = 1.93, 95% CI (1.56–2.39), $p < 0.001$], CSS [HR = 2.22, 95% CI (1.67–2.96), $p < 0.001$], RFS [HR = 1.69, 95% CI (1.29–2.22), $p < 0.001$], and PFS [HR = 1.29, 95% CI (0.54–3.07), $p < 0.001$]. For prostate cancer (PCa), a low pretreatment AGR was associated with poor BRFS [HR = 1.46, 95% CI (1.28–1.67), $p < 0.001$]. Also, a subgroup analysis, stratified by ethnicity, cancer type, cutoff value, sample size and publication year, was conducted. The results showed that worse OS and CSS were significantly associated with these factors.

Conclusion: Our meta-analysis revealed that the AGR before treatment could be used as a non-invasive predictive biomarker to evaluate the prognosis of urological cancer patients in clinical practice.

KEYWORDS

urological cancers, albumin to globulin ratio, meta-analysis, prognosis, survival

Key messages

- Many studies have reported the association between the pretreatment albumin to globulin ratio (AGR) and prognosis of urological cancers, and these conclusions remain controversial.
- Meta-analysis was conducted for evaluating the prognostic value of pretreatment AGR for patients with urological cancer.
- AGR prior to treatment can be used to predict the prognosis of patients with urological cancer.

Introduction

According to cancer statistics, in 2022 approximately 1,918,030 cancer cases will be diagnosed worldwide; cancer is still one of the leading causes of death (1). Urinary system cancers (prostate cancer, renal cancer, bladder cancer and upper tract urothelial cancer), belonging to the ten leading cancer types of diagnosed malignances; the incidence and death rates of these four urological carcinomas are increasing each year, especially in both developing and developed countries (2, 3). Over the past decades, despite considerable advances in early detection and surgical techniques, and medical therapies (e.g., chemotherapy, radiotherapy, targeted therapy, and immunotherapy) used in urinary system cancers, the 5-year survival outcome of patients diagnosed with urinary tumors remains poor; and the risk of recurrence and progression of these tumors is high (4). Numerous clinical studies have evaluated the prognosis of patients with urological cancers and have assisted clinicians in making follow-up treatment protocols using TNM stage, grade, tumor size, symptoms, and paraneoplastic syndromes (5–7). However, using the above clinical prognostic indicators alone can not accurately assess the extent of disease extent or define prognosis (7, 8). Hence, reliable, non-invasive and cost-effective pretreatment prognostic biomarkers need to be identified to evaluate the prognosis of urinary cancers and guide clinical individualized clinical treatment.

In recent years, accumulating evidence has shown that several preoperative blood-based biomarkers, such as the De Ritis ratio, neutrophil-lymphocyte ratio (NLR), serum albumin, platelet–lymphocyte ratio (PLR), and lymphocyte–monocyte ratio (LMR) have been verified as an independent prognostic indicators for patients with urinary system tumors (9–12). Recent epidemiological studies have indicated that cancer-related inflammation and nutritional status are closely associated with tumorigenesis, tumor progression, and oncological outcomes (13). The major components of serum proteins, serum albumin and globulin are valuable predictors in cancer and play a crucial role in inflammation and immunity (14, 15). The albumin-to-globulin ratio (AGR) is calculated as the albumin level divided by the globulins level. Previous studies have reported that pretreatment AGR is inversely associated with poor survival in patients with genitourinary malignant tumors (16–20). However, according to the published articles, the use of pretreatment AGR as a prognostic biomarker remains controversial. Pradere et al. (16) failed to demonstrate that upper tract urothelial carcinoma (UTUC) patients with low pretreatment AGR were associated with overall survival (OS) and recurrence-free survival (RFS). Therefore, in this context, our meta-analysis aimed to use the related published studies to explore the prognostic value of pretreatment AGR in urological cancers, to make individualized clinical decisions, and to improve patients survival.

Materials and methods

Literature search and eligibility criteria

Eligible citations were retrieved from Web of Science, PubMed, Google Scholar, and Cochrane Library up to June of 2022. Additionally, we also conducted a manual search of the references of the relevant studies. All searches were limited to human studies and no language restrictions were applied.

On the basis of our research objectives, the following search terms were used: ("albumin to globulin ratio" OR "albumin/globulin ratio" OR "AGR") and ("urinary malignancy" OR "urinary neoplasm" OR "urinary cancer" OR "urinary tumor" OR "renal malignancy" OR "renal neoplasm" OR "renal cancer" OR "renal tumor" OR "bladder malignancy" OR "bladder neoplasm" OR "bladder cancer" OR "bladder tumor" OR "upper tract urothelial malignancy" OR "upper tract urothelial neoplasm" OR "upper tract urothelial cancer"

OR "upper tract urothelial tumor" OR "prostate malignancy" OR "prostate neoplasm" OR "prostate cancer" OR "prostate tumor" OR "testicular malignancy" OR "testicular neoplasm" OR "testicular cancer" OR "testicular tumor" OR "transitional cell malignancy"). Since we included published articles, there was no need for review by an ethics committee. Our meta-analysis was conducted according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guideline (21).

Studies had to meet the following inclusion criteria: (1) patients were confirmed as urinary cancer by histopathology; (2) studies reported the correlation between pretreatment AGR and prognosis of patients with urinary cancer; (3) all the studies provided hazard ratios (HR) and related 95% confidence intervals (CIs) of survival outcomes, including overall survival (OS), cancer-specific survival (CSS), recurrence-free survival (RFS), progression-free survival (PFS), or biochemical recurrence-free survival (BRFS); and (4) studies with randomized controlled trials (RCTs), case-control studies, or cohort studies. The exclusion criteria were as follows: (1) duplicated studies; (2) conference abstracts, reviews, letters and case reports; (3) basic experiments and animal researches; and (4) studies with unavailable data.

Quality evaluation

In this study, the Newcastle-Ottawa Scale (NOS), which includes three domains (selection of the cohort, comparability of the groups, and quality of the outcomes), was used to assess the quality of the included studies (22). The NOS scale has nine stars, and a score of six or more was considered a high-quality study in our meta-analysis.

Data extraction

The primary basic information for each included study was as follows: first author's name, country, study design, sample size, intervention, age, cancer type, cut-off value for AGR, analysis method, and follow-up time. In addition, the pooled HRs and corresponding 95% CIs of the survival outcomes (OS, CSS, RFS, PFS, and BRFS) were extracted. As a common index to evaluate the prognosis of cancer patients, OS is only concerned about whether the patient dies, not the specific cause of death and the follow-up time is long. The other tumor-specific prognostic indexes, such as CSS, RFS, PFS, or/and BRFS, are supplement to OS for better manage tumor patients. Therefore, in order to better evaluate the clinical efficacy of AGR in the prognosis of urinary cancers, we made a comprehensive analysis of these indexes. If univariate and multivariate analyses were conducted in a study, we extracted the multivariate analysis data for follow-up analysis.

The above steps independently performed by two authors, and a third author resolved any discrepancies.

Statistical analysis

All the statistical data were processed by Stata 16 (StataCorp LP, College station, TX, United States of America LP, University City, TX, USA). For studies that provided only the Kaplan-Meier curves, we used Engauge Digitizer 4.1 software to extract the relevant survival data. The HRs and corresponding 95% CIs of the included articles were extracted to assess the prognostic significance of the AGR in urological cancers. Cochrane's Q test and Higgin's I^2 test were used to measure the heterogeneity among the included studies. According to the results of the heterogeneity test, the random-effects model was utilized with high heterogeneity ($I^2 \geq 50\%$ or $p < 0.1$). Otherwise, fixed-effects models were used for the analyses. Sensitivity analysis, involving the removal of each individual study, was also used to assess the reliability and stability of our survival outcomes. Additionally, Begg's test was performed to identify potential publication bias across studies if ten or more articles were included in meta-analysis. A value of P less than 0.05 was considered as a statistical significance.

Results

Search results and study characteristics

Figure 1 shows the detailed screening process used in this meta-analysis. Based on the search of electronic databases, 384 published articles were initially found, and 172 studies remained after removing 212 duplicates. After screening the titles and abstracts, 33 studies remained for further evaluation, and their full texts were read for eligibility. Twelve studies were excluded from the full-text analysis for the following reasons: nine reviews, two letters and comments, and one did not provide available data. Finally, there were 18,269 patients who were included in the 21 studies (16–19, 23–39)in this meta-analysis.

From 2017 to 2022, all the eligible studies were published, with a sample size between 104 and 6,041 participants and the cutoff values of AGR ranged from 1.11-1.6. Notably, all the included studies were only case-control, six of the 21 studies were conducted in a single center, and 15 studies were conducted in multiple centers. Of the included studies, five studies reported the correlation between pretreatment AGR and prognosis of bladder cancer (BC), seven articles explored upper tract urothelial carcinoma (UTUC), five investigated renal cell cancer (RCC), and two focused on prostate cancer (PC). Using the NOS score, eighteen articles scored 7-9 on the NOS and

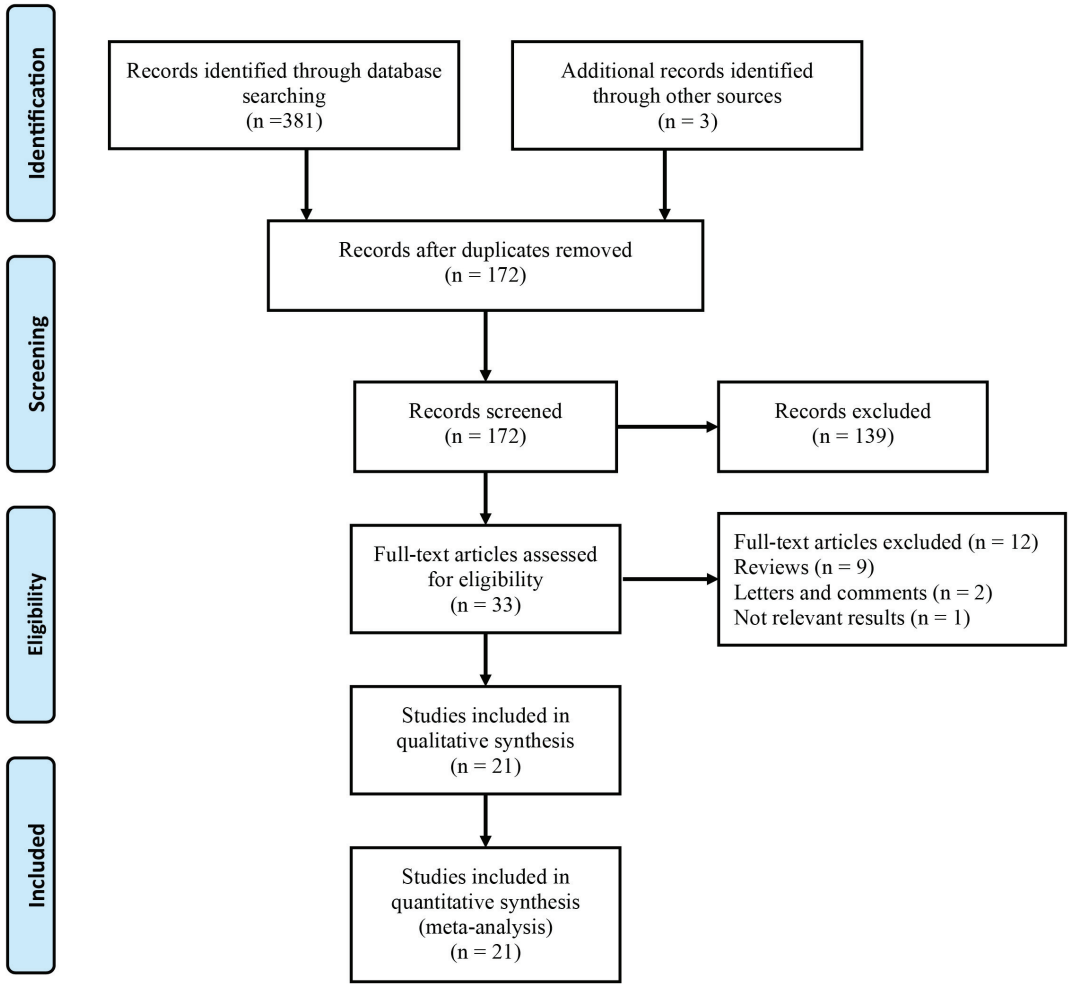

FIGURE 1
Flow diagram of the study selection process.

three studies scored 5 or 6 on the NOS, indicating high quality of the included articles. Table 1 summarizes the characteristics of the included articles.

Prognostic significance of the albumin to globulin ratio for overall survival

A total of 15 studies (16, 18, 24–35, 37) involving 9,554 patients revealed an association between AGR and OS in a multivariate analysis of urological cancers. According to Figure 2, patients with low pretreatment AGR had a worse survival outcomes than those with high AGR [HR = 1.93, 95% CI (1.56–2.39), $p < 0.001$]. Because of the high heterogeneity between the studies ($I^2 = 75.2\%$, $p < 0.001$), we used a random-effects model. To clarify the source of heterogeneity between studies, we performed subgroups analysis based on ethnicity, sample size, urothelial carcinoma (yes or no), AGR cut-off values and publication year. The results of the subgroups analysis also supported the fact that low AGR was related to poor OS and revealed that ethnicity, sample size, urothelial carcinoma (yes or no), AGR cut-off values, and publication year might be causes of high heterogeneity (Figure 3).

Prognostic significance of the albumin to globulin ratio for cancer-specific survival

Study reports from 10 studies (24, 28, 29, 31–36, 38), with 5,455 patients enrolled, indicated the prognostic value of AGR before treatment in patients with urinary system cancers on CSS. Because of Cochrane's Q test and I^2 test revealed substantial heterogeneity among studies ($I^2 = 75.6\%$, $p < 0.001$), a random-effects model was used to combine the HR of each study. Pooled analysis showed that decreased pretreatment AGR was

TABLE 1 Baseline characteristics of include studies and methodological assessment.

Authors (year)	Country	Study design	Sample size	Intervention	Age[a]	Cancer type	Stage	Cutoff	Follow-up time[c] (months)	Endpoint	Quality score
Zhang et al. (29)	China	Retrospective	187	RNU	70 (61–74)	UTUC	N	1.45	Median 78 (32-92)	OS, CSS	8
Liu et al. (36)	China	Retrospective	296	RC	61.71 ± 11.08	BC	N	1.6	Median 72.0 (49.75-115.50)	RFS, CSS	7
Liu et al. (37)	China	PSM	104	RC	NA	BC	N	1.55	Median 38 (1–90)	OS, PFS, TSS	7
Chen et al. (24)	China	PSM	592	RN and PN	NA	RCC	N	1.22	Median 42.3 (3–50)	OS, CSS	7
He et al. (18)	China	Retrospective	895	RN or PN	51.44 ± 13.44	RCC	N	1.47	Median 69.68 (95%CI: 65.73–73.63)	OS	7
Fukushima et al. (30)	Japan	Retrospective	105	RNU	74 (49–89)	UTUC	N	1.24	Median 46 (22–83)	OS, DFS	6
Otsuka et al. (31)	Japan	Retrospective	124	RNU	69 (64–75)	UTUC	N	1.4	Median 55 (28–)	OS, RFS, CSS	7
Koparal et al. (25)	Turkey	Retrospective	162	RN and PN	56.5 ± 11.8	RCC	N	1.4	Median 27.5 (6–89)	OS, DFS	6
Xu et al. (32)	China	Retrospective	620	RNU	NA	UTUC	N	1.45	Median 50 (28–78)	RFS, CSS, OS	8
Niwa et al. (19)	Japan	Retrospective	364	TUR	71 (63–77)	BC	N	1.6	Median 47 (18–89)	RFS, PFS	7
Omura et al. (33)	Japan	Retrospective	179	RNU	75 (66–79)	UTUC	N	1.25	Median 34 (17–63)	OS, CSS	7
Chung et al. (26)	Korea	Retrospective	2970	RN or RPN	55.6 ± 13.2	RCC	N	1.47	Median 26.0 (9.0–59.0)	OS, RFS	8
Oh et al. (38)	Korea	Retrospective	176	RC	68.05 ± 8.96	BC	N	1.32	Median 32.4 (0.2–95.3)	CSS, MFS	8
Quhal et al. (39)	multicenter	Retrospective	1096	TURBT	67 (58–74)	BC	N	1.41	Median 63.7 (25.3–111)	PFS, RFS	8
Miura et al. (34)	multicenter	Retrospective	2492	RNU	69 (27–97)	UTUC	N	1.4	Median 38	RFS, CSS, OS	7
Pradere et al. (16)	multicenter	Retrospective	172	NAC + RNU	68 (63–73)	UTUC	N	1.42	Median 26 (11–56)	OS, RFS	8
Taguchi et al. (35)	multicenter	Retrospective	176	pembrolizumab	71 (66–76)	Mix[b]	N + M	0.95	Median 7.5 (4–14)	OS, CSS, PFS	7
Laukhtina et al. (28)	multicenter	Retrospective	613	RN	57 (50–64)	mRCC	M	1.43	Median 31 (16–58)	OS, CSS	8
Aktepe et al. (27)	Turkey	Retrospective	163	Target therapy	60 (53–65)	mRCC	M	1.11	NA	OS, PFS	6
Aydh et al. (23)	multicenter	Retrospective	6041	RP	61(57–66)	PCa	N	1.31	Median 45 (35–58)	BRFS	8
Chung et al. (17)	Korea	Retrospective	742	RP	NA	PCa	N	1.53	NA	BRFS	8

[a]Age, Mean ± SD/Mean (Range).

[b]Mix, bladder cancer, upper tract urothelial carcinoma.

[c]Follow-up Time, median (range)/median; PSM, propensity score-matched; RNU, radical nephroureterectomy; RN, radical nephrectomy; PN, partial nephrectomy; BC, bladder Cancer; UTUC, upper tract urothelial carcinoma; RCC, renal cell carcinoma; PCa, prostate cancer; N, non-metastatic; N + M, non-metastatic + metastatic; TURBT, Transurethral resection of bladder tumor; NAC, neoadjuvant chemotherapy; OS, overall survival; CSS, cancer-specific survival; RFS, recurrence-free survival; TSS, tumor-specific survival; PFS, progression-free survival; MFS, metastasis-free survival; NA, age data was not available.

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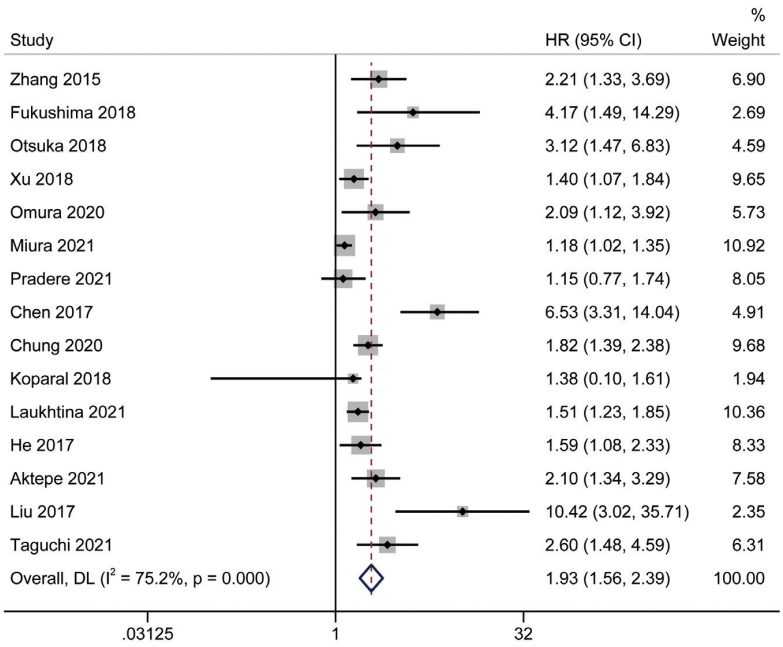

FIGURE 2
Forest plot reflects the association between AGR and OS for urological cancers.

significantly associated with shorter CSS [HR = 2.22, 95% CI (1.67–2.96), $p < 0.001$] (Figure 4). Subgroups analysis stratified by ethnicity, sample size, urothelial carcinoma (yes or no), AGR cut-off values and publication year, and results showed that low pretreatment AGR was associated with poor CSS (Figure 5).

Prognostic significance of the albumin to globulin ratio for recurrence-free survival

Eight studies (16, 19, 26, 31, 32, 34, 36, 39) assessed the prognostic impact of AGR before treatment on RFS using multivariate analysis. Since the statistics showed considerable inter-study heterogeneity ($I^2 = 86.4\%$, $p < 0.001$), a random-effects model was used. The pooled meta-analysis results showed that low pretreatment AGR could predict inferior RFS independently [HR = 1.69, 95% CI (1.29–2.22), $p < 0.001$] (Figure 6).

Prognostic significance of the albumin to globulin ratio for progression-free survival and biochemical recurrence-free survival

Limited related data from four studies (19, 35, 37, 39) and two studies (17, 23) were suitable for PFS and BRFS analyses, respectively. Analysis of datasets revealed an association between low AGR and worse PFS [HR = 1.29, 95% CI (0.54–3.07), $p < 0.001$] (Figure 7A), and poor BRFS [HR = 1.46, 95% CI (1.28–1.67), $p < 0.001$] (Figure 7B).

Sensitivity analysis

In this meta-analysis, we conducted a sensitivity analysis of OS and CSS outcomes to ascertain the strength of our results. The pooled HRs of OS and CSS were not significantly affected when the study was removed. Therefore, we believe that our results are reliable (Figure 8).

Publication bias

Figure 9 presents the funnel plots for OS and CSS, and asymmetry was observed by visual inspection of the Begg's funnel plots. Therefore, a potential publication bias may have was existed for the association of pretreatment AGR, OS, and CSS based on funnel plots. Our Begg's statistical tests aslo indicated that there was a significant publication bias (OS, $p = 0.018$; CSS, $p < 0.001$).

Discussion

Recurrence and metastasis of urinary tumors are common and seriously affect both the prognosis and quality of life

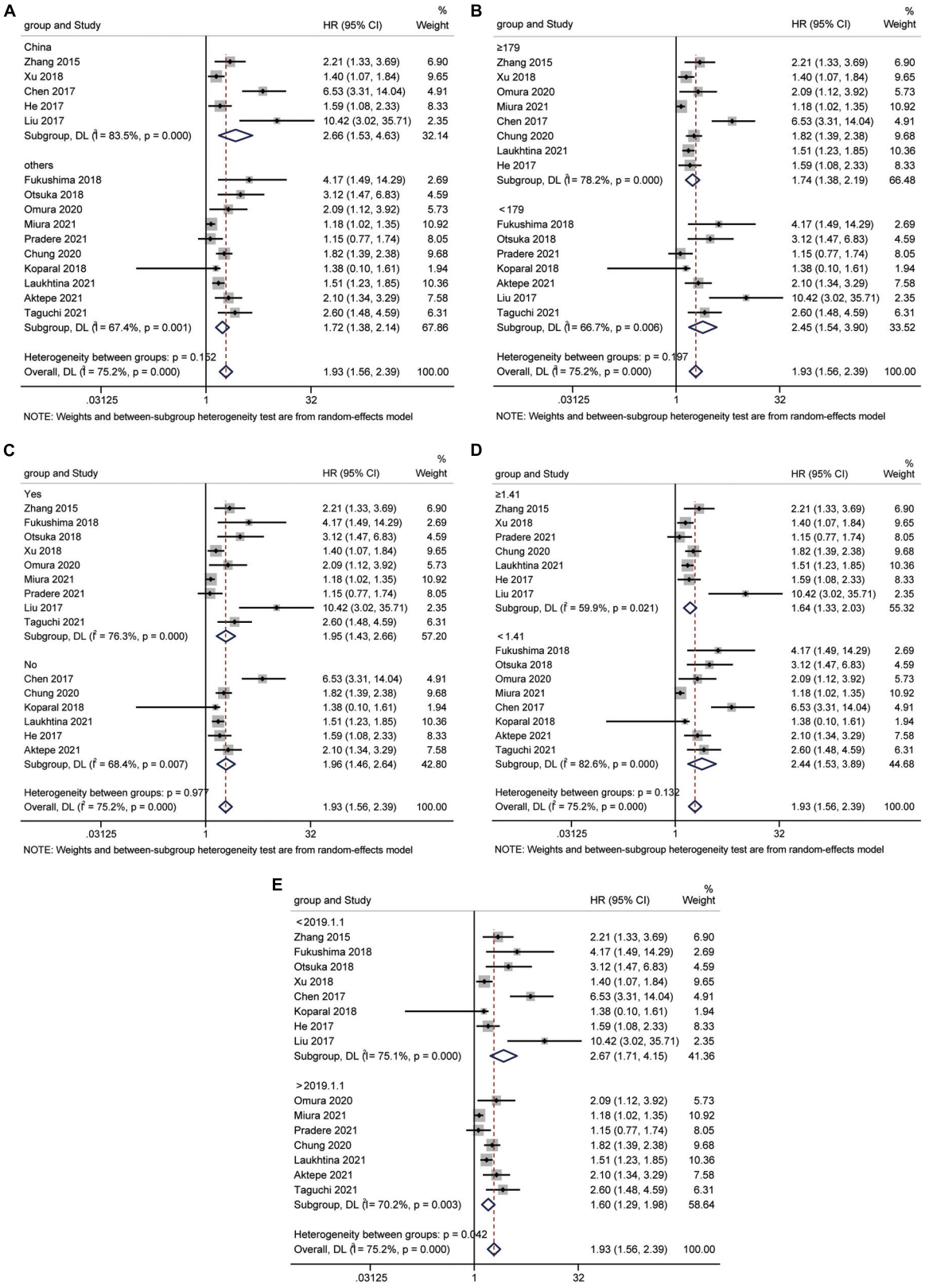

FIGURE 3
Forest plot for subgroup analysis of OS and AGR. **(A)** Subgroup analysis of OS and AGR according to ethnicity; **(B)** Subgroup analysis of OS and AGR according to sample size; **(C)** Subgroup analysis of OS and AGR according to urothelial carcinoma (yes or no); **(D)** Subgroup analysis of OS and AGR according to cut-off values; **(E)** Subgroup analysis of OS and AGR according to publication year.

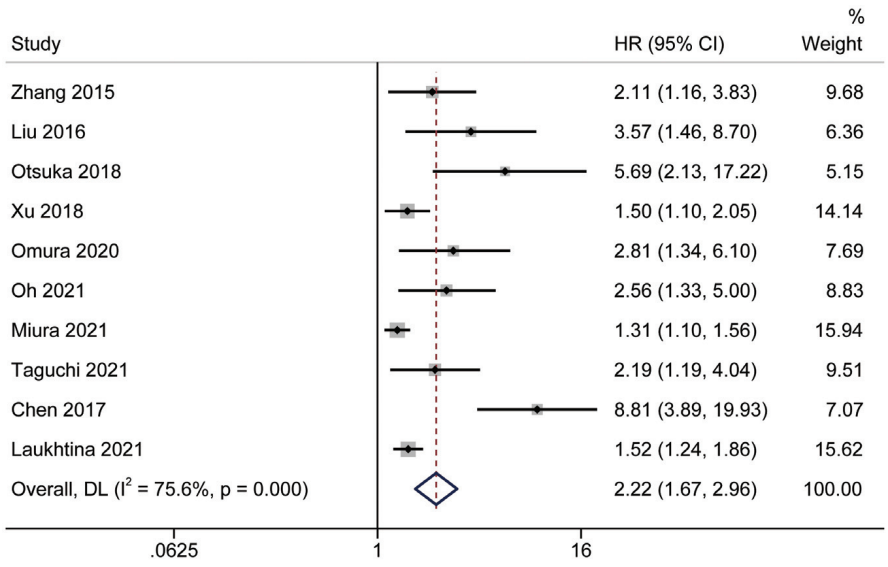

FIGURE 4
Forest plot reflects the association between AGR and CSS for urological cancers.

of patients. Approximately 75% of high-grade non- muscle-invasive bladder cancer (NMIBC) cases would relapsed, progressed or died within 5 years (40). For urothelial cancer, even if the patients undergo radical surgery, the 5-year survival rate is less than 50% (41). Furthermore, 20% to 30% of patients with RCC undergoing curative resection have distant metastasis during follow-up, and the 5-year survival rate is very low (5-10%) (42). Therefore, it is of great significance to identify the predictive indicators that affect the prognosis and treatment decision-making for urinary cancer. To our knowledge, this is the first and most comprehensive meta-analysis to evaluate the prognostic impact of AGR on UC.

As a promising blood marker, some studies have reported a relationship between pretreatment AGR and urological cancers up to now. Owing to the inconsistent results of these studies, we iperformed a meta-analysis of twenty-one published studies involving 18,269 patients to further evaluate the potential value of AGR for predicting the survival of patients with urinary cancer. Based on the pooled data, our results are in line with the most of the related published literatures, which demonstrated that low pretreatment AGR is an independent predictor of survival outcomes. With a decrease in AGR, patients with urinary system cancer (BC, UTUC, RCC, and PCa) had worse OS, CSS, RFS, PFS, and BRFS outcomes. We then performed a subgroups analysis of OS and CSS based on ethnicity, cancer type, cutoff value, sample size or publication year. The results of the subgroup analysis were consistent with meta-regression analyses. Finally, sensitivity analyses also demonstrated the robustness of our outcomes. Albumin and globulins are the two most abundant proteins in human blood plasma, and can be easily and cost-effectively measured. Thus, AGR can be used as a competent prognostic biomarker in patients with urological cancer.

Growing evidence suggests that inflammation in the tumor microenvironment plays a critical role in tumor growth, progression and metastasis (43). Tumor growth, necrosis and hypoxia trigger the production of a series of inflammatory factors, such as tumor necrosis factor (TNF), interleukin-1 and interleukin-6, which increase vascular permeability by damaging vascular endothelial cells (44). In 2014, Duran and his colleagues first demonstrated that the AGR was a strong prognostic indicator of poor survival outcomes in lung adenocarcinoma patients (45). Although the specific mechanism is not clear, it is at least related to these two indicators. Albumin is the main component of total serum protein, and its level either reflects the body's nutritional status or represents the systemic inflammation (18). Cytokines and chemokines produced by tumor cells can suppress albumin production and lead to malnutrition, which accelerates disease progression (46). Moreover, studies have reported that albumin plays an important role in delivering chemotherapy drugs to cancer patients, and this mechanism affects the survival outcomes (47). Furthermore, globulins, including complementary components, C-reactive protein (CRP), and immunoglobulin, is also involved in the inflammatory responses and immunosuppression against cancer cells in the human body (48). Chronic inflammation can affect not only the tumor growth but also angiogenesis and cancer migration (49). Studies have shown that some inflammatory markers, CRP and NLR, were closely related to the prognosis of cancer patients (50, 51). Equally, the AGR is also a inflammatory biomarker and decrease of AGR reflects the malnutrition and inflammatory response. Because of the AGR

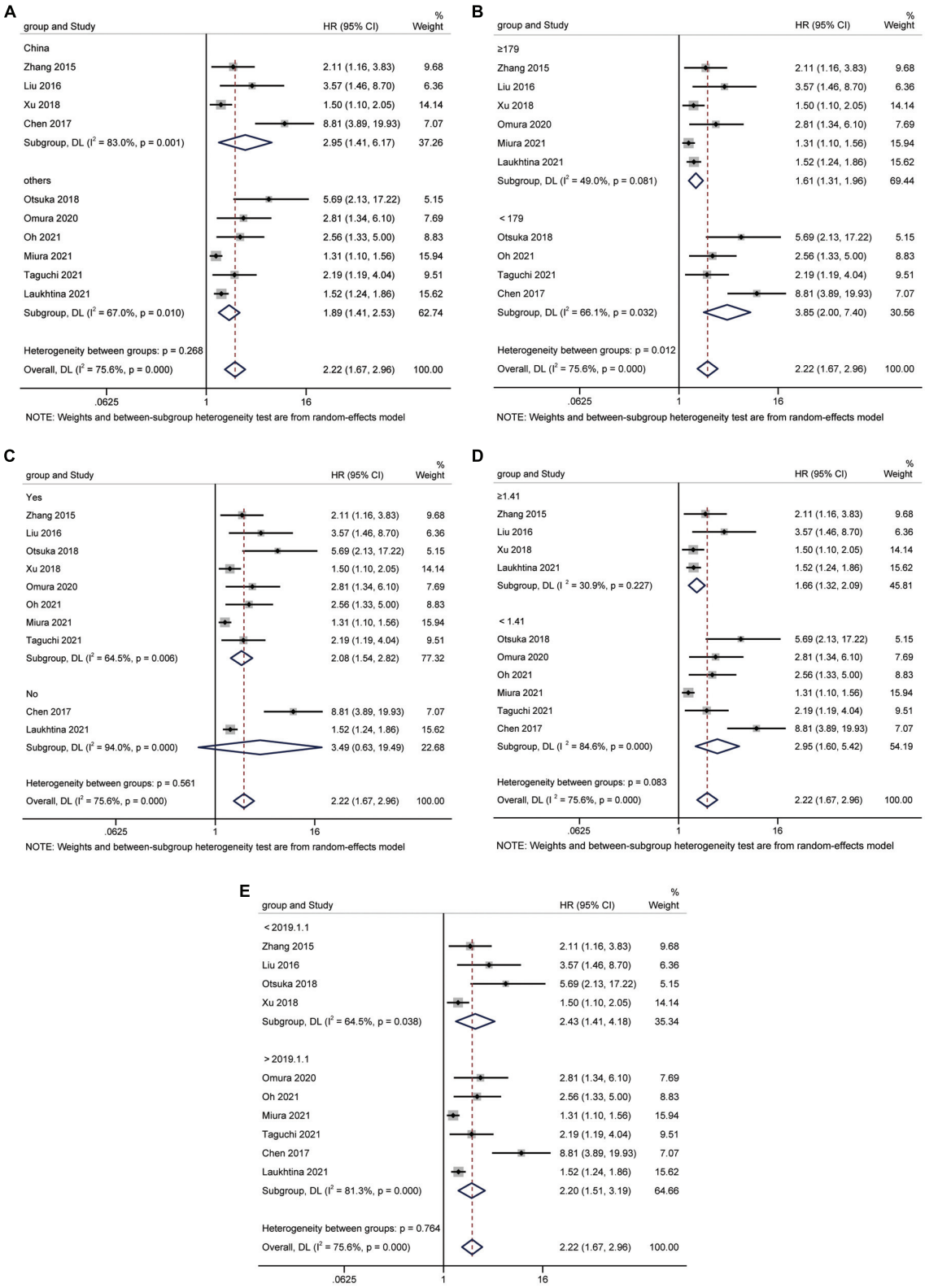

FIGURE 5
Forest plot for subgroup analysis of CSS and AGR. **(A)** Subgroup analysis of CSS and AGR according to ethnicity; **(B)** Subgroup analysis of CSS and AGR according to sample size; **(C)** Subgroup analysis of CSS and AGR according to urothelial carcinoma (yes or no); **(D)** Subgroup analysis of CSS and AGR according to cut-off values; **(E)** Subgroup analysis of CSS and AGR according to publication year.

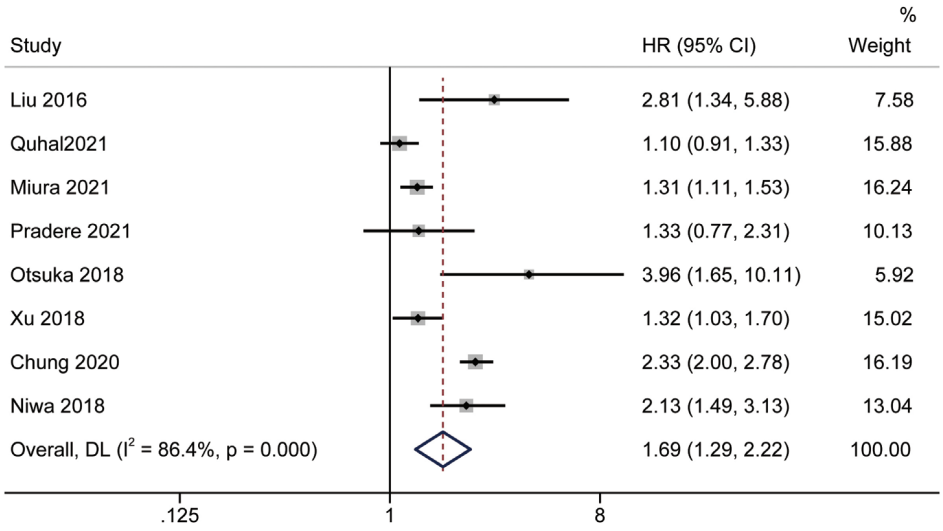

FIGURE 6
Forest plot reflects the association between AGR and RFS for urological cancers.

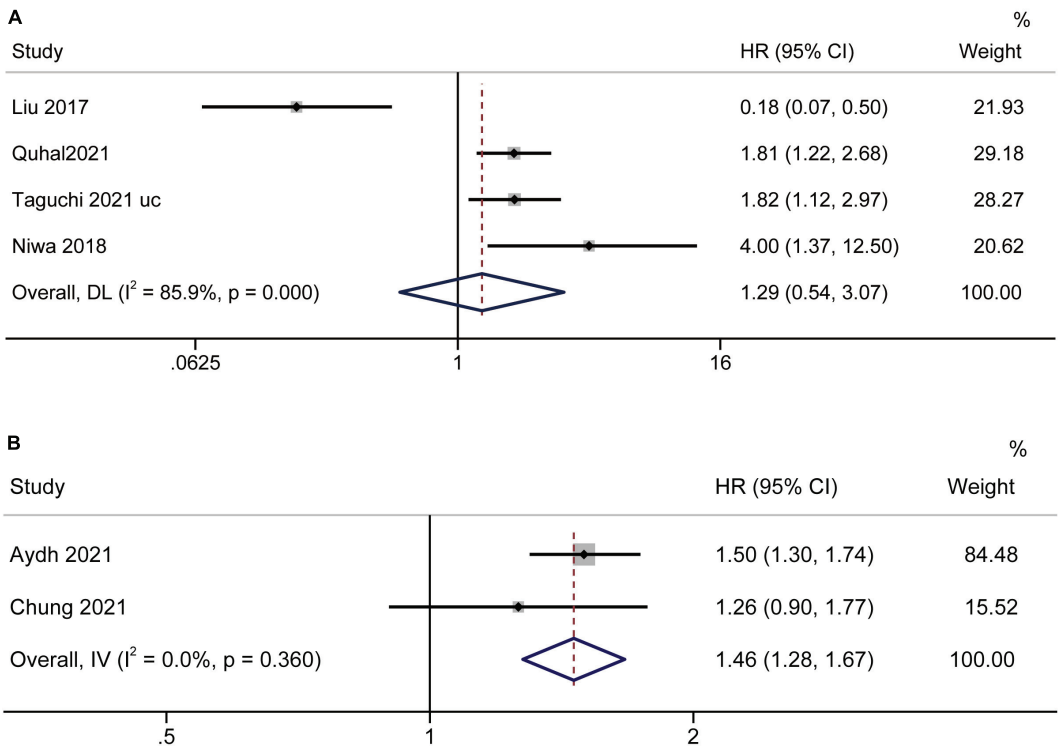

FIGURE 7
Forest plot reflects the association between AGR and PFS/BRFS for urological cancers. **(A)** AGR and PFS; **(B)** AGR and BRFS.

incorporates the advantages of albumin and globulin, and it is more likely to predict the poor survival in cancer patients.

Previous meta-analyses have explored the correlation between AGR and different organs and systems. A meta-analysis conducted by Li et al. (52) discovered that a decreased AGR suffer from worse OS and DFS in patients with lung cancer. In colorectal cancer, Ma et al. (53) provided evidence that low pretreatment AGR was related to poor OS (HR = 2.07, $P < 0.01$) and DFS/PFS (HR = 2.10, $P = 0.01$), and advanced clinicopathological features, including age, tumor size, node

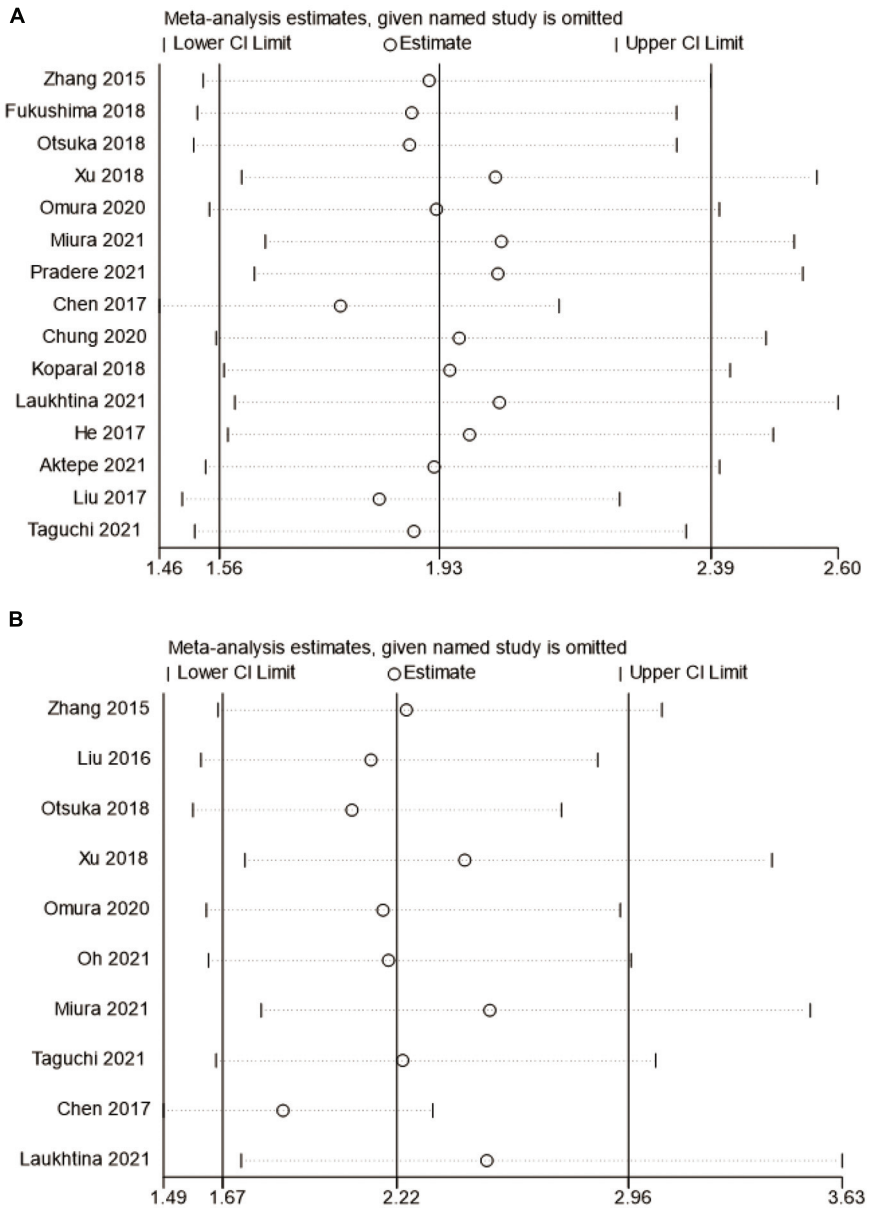

FIGURE 8
Forest plot for sensitivity analysis. **(A)** overall survival and **(B)** cancer-specific survival.

metastasis stage, and tumor depth. Additionally, many recent studies have explored the association between the AGR and genitourinary cancers. Omura and his colleagues performed a retrospective study involving 179 patients with UTUC who underwent radical nephroureterectomy and revealed that a low AGR could predict the prognosis of patients with non-metastatic UTUC (33). A multicenter research team found that the UTUC patients with a low pretreatment AGR had a markedly shorter OS and RFS than those with a high AGR patients (16). In Korea, Chung's study proved that the association between the preoperative serum AGR and the poor prognosis in patients with RCC in a large cohort (26). For non-metastatic PCa patients who received radical prostatectomy (RP), the findings of Aydh et al. and Chung et al. indicated that the pretreatment AGR can be a useful serological marker for predicting the BRFS and adverse pathology (17, 23). In 2021, Zhang et al. (48) verified that the AGR combined with other indices [C-reactive protein/albumin ratio (CAR), neutrophil-lymphocyte ratio (NLR), and other clinicopathological features] could predict OS and PFS in BC patients after radical cystectomy. This is the first meta-analysis to assess the prognostic value of the AGR in

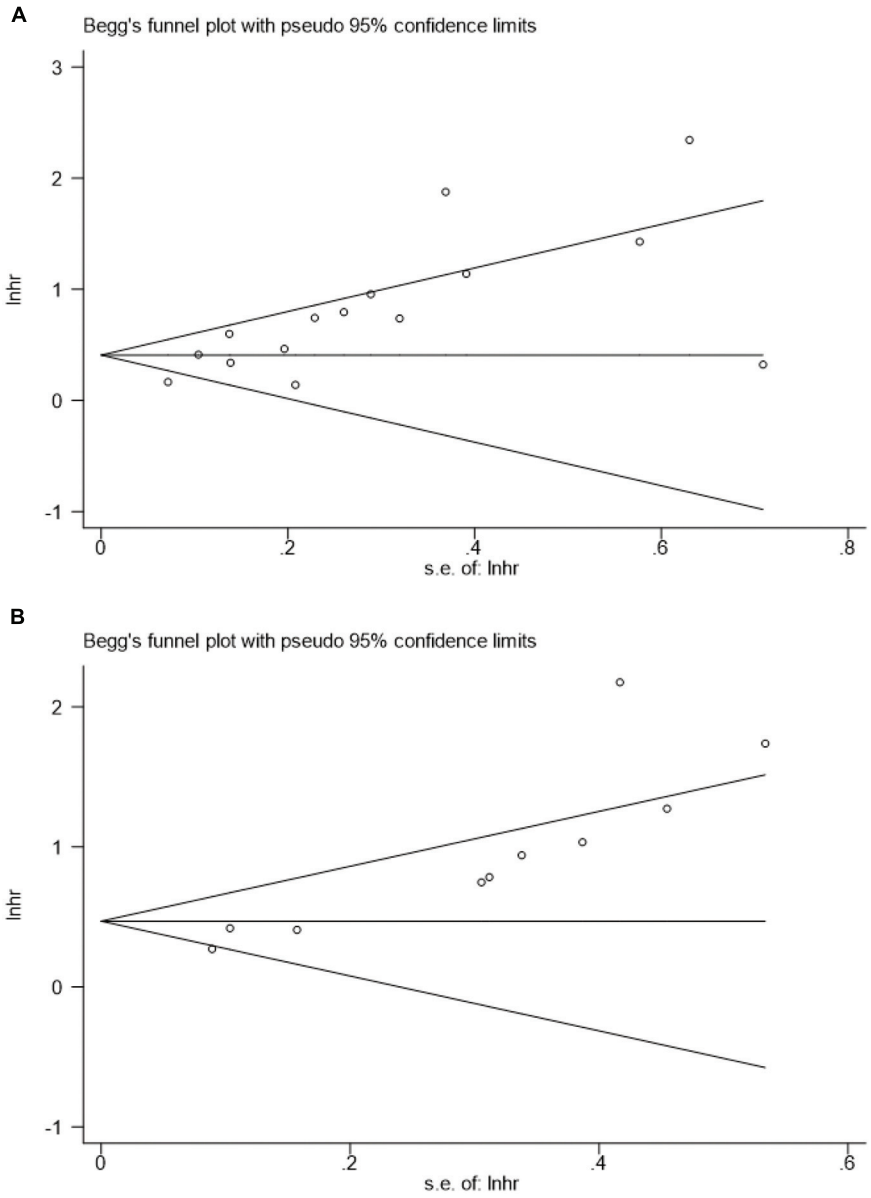

FIGURE 9
Begg's test for **(A)** overall survival and **(B)** cancer-specific survival.

urinary system cancers. Our results further showed that a low pretreatment AGR indicates poor prognosis, which is in line with previous studies.

Although this meta-analysis provides evidence regarding the prognostic value of AGR in patients with urological cancer, several limitations cannot be avoided. First, most of the included studies were single-center and retrospective studies; therefore, the heterogeneity between studies is inevitable. Second, because of different treatment strategies for various cancers may introduce bias in the results. Third, the cutoff threshold of AGR selected by different studies was different, making it difficult to determine the optimal cutoff value. Fourth, only common urological tumors were included in this study, but the correlation between AGR and the prognosis of other genitourinary tumors is still unclear. Finally, considering the limitation of many factors affecting the prognosis of urinary cancers, the evaluation effectiveness of this index needs to be verified. Therefore, further studies need to be conducted.

Conclusion

In summary, our study proved that a low AGR before treatment is associated with inferior OS, CSS, RFS, PFS, and

BRFS outcomes in urinary system cancers. As a non-invasive, effective, and cost-effective indicator, the AGR can be used to predict the prognosis of patients with urological cancer. However, further large-scale prospective studies with larger sample sizes are needed.

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 author/s.

Author contributions

LT and JW conceived and designed the experiments. ZX, XF, JL, and XY analyzed the data. JL, JS, and HW contributed the reagents, materials, and analysis. ZX and XF wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Gut microbial evidence chain in high-salt diet exacerbates intestinal aging process

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Although excessive salt consumption appears to hasten intestinal aging and increases susceptibility to cardiovascular disease, the molecular mechanism is unknown. In this study, mutual validation of high salt (HS) and aging fecal microbiota transplantation (FMT) in C56BL/6 mice was used to clarify the molecular mechanism by which excessive salt consumption causes intestinal aging. Firstly, we observed HS causes vascular endothelial damage and can accelerate intestinal aging associated with decreased colon and serum expression of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and increased malondialdehyde (MDA); after transplantation with HS fecal microbiota in mice, vascular endothelial damage and intestinal aging can also occur. Secondly, we also found intestinal aging and vascular endothelial damage in older mice aged 14 months; and after transplantation of the older mice fecal microbiota, the same effect was observed in mice aged 6–8 weeks. Meanwhile, HS and aging significantly changed gut microbial diversity and composition, which was transferable by FMT. Eventually, based on the core genera both in HS and the aging gut microbiota network, a machine learning model was constructed which could predict HS susceptibility to intestinal aging. Further investigation revealed that the process of HS-related intestinal aging was highly linked to the signal transduction mediated by various bacteria. In conclusion, the present study provides an experimental basis of potential microbial evidence in the process of HS related intestinal aging. Even, avoiding excessive salt consumption and actively intervening in gut microbiota alteration may assist to delay the aging state that drives HS-related intestinal aging in clinical practice.

KEYWORDS

high-salt diet, intestinal aging, gut microbiota, machine learning, signal transduction

Introduction

Aging is an unavoidable process in human life activities that involves the gradual decline in the function of various organ systems, which is reflected at the cellular, tissue, organ and systemic levels and further leads to the onset, development and death of many diseases (1, 2). Even, aging is a risk factor for chronic diseases, among others, senescence mechanisms have become a target of huge research on the topic of the aging process (1). Intestinal aging is an important element of the process, and it is also said that aging starts in the gut. As the aging process is accompanied by the accumulation of damage to the body, it leads to reduced function and vulnerability to disease (3, 4). The physiological and immune functions of the intestine gradually decline with increasing age, and the structure of the diet and gut microbiota also change accordingly (5). To our knowledge, aging is accompanied by degenerative changes in the intestinal tract, including histopathological changes in the intestinal tract, weakened intestinal contraction and peristalsis, reduced secretion, decreased levels of various digestive enzymes, resulting in reduced intestinal digestion and absorption, reduced gut microbiota, increased intestinal permeability, causing intestinal dysfunction and chronic inflammation of the intestinal tract (6).

Only a few of the various processes that make up the complex process of aging and act as important entry points for age-related diseases include immunosenescence and inflammaging. Additionally, diet, prebiotics, probiotics, and synbiotics may extend longevity through gut microbiota manipulation (7). The microbiota-targeted dietary and probiotic interventions have been shown to favorably affect the host health and aging by an enhancement of antioxidant activity, improving immune homeostasis, suppression of chronic inflammation, regulation of fat deposition and metabolism and prevention of insulin resistance. The function of gut microbiota in aging processes is discussed in recent research findings, with a focus on the therapeutic potential of microbiome-targeted therapies in anti-aging therapy (8).

High salt (HS) is a risk factor for a variety of diseases, and HS may play a role in the development of gastrointestinal disorders *via* intestinal microenvironmental remodeling, adding to a better understanding of HS's complicated pathogenic function in gastrointestinal diseases (9). Thus, HS is a major cause of many chronic and age-related deficiencies such as cardiac hypertrophy, exercise disorders, and death (10). Growing data suggests that the gut microbiota is at the root of many age-related changes, such as immune system dysregulation and disease vulnerability. Throughout its life cycle, the gut microbiota has undergone significant changes, and aging-related processes may have an impact on the gut microbiota and the metabolic changes that go along with it (11). The connection between HS, the gut microbiota, and intestinal aging hasn't been well explored, though. To accomplish this, we employed older animals for further verification after performing 16S rRNA gene sequencing on bacterial DNA taken from HS-induced mice and preliminary verification using FMT of HS-induced mice. To prove that HS promotes intestinal aging through the gut microbiota, fecal microbiota were implanted in older mice. We looked at relationships between these microbial fingerprints and biological age related microbiota genera, which are excellent predictors of mortality, morbidity, and other age-related events.

Abbreviations: HS, High salt; FMT, fecal microbiota transplantation; MDA, malondialdehyde; CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; NO, nitric oxide; ET-1, endothelin-1; AngII, angiotensin II; VEGF, vascular endothelial growth factor; VK2, vitamin k2; OTUs, operational taxonomic units.

Methods

Design and grouping

A total of 24 C56BL/6 mice (6–8 weeks old, male, provided by Changzhou cavens experimental animal Co., Ltd.) were randomly divided into normal control group (CON), high salt group (HS) and fecal microbiota transplantation (FMT) of HS group mice to normal control mice group (FMT-HS). Meanwhile, sixteen 14-month-old C56BL/6 mice (male, provided by Changzhou cavens experimental animal Co., Ltd.) were randomly designed as older control group (CON-O) and fecal microbiota of older mice transplanted to normal control mice group (FMT-O), with 8 mice in each group for experimental validation. The ethics committee of Jiangnan University approved the animal experiment which was carried out in the university's animal facility (NO. 20211015c0650220).

FMT preparation

Fresh feces were collected from HS group mice and stored into a 50 ml centrifuge tube every day. Then the collected feces were dissolved using normal saline in the ratio of 1:10 according to our previous studies (12). Thus, the fecal samples were centrifuged (3,000 rpm for 5 min) after sufficient mixing. The fecal bacterial suspension was transferred and gained into a sterile centrifuge tube and then were administered by gavage to mice within 2 h (12).

Intervention

In this study, an 8% HS (NaCl) diet for 4 weeks was used in the HS group, while an 0.4% salt (NaCl) diet was used in other groups. Nantong Troffer Feed Technology Co., Ltd. (Nantong,

China) provided the HS feed [production license: (2014) 06092], which was then sterilized by Nantong Michael Irradiation Co., Ltd. (Nantong, China). The mice in FMT-HS and FMT-O groups were respectively gavaged using FMT of the mice in HS group mice or older control group (100 μl/d per mouse), while the mice in other groups were administered by gavage with 100 μl normal saline.

Measurement of intestinal aging-related factors in colon and serum by enzyme-linked immunosorbent assay

To reduce suffering, isoflurane anesthesia was administered to all mice after 4 weeks. After that, blood was extracted from the eyeball and centrifuged after 2–4 h for 5 min at 3,000 rpm. The supernatant was then sub packed for storage into 1.5-ml sterilized EP tubes. The colon tissue (1–2 cm) was gathered and stored in the EP tubes. Lastly, the levels of malondialdehyde (MDA), catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in the colon and serum were assessed in accordance with the kit's instructions, which were bought from MEIMIAN (Yancheng, China).

Detection of vascular endothelial function-related factors in serum by ELISA

Then, using kits given by MEIMIAN (Yancheng, China) and following the operating instructions, the levels of nitric oxide (NO), endothelin-1 (ET-1), angiotensin II (AngII), vascular endothelial growth factor (VEGF), and vitamin k2 (VK2) were determined.

16S rRNA gene sequencing, gut microbial analysis of intestinal contents

The intestinal contents of all mice were stored, and the intestinal contents of 6 mice in each group were randomly selected for subsequent analysis. The intestinal contents were used to extract microbial DNA through E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.). The final DNA concentration and purification was assessed using NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was estimated using 1% agarose gel electrophoresis. The V3-V4 hypervariable portions of the bacterium 16S rRNA gene were amplified by a thermocycler PCR system (GeneAmp 9700, ABI, USA) with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR products were extracted from a 2% agarose gel, purified with a AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified with QuantiFluorTM-ST (Promega, USA) according to the manufacturer's instructions.

The purified amplicons were sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) at an equimolar ratio (Shanghai, China). The operational taxonomic units (OTUs) were clustered using UPARSE (http://drive5.com/uparse/) with a unique "greedy" technique that performs chimera filtering and OTU clustering at the same time. Finally, the RDP Classifier algorithm was used to compare the taxonomy of each 16S rRNA gene sequence to the 16S rRNA database [Silva (SSU123)].

Prediction model for intestinal aging based on microbiota genera with relative abundance difference

To develop a system for diagnosing intestinal aging, we explored a machine learning technique based on the significantly different microbiota. Machine learning is the process through which computer systems gradually increase their capacity to execute specified tasks by using computer algorithms and statistical models (12). In order to generate predictions or judgments without explicitly programming to carry out tasks, machine learning creates a mathematical model using sample data, known as "training data." In this work, a model of intestinal aging was created using the LDA linear judgment analysis approach. CON-O and CON group made up the necessary training set for the modeling process. After that, a different set of samples was utilized as a test set to assess the model's capacity to detect intestinal aging brought on by excessive salt intake. The mass package of the R language were used to analyze this section.

Statistical analysis

GraphPad Prism 8.3 was used to analyze all data, which were all expressed as mean standard error (SEM). The Kruskal-Wallis test or one-way ANOVA were used for all analyses. $P < 0.05$ denotes a meaningful difference. The Majorbio I-Sanger Cloud Platform was utilized to examine the 16S rRNA gene sequencing data (www.i-sanger.com).

Results

HS induced intestinal aging process in mice related to gut microbiota

We first measured intestinal aging-related factors (SOD, GSH-Px, CAT, MDA, pentosidine) in colon and serum in CON,

HS, FMT-HS groups (Figures 1A–I). As expected, significantly decreased levels of SOD and GSH-Px, significantly increased level of MDA in colon were found in HS group after HS induced for 4 weeks, which were also found in FMT-HS group mice (Figures 1A,B,D). Nevertheless, no significant difference were found in the level of CAT in colon (Figure 1C). Meanwhile, significantly decreased levels of SOD, GSH-Px and CAT, significantly increased levels of MDA and pentosidine in serum were found in HS group after HS induced for 4 weeks, which were also found in FMT-HS group mice (Figures 1E–I). Thus, we measured the changes of vascular endothelial function-related factors (NO, ET-1, AngII, VEGF, VK2) in serum in CON, HS, FMT-HS groups (Figures 1I,J). Remarkably, significantly decreased levels of NO and VK2, significantly increased levels of ET-1, VEGF in serum were found in HS group after HS induced for 4 weeks, the significant changes in the levels of NO, VK2, ET-1, AngII, were found in FMT-HS group mice (Figures 1J–N). Nevertheless, increased trends were found in the level of AngII in serum in HS group and the level of VEGF in FMT-HS group (Figures 1L,M). These results suggest that HS induced intestinal aging process in mice related to gut microbiota.

HS significantly changed gut microbial diversity, which was transferable by FMT

To test how HS induced intestinal aging process in mice related to gut microbiota, we measured the changes in gut microbial diversity. As for gut microbiota, the alpha diversity were examined by indices such as chao1, ace, shannon, simpson, shannoneven and simpsoneven. Richness of the gut microbial community is reflected by the indices chao1 and ace, diversity of the gut microbial community is reflected by the indices shannon and simpson, and evenness of the gut microbial community is shown by the indices simpsoneven and shannoneven. The indices of chao1, ace, simpson in HS goup were found significantly lower than that of the CON group; the indices of shannoneven and simpsoneve were found significantly higher than that of the CON group (Figures 2A–F). While the index of simpson in FMT-HS group was also found significantly lower than that of the CON group, the indices of shannoneven and simpsoneven in FMT-HS group were also significantly higher than that of the CON group (Figures 2A–F). Nevertheless, no significant difference was found in the index of shannon (Figure 2C). The changes of the six indices indicate HS significantly changed gut microbial alpha diversity.

Beta diversity analysis is to explore the similarity or difference of community composition among different groups of samples by comparing and analyzing the species diversity among different habitats or microbial communities. Based on the principal co-ordinates analysis (PCoA), PC1 accounted for 35.08% of the total variation and PC2 accounted for 18.85%, which revealed the microbial community of the CON group differed significantly from that of the HS and FMT-HS groups ($R^2 = 0.3784$, $p = 0.001$; Figure 2G). All these findings indicate HS induced intestinal aging process in mice related to gut microbial diversity.

HS significantly changed gut microbial composition, which was screened by FMT

To explore how the microbial characteristics associate with the HS-related intestinal aging process, we calculated the differential relative abundance of characteristics among CON, HS and FMT-HS groups. Our study found that the CON group contained 1,126 OTUs, the HS group 572, and the FMT-HS group 654 (Figure 3A). The phyla-level and genera-level were then selected to perform further analysis (Figures 3B,C). As shown in Figure 3B, there were 38.43%, 32.04%, 29.04% Firmicutes in the CON, HS and FMT-HS group. There were 47.89%, 58.41%, 60.34% Bacteroidetes in the CON, HS and FMT-HS groups. In Figure 3C, the top 25 genera are displayed in the CON, HS, and FMT-HS groups. For example, there were 23.91%, 37.69%, 34.54% *norank_f__Muribaculaceae* in the CON, HS and FMT-HS groups; meanwhile, there were 8.07%, 6.67%, 4.34% *Dubosiella* in the CON, HS and FMT-HS groups. Our data indicate that HS can regulate the gut microbial community relative abundance, even increase Bacteroidetes and *norank_f__Muribaculaceae*, whereas decrease Firmicutes and *Dubosiella*. To investigate further how HS induced intestinal aging process in mice related to gut microbial composition, a total of 32 significantly different genera of the microbial community among the three groups were calculated (Supplementary Table 1), and the top 20 genera were shown as Figure 3D. Taken together, all these results reveal that HS induced intestinal aging process in mice related to gut microbial composition.

Intestinal aging-related factors imbalance was also found in older mice, which was transferable by FMT

To verify whether HS induced intestinal aging process in mice related to gut microbiota, we explored the intestinal aging-related factors (SOD, GSH-Px, CAT, MDA, pentosidine) in colon and serum in CON, CON-O, FMT-O groups (Figures 4A–I). As seen in Figures 4A–I, the level of SOD in colon in CON group was lower than that in CON-O and FMT-O groups, however, there was no statistically significant difference. The level of GSH-Px in colon in CON group was significantly higher than that in CON-O and FMT-O groups. No statistically significant difference was found in the level of CAT in colon among the

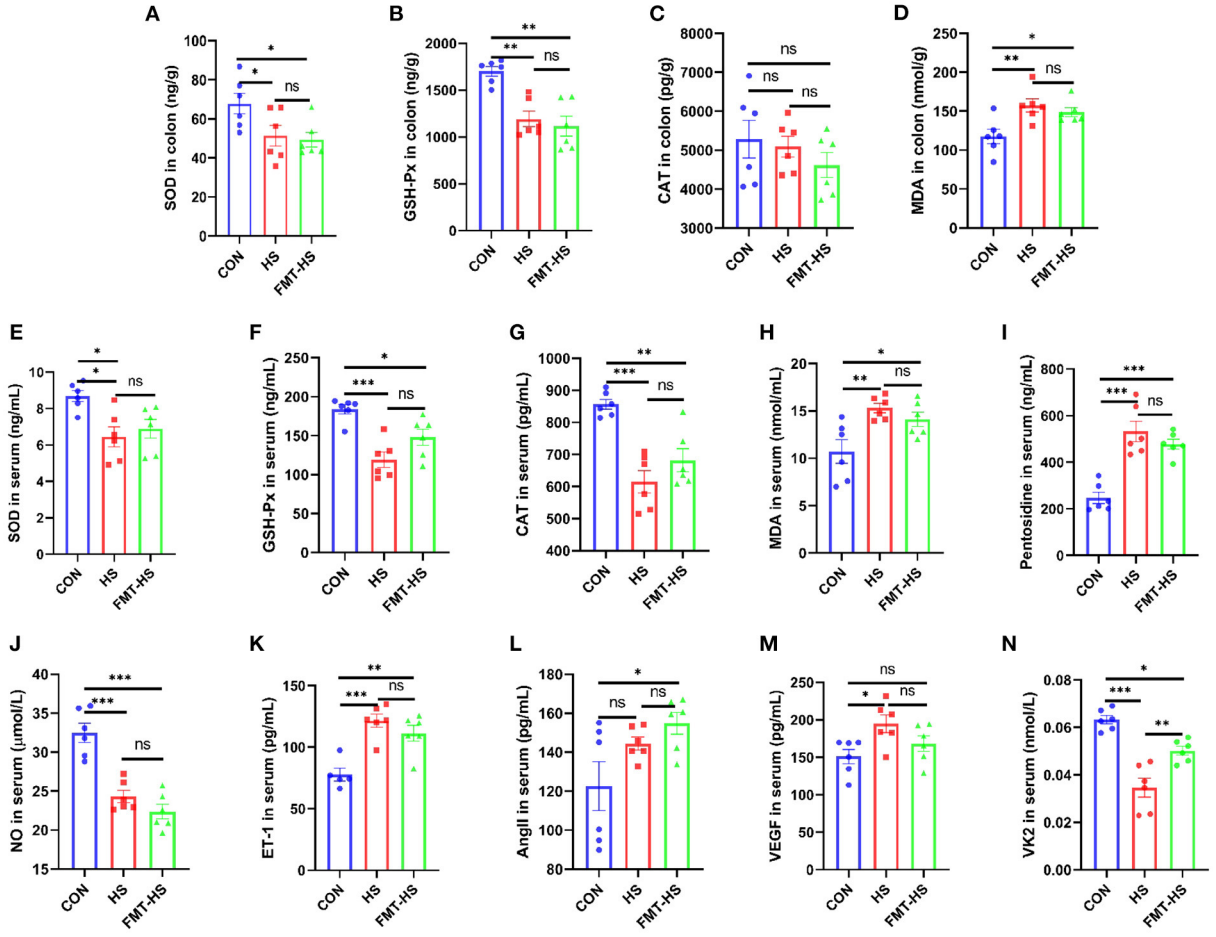

FIGURE 1
High salt (HS) induced intestinal aging-related factors imbalance in mice, which was transferable by fecal microbiota transplantation. The changes of aging-related factors in colon and serum **(A–I)**. The changes of vascular endothelial function-related factors in serum **(J–N)**. SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde; NO, nitric oxide; ET-1, endothelin-1; AngII, angiotensinII; VEGF, vascular endothelial growth factor; VK2 vitmain k2, respectively. CON, natural diet; HS, 8% salt diet; FMT-HS, gut microbiota of HS group mice transplanted to CON group mice. All data are expressed as mean ± standard error (SEM). One-way ANOVA with Tukey *post hoc* test was conducted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no significance.

three groups. In addition, the levels of GSH-Px and CAT in serum in CON group were found significantly higher than that in CON-O and FMT-O groups (Figures 4F,G). The level of pentosidine in colon in CON group was found significantly lower than that in CON-O and FMT-O groups (Figure 4I). Nevertheless, no statistically significant difference were found in the levels of SOD and CAT in serum (Figures 4E,H). Thus, the levels of NO and VK2 in serum in CON group were found significantly higher than that in CON-O and FMT-O groups, the level of ET-1 in serum in CON group was found significantly lower than that in CON-O and FMT-O groups (Figures 4J,K,N). Nevertheless, statistically significant difference were found in the level of AngII and VEGF in serum among the three groups (Figures 4L,M). These results suggest that intestinal aging process in mice is related to gut microbiota.

Significant change of gut microbial diversity was also found in older mice, which was transferable by FMT

To test the role of gut microbiota in intestinal aging process in mice, we observed the changes in gut microbial diversity. As for alpha diversity, we found the indices of ace and simpson in CON group were evidently higher than that of the CON-O and FMT-O groups (Figures 5B,D). Nevertheless, neither statistically significant difference were found in the index of ace between CON and FMT-O groups, nor in the index of simpson between CON and CON-O groups (Figures 5B,D). The indices of chao1, shannon, shannoneven and simpsoneve in CON group were evidently lower than that of the CON-O and FMT-O groups (Figures 5A,C,E,F). However, there were no statistical difference

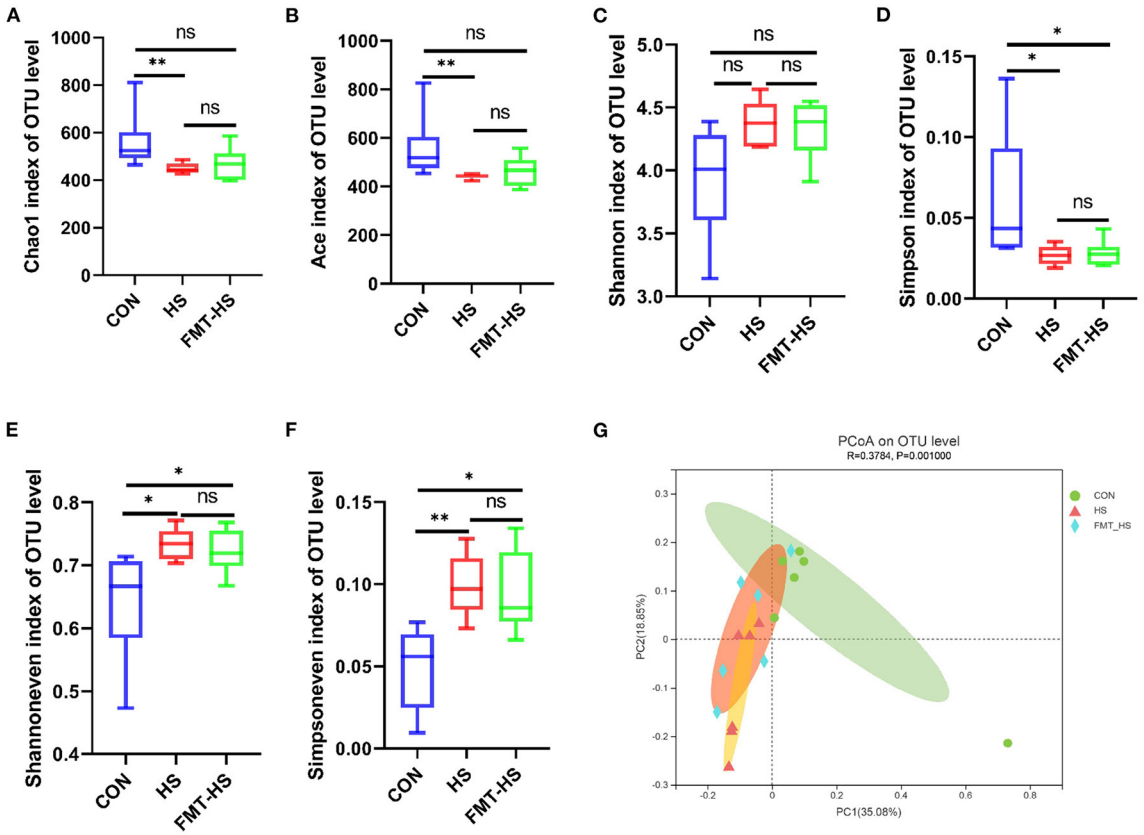

FIGURE 2
High salt (HS) dramatically altered gut microbial diversity, and fecal microbiota transplantation was able to transmit these changes. The changes of α-diversity **(A–F)**. PCoA plot in β-diversity **(G).** The closer two sample points are, the more similar the composition of the two sample species. Different colored points and shapes indicate samples from different groups. All data are expressed as mean ± standard error (SEM). One-way ANOVA with Tukey *post hoc* test was conducted. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$; ns, no significance.

in the indices of chao1, shannon, shannoneven between CON and CON-O groups, (Figures 5B,D). Indeed, the changes of intestinal aging process in mice are related to gut microbiota according to the changes of gut microbial alpha diversity.

Based on the principal co-ordinates analysis (PCoA) in beta diversity, PC1 accounted for 30.16% of the total variation and PC2 accounted for 22.98% (Figure 5G). The distance in PCoA between the CON and CON-O groups was relatively close, which revealed the microbial community of the CON group differed significantly from that of the CON and CON-O groups ($R^2 = 0.7025$, $p = 0.001$; Figure 5G). All these findings indicate intestinal aging process in mice is related to gut microbiota.

Significant change of gut microbial composition was also found in older mice, which was screened by FMT

To investigate how the microbial characteristics associated with the intestinal healthy aging in mice, we calculated the differential abundance of characteristics among CON, CON-O and FMT-O groups. We found that the 1,126, 617, 618 OTUs in CON, CON-O, and FMT-O group (Figure 6A). The top phyla-level and genera-level were shown as in Figures 6B,C. There were 38.43%, 46.10%, 36.60% Firmicutes in the CON, CON-O, and FMT-O groups, while there were 47.89%, 38.14%, 36.60% Bacteroidetes in the CON, CON-O, and FMT-O groups. In Figure 6C, the top 30 genera were analyzed in the CON, HS, and FMT-HS groups. Such as 23.91%, 27.38%, 30.99% *norank_f__Muribaculaceae* were found in the CON, CON-O, and FMT-O groups; meanwhile, 8.07%, 3.65%, 3.30% *Dubosiella* were found in the CON, HS and FMT-HS groups. Our data indicate that there was difference in the gut microbial community abundance in intestinal aging process. To further investigate the changes of gut microbial composition in intestinal aging process in mice, a total of 52 significantly different genera of the microbial community were found among the three groups (Supplementary Table 2), and the top 20 were shown as Figure 6D. Taken together, all these results reveal the differential abundance of microbial characteristics exist in the intestinal aging process in mice.

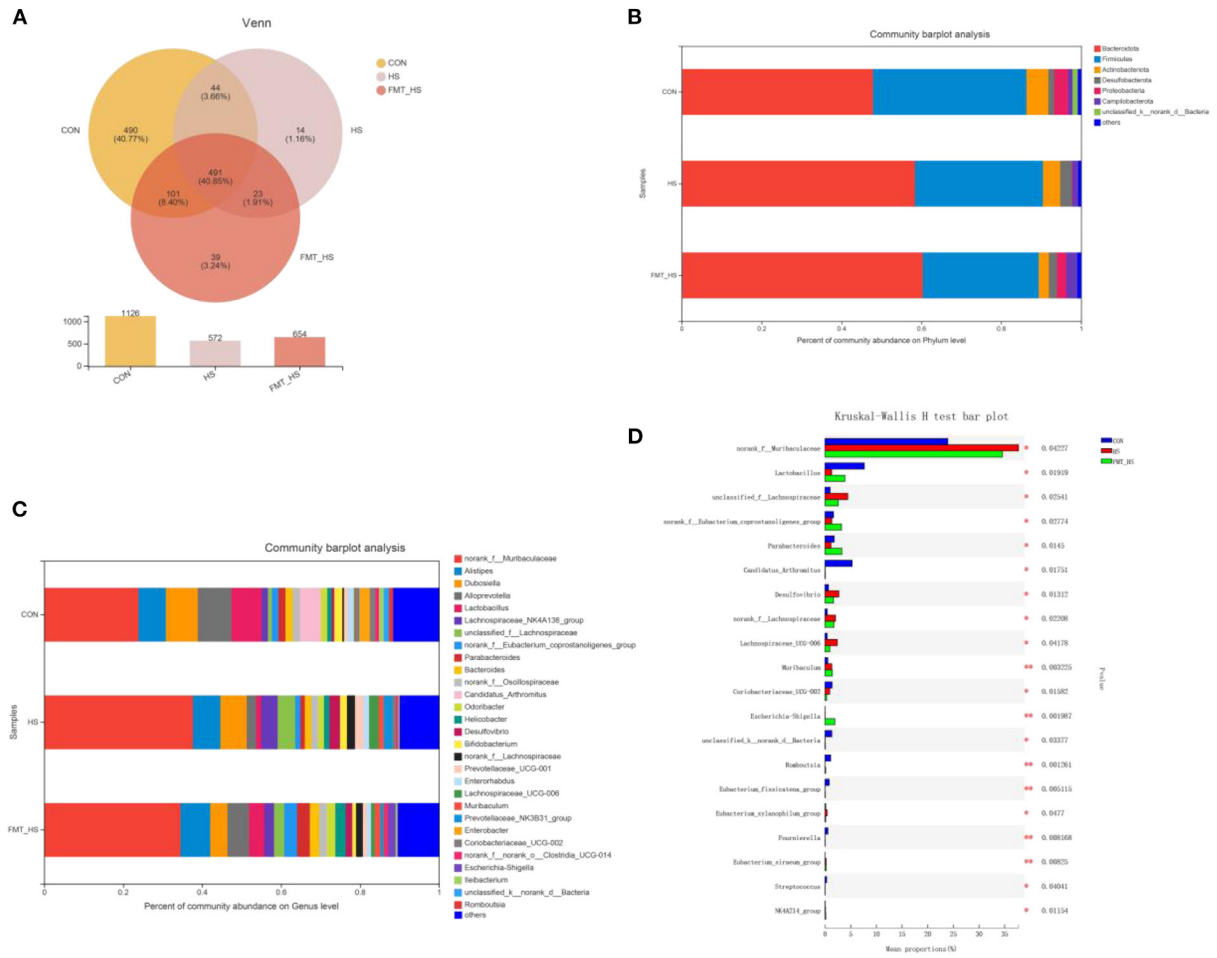

FIGURE 3
High salt (HS) significantly changed gut microbiota composition, which was screened by fecal microbiota transplantation ($n = 6$). The down-regulated OTU number is shown in here **(A)**. Phylum-level abundance as a percentage of total community abundance **(B)**. The top 30 in community abundance in terms of genera percent **(C)**. The significantly different genera **(D)**. Kruskal-Wallis test with Dunn *post hoc* test was used for statistical tests. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$.

HS significantly induced intestinal aging-related factors imbalance, in which microbiota genera with relative abundance difference were screened by FMT and verified in older mice

As gut microbial compositions were associated with aging status, we sought to investigate the microbial features observed in HS induced intestinal aging mice. To achieve that, we found the 16 common different genera in HS-related 32 significantly different genera (Supplementary Table 1) and age-related 52 significantly different genera (Supplementary Table 2; Figure 7A). In particular, a total of 8 genera with increased relative abundance (such as *unclassified_f__Lachnospiraceae*, *Desulfovibrio*, *norank_f__Lachnospiraceae*, *Lachnospiraceae_UCG-006*, *Escherichia-Shigella*, *Eubacterium_siraeum_group*, *NK4A214_group*, *Anaerofustis*, *Butyricicoccus*) and 4 genera with decreased relative abundance (*Lactobacillus*, *Coriobacteriaceae_UCG-002*, *Glutamicibacter*, *Paenibacillus*) were screened in HS induced mice (Figure 7B). Additionally, a total of 9 genera with higher relative abundance (*unclassified_f__Lachnospiraceae*, *Desulfovibrio*, *Lachnospiraceae_UCG-006*, *norank_f__Lachnospiraceae*, *Escherichia-Shigella*, *Eubacterium_siraeum_group*, *NK4A214_group*, *Butyricicoccus*, *Anaerofustis*) and 3 genera with lower relative abundance (*Parabacteroides*, *Glutamicibacter*, *Paenibacillus*) were found in older mice. Intriguingly, a total of 11 genera were screened in HS induced mice and verified in older mice. The correlation analysis showed the close relationship between the 11 genera and the

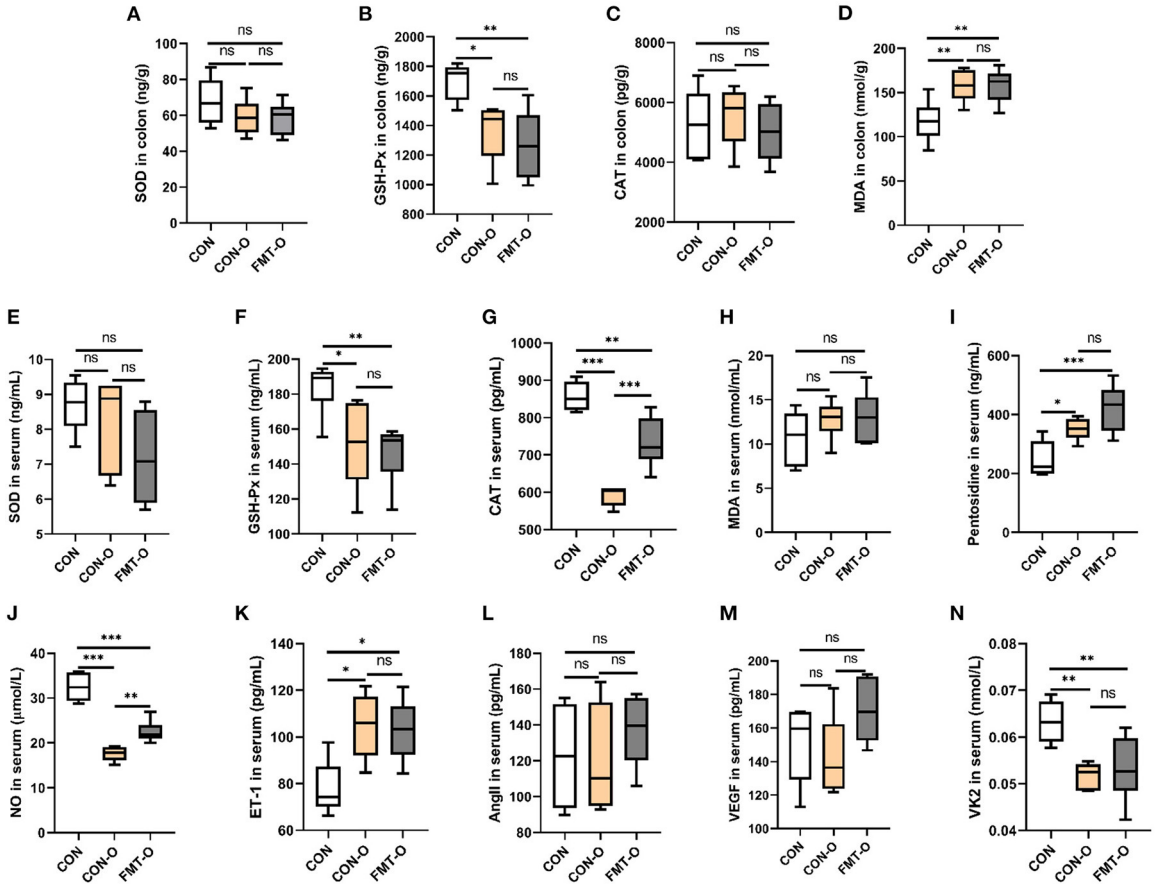

FIGURE 4
Intestinal aging-related factors imbalance was found in older mice, which was transferable by fecal microbiota transplantation. The changes of aging-related factors in colon and serum **(A–I)**. The changes of vascular endothelial function-related factors in serum **(J–N)**. SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; NO, nitric oxide; ET-1, endothelin-1; AngII, angiotensinII; VEGF, vascular endothelial growth factor; VK2, vitmain k2, respectively. CON, natural diet; HS, 8% salt diet; FMT-HS, gut microbiota of HS group mice transplanted to CON group mice. All data are expressed as mean ± standard error (SEM). One-way ANOVA with Tukey *post hoc* test was conducted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no significance.

intestinal aging-related factors (Figure 7C). The network of the significantly different genera were conducted as shown in Figure 7D. These results suggest the 11 genera may be the microbiota genera with relative abundance difference in HS induced aging process.

Prediction model for intestinal aging based on microbiota genera with relative abundance difference

We explored a machine learning technique to develop a system for diagnosing intestinal aging based on the 11 genera (Figure 8). The correlation coefficient among the 11 genra are shown in Figure 8A. The data suggest that there is no over fitting of the variables (11 microbiota genera with relative abundance difference) in the model establishment. Moreover, LDA model was established, revealing the contribution of 11 variables (Figure 8B). Thus, we found ROC obtained using only microbiota genera with relative abundance difference in the testing dataset was 0.7813 (95% CI, 0.6536–0.9089, $p = 0.0008$) (Figure 8C). These findings suggest the machine learning model for HS-related intestinal aging based on microbiota genera with relative abundance difference is full of diagnostic ability, which providing better understanding of the complex interaction between HS and intestinal aging.

Signal transduction pathways enriched by the significantly different genera in HS induced aging process

To examine potential gut microbial molecular mechanism, the signal transduction pathways were enriched by the

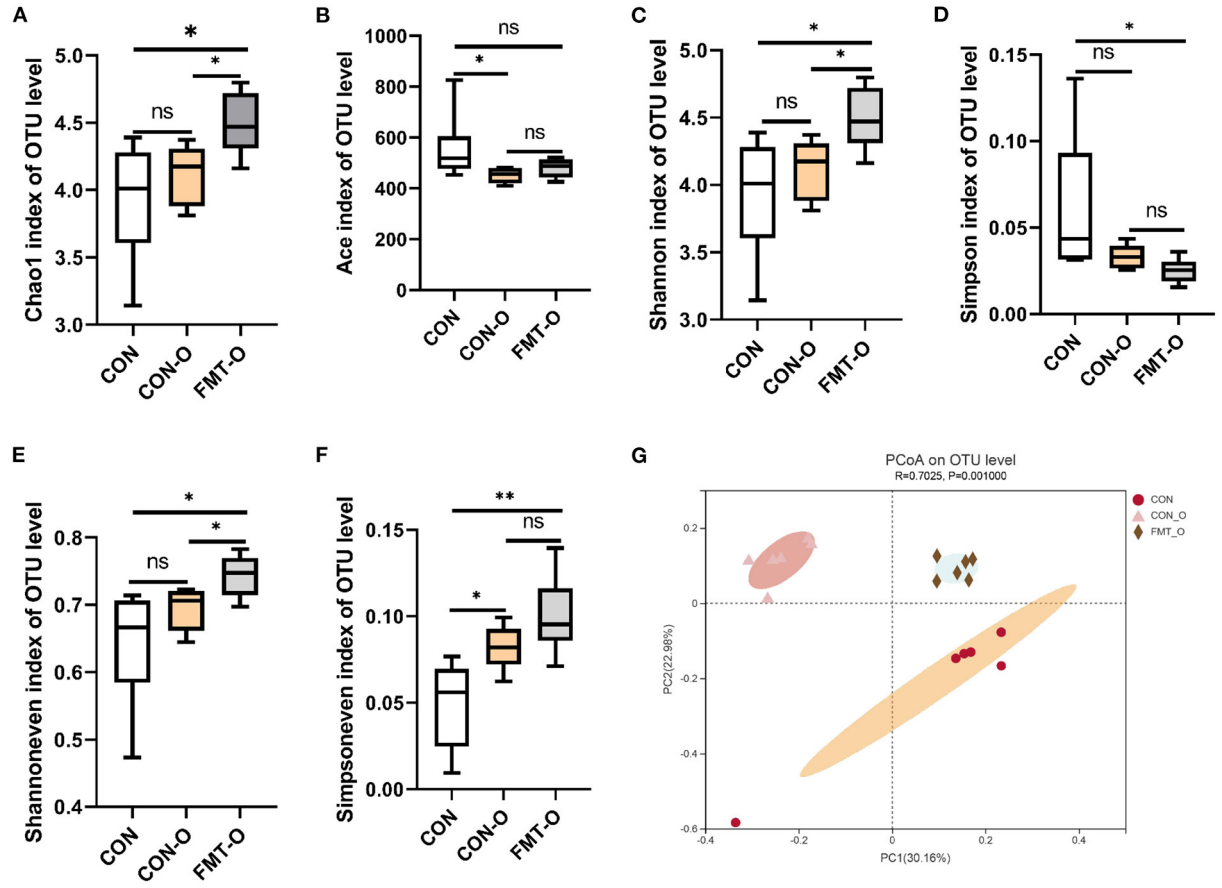

FIGURE 5
Significant change of gut microbial diversity was found in older mice, which was transferable by fecal microbiota transplantation. The changes of α-diversity **(A–F)**. PCoA plot in β-diversity **(G).** The closer two sample points are, the more similar the composition of the two sample species. Different colored points and shapes indicate samples from different groups. All data are expressed as mean ± standard error (SEM). One-way ANOVA with Tukey *post hoc* test was conducted. *$p < 0.05$, **$p < 0.01$; ns, no significance.

significantly different genera using PICRUSt2. A total of 11 signaling pathways were enriched and identified as shown in Figures 9, 10. Notably, compared with the CON group, increased relative abundance (enriched in the signaling pathways in two-component system, HIF-1 signaling pathway, AMPK signaling pathway, phosphatidylinositol signaling system, PI3K-Akt signaling pathway, MAPK signaling pathway-plant, FoxO signaling pathway, phospholipase D signaling pathway, MAPK signaling pathway-fly, MAPK signaling pathway-yeast and cAMP signaling pathway) was found in HS and FMT-HS groups (Figure 9). However, there was no statistical significance in the relative abundance in MAPK signaling pathway-fly signaling pathway between CON and HS groups (Figure 9I). To better understand these findings, compared with the CON group, higher relative abundance were also significantly enriched in the above pathways in CON-O and FMT-O groups Figure 9. Based on the whole findings, the 11 signaling pathways may be potential gut microbial molecular mechanism in the HS induced aging process.

Correlation between the significantly different genera and signal transduction pathways

In addition, the spearman correlation analysis was used to assess the link between the substantially different genera and the signal transduction pathways. As illustrated in Figure 11, *Eubacterium_siraeum_group* and *NK4A214_group* were considerably negatively connected with the cAMP signaling pathway but strongly favorably correlated with all other signaling pathways. With the exception of the cAMP signaling pathway, the other signaling pathways were significantly positively linked with *unclassified_f__Lachnospiraceae*, *Desulfovibrio*, *norank_f__Lachnospiraceae* and *Lachnospiraceae_UCG-006*. Furthermore, the two-component system, phosphatidylinositol signaling system, PI3K-Akt signaling pathway, MAPK signaling pathway-yeast, and phospholipase D signaling pathway were strongly connected to 10 of the 11 significantly different genera,

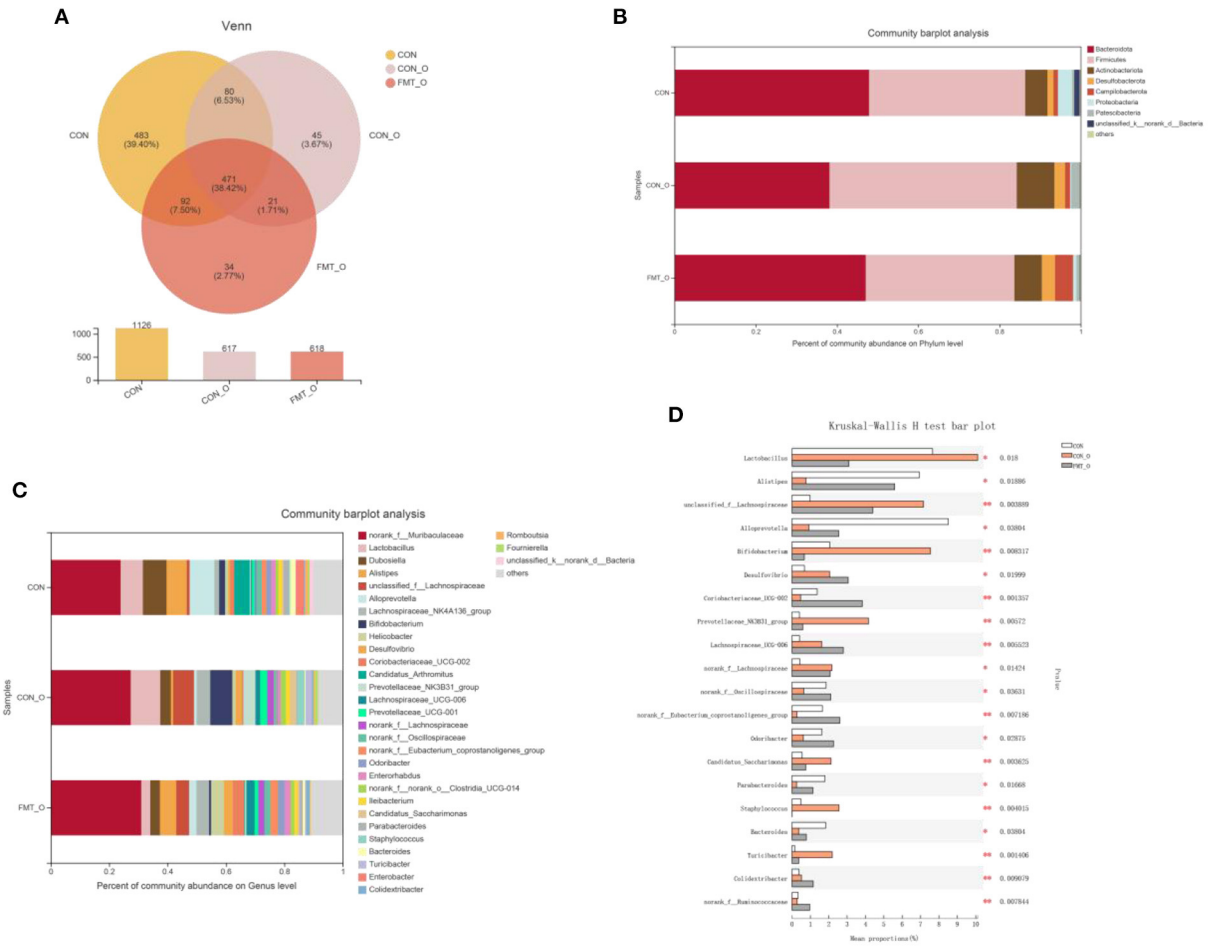

FIGURE 6
Significant change of gut microbial composition was found in older mice, which was screened by fecal microbiota transplantation ($n = 6$). The down-regulated OTU number is shown in here **(A)**. Phylum-level abundance as a percentage of total community abundance **(B)**. The top 30 in community abundance in terms of genera percent **(C)**. The significantly different genera **(D)**. Kruskal-Wallis test with Dunn *post hoc* test was used for statistical tests. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

which suggest most possible mechanisms of differential bacterial response to host.

Discussion

Globally, average life expectancy has increased dramatically in recent decades, resulting in a proportionately larger aging population. Although chronological age is currently the most extensively recognized indication of aging, it gives little information on the quality of life during the intestinal aging process. Understanding how to promote healthy intestinal aging will be critical to extending one's life. There is mounting evidence that the gut microbiota is inextricably related to HS and the aging process. Here, mice were fed an 8% HS diet to investigate HS accelerated intestinal aging, and FMT were employed to confirm the association between HS accelerated aging and gut microbiota. Furthermore, young and older mice were utilized to assess gut microbial diversity, and FMT were employed to confirm and discover the crucial function of gut microbiota, as well as to search for evidence of gut microbiota in HS accelerated intestinal aging.

Since oxidative stress-related enzymes are frequently used to evaluate aging, we chose 4 redox enzymes (SOD, GSH-Px, CAT, MDA, pentosidine) as the primary markers for the initial evaluation of aging in this study (13, 14). The increase in cardiovascular disease in aging is partly due to vascular endothelial cell senescence and associated vascular dysfunction, and aging is also often accompanied by vascular endothelial cell aging, so we selected 5 key indicators of vascular endothelial function (NO, ET-1, AngII, VEGF, VK2) to aid in the assessment of aging (12, 15). Advanced glycation end products (AGEs) can

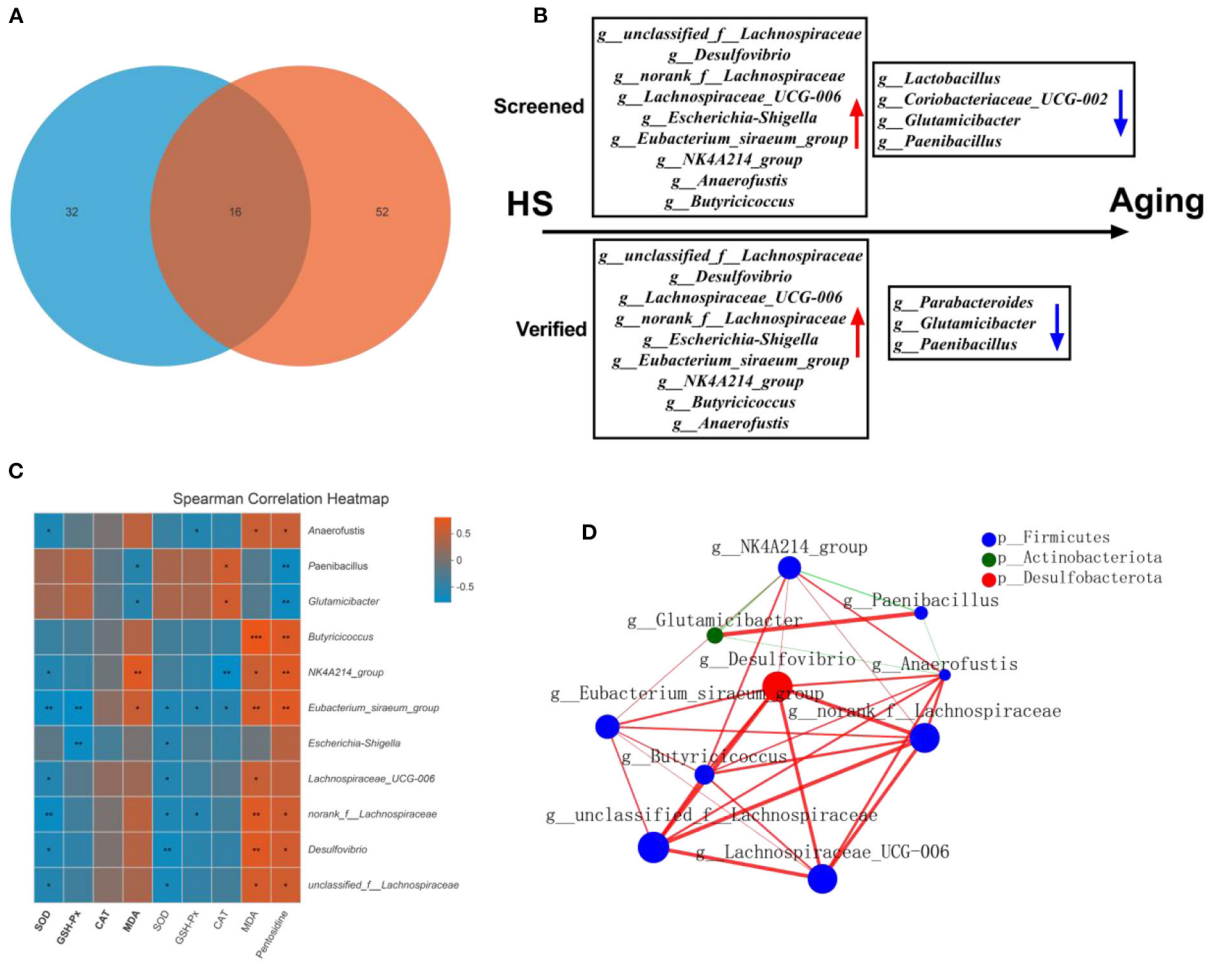

FIGURE 7
High salt (HS) significantly induced intestinal aging-related factors imbalance, in which key genera were screened by fecal microbiota transplantation and verified in older mice ($n = 6$). The common genra are shown in here **(A)**. The screened and verified gut microbiota in the process of HS induced intestinal aging **(B)**. The correlation between significantly different 11 in community abundance in terms of genera and intestinal aging-related factors **(C)**. The network of the significantly different genera **(D)**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

continuously accumulate with food intake and self generation, and participate in the progress of aging and related diseases (16, 17). Pentosidine is one of the components of AGEs. VK2 is considered to be related to vascular function in recent years (18, 19), in addition to the role of the latest inhibitor of ferroptosis (20). We evaluated the previous the oxidative stress indicators in the intestinal tissue, serum and the previous vascular endothelial factors in serum. In order to ensure the reliability of the results, we also selected pentosidine and VK2, which reveal that HS diet accelerated the aging of the intestinal tract in mice as much as possible from the biochemical indicators. Consistent with other study (21), our work suggests too much salt may speed up the intestinal aging process.

A prior research (22) similarly showed that the main cause of the chronic hepatic steatosis and inflammation that results in cardiovascular damage under HS loading is SIRT3 suppression induced by histone modification. Additionally, a HS diet promotes lung metastasis, speeds up the formation of breast cancer, and raises the number of Th17 cells in the body. Increased Th17 cells may accelerate the spread of breast cancer by secreting IL-17F, which causes breast cancer cells to activate the MAPK signaling pathway (23). The gut microbiota has been shown in recent years to be intermediate in the physiological responses induced in the host by a high salt diet (24–26). HS diet exacerbates colitis in mice by regulating gut microbial community, especially decreasing *Lactobacillus* levels and butyrate production (27). HS diet regulates a variety of intestinal bacteria, not necessarily all of which can participate in intestinal aging. Therefore, we initially observed the changes of gut microbiota community induced by HS diet, and then conducted preliminary screening and further validation, using the strategy of FMT. FMT can be used to treat recurrent

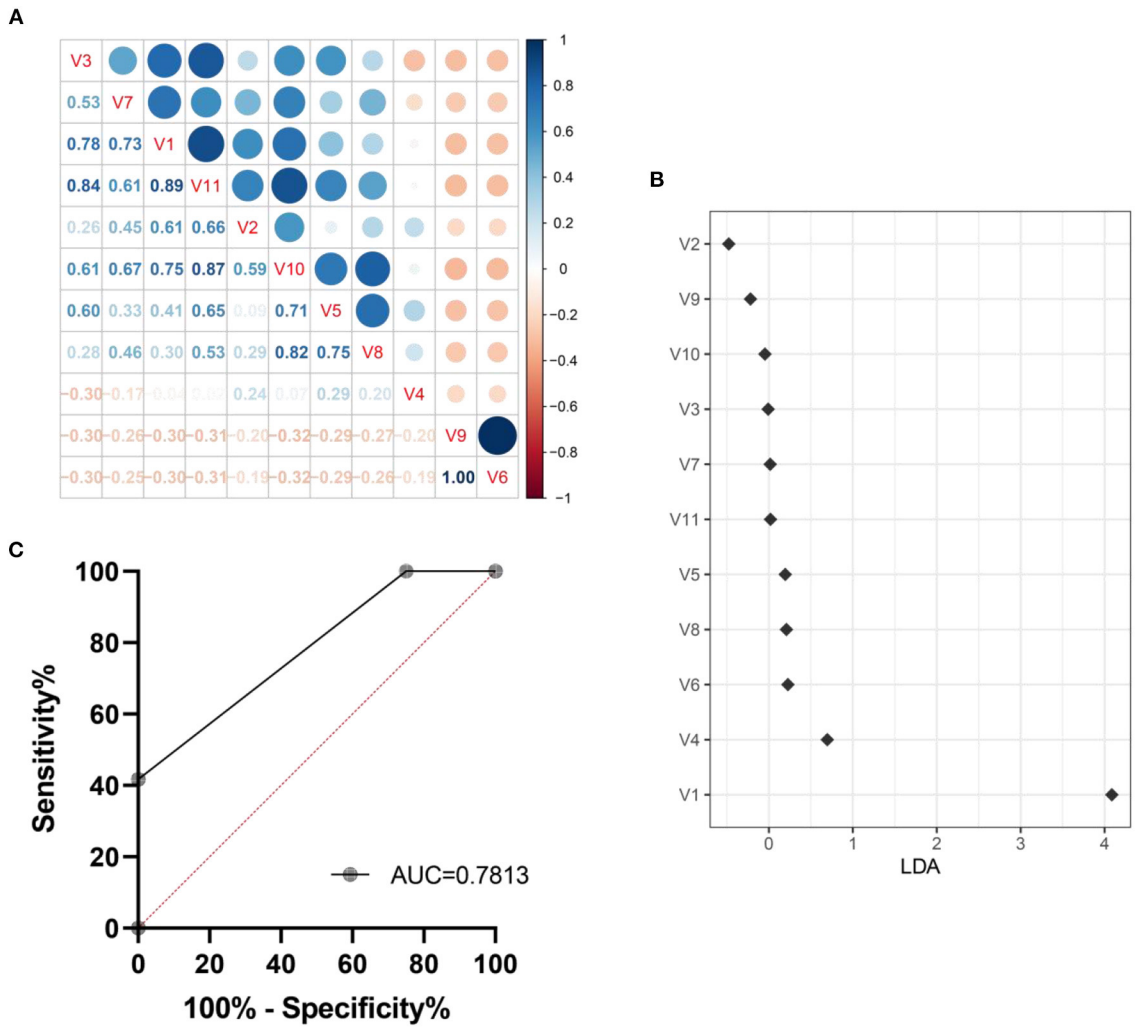

FIGURE 8
Machine learning model for intestinal aging was constructed and verified based on the significantly different genera in high salt (HS) induced mice. The correlation coefficient among genra are shown in here **(A)**. The LDA linear judgment analysis **(B)**. Evaluation of diagnostic ability based on ROC training set verification **(C)**. V1-V11: *unclassified_f__Lachnospiraceae*, *Desulfovibrio*, *Lachnospiraceae_UCG-006*, *norank_f__Lachnospiraceae*, *Escherichia-Shigella*, *Eubacterium_siraeum_group*, *NK4A214_group*, *Butyricicoccus*, *Anaerofustis*, *Parabacteroides*, *Glutamicibacter*, *Paenibacillus*.

Clostridium difficile infection as a therapeutic approach to restore the gut microbiota, since the gut microbiota can partially transfer the intestinal features of the host (28). Furthermore, a great number of clinical trials are investigating the use of FMT in additional disorders associated with the gut microbiota (28). Our previous study used the FMT method to screen and verify the differential microbiota of high salt diet interacting with ATF4 (12). In the current study, our study identified a total of 32 bacterial genera in mouse intestinal contents associated with HS-induced aging that could be significantly associated with aging *via* FMT delivery to control mice. The gut microbiota plays an important role in the physiological succession during the life cycle, and in particular, changes in the gut microbiota are closely related to aging-related diseases, and anti-aging targeting the gut microbiota is an encouraging strategy (29). The current study again reveals that a high salt diet led to intestinal aging in mice associated with gut microbiota, further validating the previous relationship that aging and gut microbiota are closely linked. Previous study suggested that HSD disrupts the balance of the intestinal microbiota primarily by depleting lactic acid-producing bacteria in a dose-dependent manner, and that these are important for salt-sensitive inflammatory diseases (30).

Differences in age are the gold standard for aging. To further characterize the changes in gut microbiota during high salt-induced aging, our study used older mice to reveal evidence of high salt-induced gut microbiota, examining which

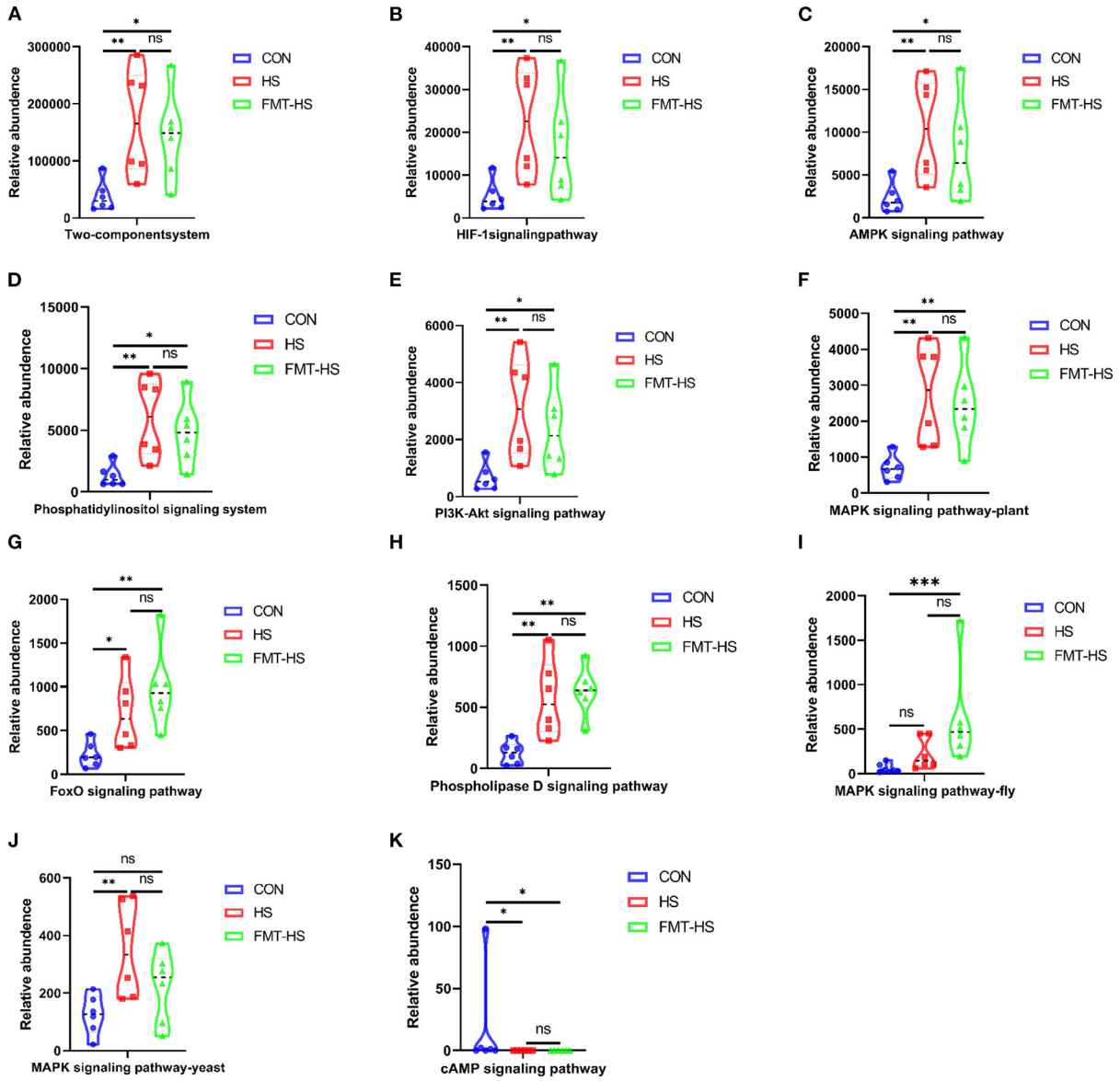

FIGURE 9
Signal transduction pathways enriched by the significantly different genera in high salt (HS) induced mice which were screened by fecal microbiota transplantation ($n = 6$). The relative abundance of Two-component system, HIF-1 signaling pathway, AMPK signaling pathway, Phosphatidylinositol signaling system, PI3K-Akt signaling pathway, MAPK signaling pathway-plant, FoxO signaling pathway, Phospholipase D signaling pathway, MAPK signaling pathway-fly, MAPK signaling pathway-yeast and cAMP signaling pathway **(A–K)**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no significance.

of the previous 32 bacteria were screened and validated in experiments with older mice. FMT from aged mice to young mice was previously reported to result in disruption of intestinal epithelial barrier integrity, accelerated aging-related systemic inflammation, and reduced levels of key functional visual proteins; in contrast, FMT from young mice reversed these aging-related features in aged mice (31). As in previous studies, our ork also found that FMT from older mice transmitted aging, suggesting that gut microbiota is involved in aging. Finally, this study found that there were 52 significant differences between bacteria and aging in older mice, which illustrate the detailed bacteria in aging process. Combining the two experimental studies before and after, we identified, screened and validated 11 microbiota genera with relative abundance difference (*unclassified_f__Lachnospiraceae*, *Desulfovibrio*, *Lachnospiraceae_UCG-006*, *norank_f__Lachnospiraceae*, *Escherichia-Shigella*, *Eubacterium_siraeum_group*, *NK4A214_group*, *Butyricicoccus*, *Anaerofustis*, *Parabacteroides*,

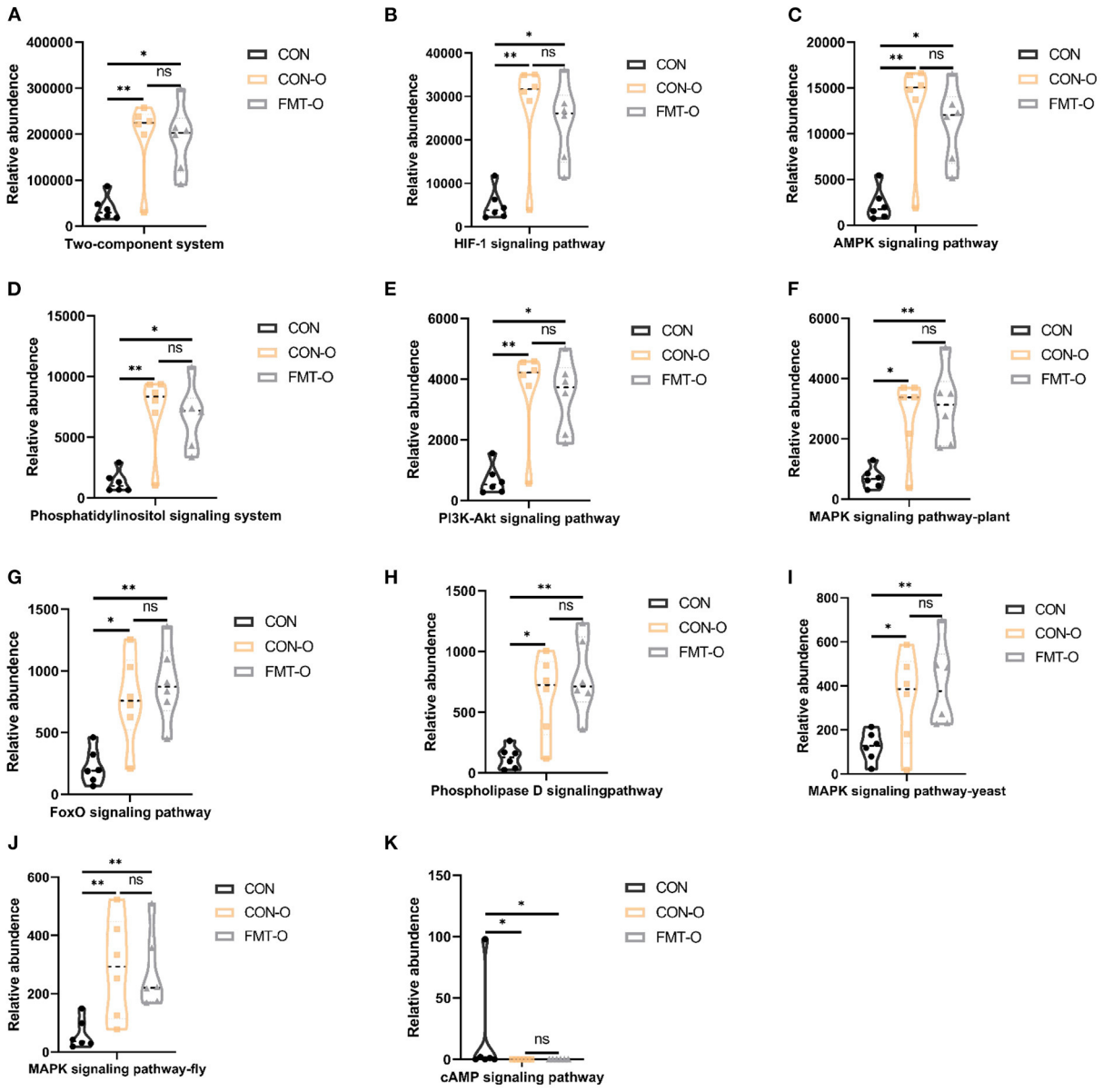

FIGURE 10
Signal transduction pathways enriched by the significantly different genera in older mice which were screened by fecal microbiota transplantation ($n = 6$). The relative abundance of Two-component system, HIF-1 signaling pathway, AMPK signaling pathway, Phosphatidylinositol signaling system, PI3K-Akt signaling pathway, MAPK signaling pathway-plant, FoxO signaling pathway, Phospholipase D signaling pathway, MAPK signaling pathway-fly, MAPK signaling pathway-yeast and cAMP signaling pathway **(A–K)**. *$p < 0.05$, **$p < 0.01$; ns, no significance.

Glutamicibacter, *Paenibacillus*) associated with high salt-induced intestinal aging, information on which has been elucidated in Figure 7. In this work, a machine learning model for intestinal aging was constructed and verified based on the significantly different genera in HS induced mice, which indicates the 11 microbiota genera with relative abundance difference are of potential diagnostic value in HS-related intestinal aging. Similar to earlier research, our study alos completed the model based on gut microbiota for the first time to evaluate the status of intestinal aging induced by excessive salt (32, 33). Aging is determined by complex interactions among genetic and environmental factors. Increasing evidence suggests that the gut microbiome lies at the core of many age-associated changes, including immune system dysregulation and susceptibility to diseases (11). After studying the mechanisms involved in order to reveal the changes in host function caused

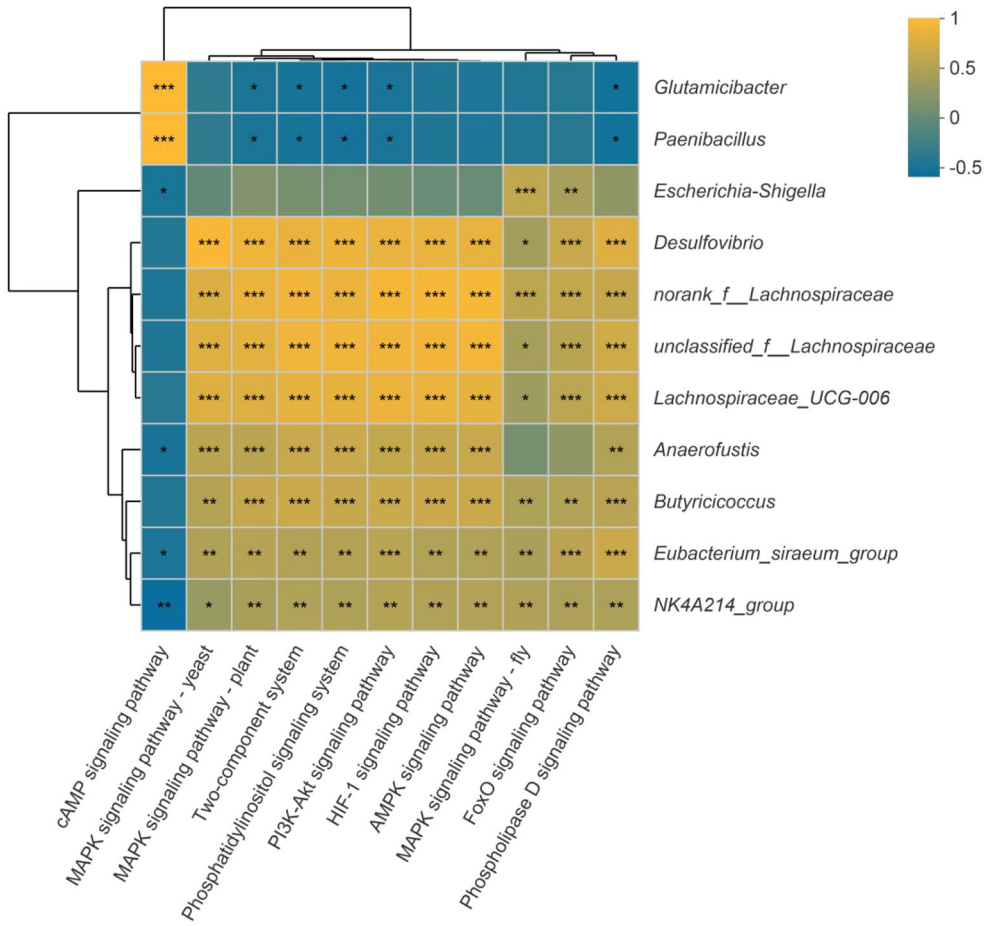

FIGURE 11
Correlation between the significantly different genera and signal transduction pathways. Dark yellow reveals a positive correlation, while dark blue reveals a negative correlation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

by the 11 differential bacteria, we identified 10 signaling pathways (two-component system, HIF-1 signaling pathway, AMPK signaling pathway, phosphatidylinositol signaling system, PI3K-Akt signaling pathway, MAPK signaling pathway-plant, FoxO signaling pathway, phospholipase D signaling pathway, MAPK signaling pathway-fly, MAPK signaling pathway-yeast and cAMP signaling pathway) that were significantly differentially enriched in this process, suggesting that these signaling pathways are associated with HS-induced intestinal aging. Moreover, *Eubacterium_siraeum_group* and *NK4A214_group* were considerably negatively connected with the cAMP signaling pathway but strongly favorably correlated with all other signaling pathways, which reveal *Eubacterium_siraeum_group* and *NK4A214_group* may be the core bacteria in HS related aging. Decreasing *Eubacterium_siraeum_group* has been found in *Lactobacillus plantarum* (LP)-derived postbiotics on ameliorating *Salmonella*-related neurological dysfunctions (34). trans-anethole impaired intestinal barrier and intestinal inflammation was also found associated with *NK4A214_group* (35). Additionally, the two-component system, phosphatidylinositol signaling system, PI3K-Akt signaling pathway, MAPK signaling pathway-yeast, and phospholipase D signaling pathway were considered as the most possible mechanisms of differential bacterial response to host in HS related intestinal aging. Notably, two-component system (36), phosphatidylinositol signaling system (37), PI3K-Akt signaling pathway (38), MAPK signaling pathway-yeast (39), phospholipase D signaling pathway (40) involve the regulation of physiological and pathological activities.

Dietary intervention is regarded as a low-cost, broad-spectrum preventative technique for slowing aging (41). Previous research suggests that increasing circulating TMAO levels throughout the aging process may worsen EC and vascular aging, which is likely due to inhibition of SIRT1 expression and increased oxidative stress, and hence activation of the p53/p21/Rb pathway (42). However, although our study

systematically revealed relevant evidence of microorganisms in the process of high salt accelerated intestinal aging through observation, screening and validation in two animal experiments, there are several shortcomings here: (1) lack of further validation in clinical experiments. The relevant evidence of gut microbiota we found has been revealed indirectly, but systematic and direct clinical evidence needs to be further investigated. All these considerations will provide clear research ideas for future studies. (2) Lack of further studies at the level of bacterial strains. Our study revealed the characteristics of gut microbiota changes, but it is not possible to isolate the strains yet. These will also provide directions for future studies. Furthermore, it was reported that oral administration of *Akkermansia* (strains) sufficiently ameliorated the senescence-related phenotype in the intestinal systems in aged mice and extended the health span (43). (3) Lack of further molecular biological level testing of molecular mechanisms. In fact, although further molecular mechanisms have been reported, it would be more convincing to perform quantitative or qualitative experimental assays.

Conclusion

To summarize, we investigated the influence of age-related changes in gut microbiota on the progression of intestinal aging in HS induced mice in order to identify a microbial profile linked with intestinal aging. Our results suggest a potential relationship between specific gut microbiota and HS in intestinal aging status, which encourages further investigation to validate causality and the potential of future microbiota-targeted therapeutics to support healthy intestinal aging. The study concluded that maintaining a healthy gut microbiota is essential for preventing intestinal aging and that both target gut tissue and a healthy microbiota can aid in preventing or delaying the onset of diseases associated with intestinal aging brought on by HS.

Data availability statement

The data presented in the study are deposited in the NCBI SRA repository, accession number PRJNA885812. The datasets generated and analyzed during the current study are available in the (Supplementary tables) repository, (https://figshare.com/, doi: 10.6084/m9.figshare.21062974).

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of Jiangnan University approved the animal experiment which was carried out in the university's animal facility (NO. 20211015c0650220).

Author contributions

T-hL, C-yZ, and Y-zX participated in study design. T-hL and LZ conducted animal experiment operation. C-yZ, T-hL, and Y-lR helped to draft and revise the manuscript. T-hL, X-yL, T-lW, Y-yD, and Y-yS carried out the statistical analysis of data. All authors contributed to the article and approved the submitted version.

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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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Ferroptosis-related differentially expressed genes serve as new biomarkers in ischemic stroke and identification of therapeutic drugs

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Background: Iron is an essential nutrient element, and iron metabolism is related to many diseases. Ferroptosis is an iron-dependent form of regulated cell death associated with ischemic stroke (IS). Hence, this study intended to discover and validate the possible ferroptosis-related genes involved in IS.

Materials and methods: GSE16561, GSE37587, and GSE58294 were retrieved from the GEO database. Using R software, we identified ferroptosis-related differentially expressed genes (DEGs) in IS. Protein-protein interactions (PPIs) and enrichment analyses were conducted. The ROC curve was plotted to explore the diagnostic significance of those identified genes. The consistent clustering method was used to classify the IS samples. The level of immune cell infiltration of different subtypes was evaluated by ssGSEA and CIBERSORT algorithm. Validation was conducted in the test sets GSE37587 and GSE58294.

Results: Twenty-one ferroptosis-related DEGs were detected in IS vs. the normal controls. Enrichment analysis shows that the 21 DEGs are involved in monocarboxylic acid metabolism, iron ion response, and ferroptosis. Moreover, their expression levels were pertinent to the age and gender of IS patients. The ROC analysis demonstrated remarkable diagnostic values of LAMP2, TSC22D3, SLC38A1, and RPL8 for IS. Transcription factors and targeting miRNAs of the 21 DEGs were determined. Vandetanib, FERRIC CITRATE, etc., were confirmed as potential therapeutic drugs for IS. Using 11 hub genes, IS patients were categorized into C1 and C2 subtypes. The two subtypes significantly differed between immune cell infiltration, checkpoints, and HLA genes. The 272 DEGs were identified from two subtypes and

their biological functions were explored. Verification was performed in the GSE37587 and GSE58294 datasets.

Conclusion: Our findings indicate that ferroptosis plays a critical role in the diversity and complexity of the IS immune microenvironment.

KEYWORDS

ferroptosis, ischemic stroke, biomarkers, immune microenvironment, subtypes

Introduction

There are genetic and environmental risk factors that interact to cause ischemic stroke (IS). Society and families are burdened by IS because it is the leading cause of disability (1). IS patients must continue taking medication for a long period after stroke onset, bringing about huge financial, mental, and time-wise burdens. IS risk factors include hypertension, diabetes, hyperlipidemia, and smoking. However, the molecular mechanism remains undetermined. Studies showed that early IS diagnosis can positively impact therapeutic outcomes and prognoses (2). Therefore, a better understanding of IS and identifying new biomarkers and therapeutic targets are urgently needed.

Iron is the most abundant trace element in the human body and is also considered indispensable for IS development (3). Ferroptosis is a unique type of programmed cell death distinguished by excessive iron buildup and lipid peroxidation (4). A recent study suggested that ferroptosis played an essential role in tumorigenesis and cancer progression (5). Additionally, ferroptosis is highly involved in many other diseases, such as IS and heart diseases (6). Moreover, research also proved that ferroptosis-related gene signatures could be used as a biomarker to diagnose, predict, and treat multiple diseases (7, 8). Nevertheless, the function of ferroptosis-related genes in IS is yet unclear.

In addition, stroke is often followed by post-stroke infection due to systemic immunosuppression, which has a worse outcome (9). It has been shown that immunomodulatory approaches, such as T-cell transfer and activators of natural killer T cells (NKTs), can reduce post-stroke immunosuppression (10). Immunomodulatory approaches can effectively manage stroke and its complications by targeting multiple elements of the immune system (11). Several well-known drugs, like azithromycin and metformin, can change the innate immune response. Both of these drugs are known to protect the brain after a stroke (12). So, it is important to figure out how immunosuppression works in stroke so that these drugs can be used to treat people. However, the immune mechanisms implicated in IS and IS-associated systemic immunosuppression are still poorly understood.

In this study, by comparing IS and normal samples in the GSE16561 dataset, differentially expressed genes (DEGs) were identified, which were then intersected with ferroptosis-related genes in the FerrDb database. We performed an enrichment analysis, as well as an investigation of expression levels and clinical significance. Unsupervised cluster analysis was performed on patients based on hub gene expression, and the characteristics of the immune microenvironment among different subtypes were analyzed. Validation was carried out in the GSE37587 and GSE58294 datasets (**Figure 1**).

Materials and methods

Data source

Data chips, microarrays, and gene expression data from GEO[1] are available for research and analysis (13). Datasets were included and excluded according to the following criteria: (i) genomic wide expression mRNA microarray data had to be included, (ii) IS samples were required to be included, and (iii) specimen numbers must be greater than 30.

Three gene expression datasets, GSE16561 (14) and GSE37587 (15), derived from the GEO (GPL6883, Illumina HumanRef-8 v3.0 expression bead chip, array, Homo sapiens) were obtained with corresponding clinical data. GSE58294 (16) derived from the GEO (GPL570, Affymetrix Human Genome U133 Plus 2.0 Array, Homo sapiens) were obtained with corresponding clinical data. Data of the datasets were extracted from the total RNA of whole blood. Altogether, 39 IS and 24 normal whole blood samples were obtained from the GSE16561 cohort, 68 IS whole blood samples were obtained from the GSE37587 cohort, and 69 IS and 23 normal whole blood samples were obtained from the GSE16561 cohort.

A total of 388 ferroptosis-related genes were found in the FerrDb database[2] (17; **Supplementary Table 1**) after removing duplicates. These genes include drivers, suppressors, and markers.

1 http://www.ncbi.nlm.nih.gov/geo

2 http://www.zhounan.org/ferrdb/current/

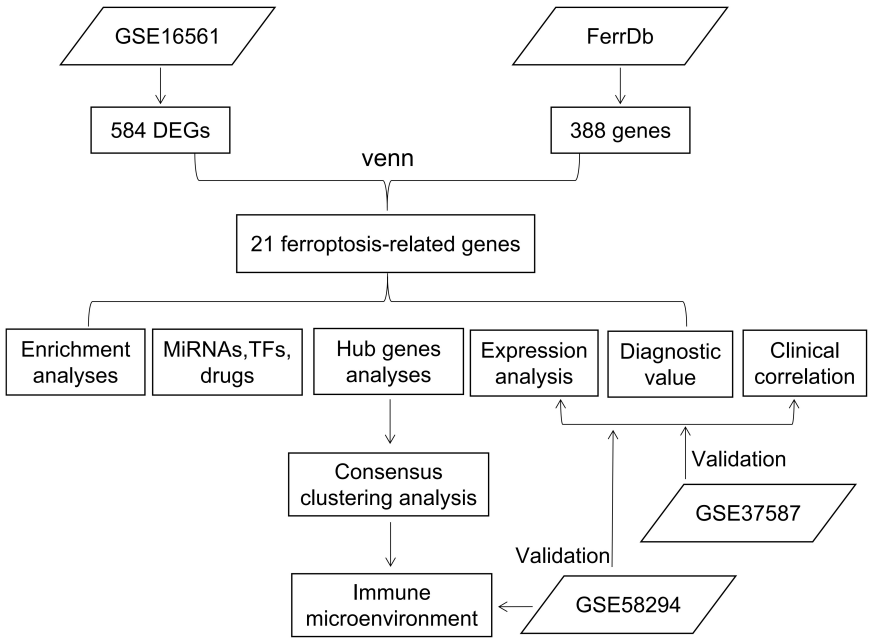

FIGURE 1
Workflow chart. DEGs, differentially expressed genes; TFs, transcriptional factors.

Quality control

GSE16561

The raw expression profile GSE16561_RAW.tar was downloaded from the GEO database. Probes were annotated to their respective gene symbols *via* the GPL6883 platform file. Mean expression levels were used to compute gene symbols from several probes. Quantile normalization and log2 transformations were applied to raw data. Two abnormal IS samples (3100193_Stroke and 3100137_Stroke) were excluded based on principal component analysis (PCA). The following analysis included 37 IS and 24 normal samples. The GSE58294 dataset was also preprocessed according to the above process.

GSE37587

The raw expression profile GSE37587_non-normalized.txt.gz was downloaded from the GEO database. Probes were annotated to their corresponding gene symbols using the GPL6883 annotation file. Mean expression levels were used to compute gene symbols from several probes. Following the log2-transformation and quantile normalization, the raw expression files of the GSE16561 and the GSE37587 were combined. The batch correction was performed with the ComBat algorithm of the "sva" R package.[3] The final dataset comprised 68 IS samples from GSE37587 and 24 normal samples from GSE16561. According to PCA, 3 abnormal IS samples were deleted (GSM922927, GSM922908, and GSM922905), thus 65 IS samples and 24 normal samples were included in the subsequent analysis. At the same time, a total of 107 IS samples from the two data sets were used for cluster analysis.

Identification of ferroptosis-related differentially expressed genes and functional analysis

Limma[4] (18) was used to identify DEGs between IS and normal samples in the GSE16561 (adjustment $p < 0.05$ and $|\log2FC| > 0.5$). Heat maps were generated using the R package "pheatmap,"[5] exhibiting the top 20 genes with the most significant upregulation or downregulation, respectively. Twenty-one ferroptosis-related DEGs were obtained with a Venn diagram using the R package "Venn,"[6] and their expression correlation was calculated with the R package "corrplot"[7] and visualized using the "circlize" package[8] (19). The ability of the 21 ferroptosis-related DEGs to distinguish between

3 https://bioconductor.org/packages/sva/

4 https://bioconductor.org/packages/limma/
5 https://CRAN.R-project.org/package=pheatmap
6 https://CRAN.R-project.org/package=venn
7 https://CRAN.R-project.org/package=corrplot
8 https://CRAN.R-project.org/package=circlize

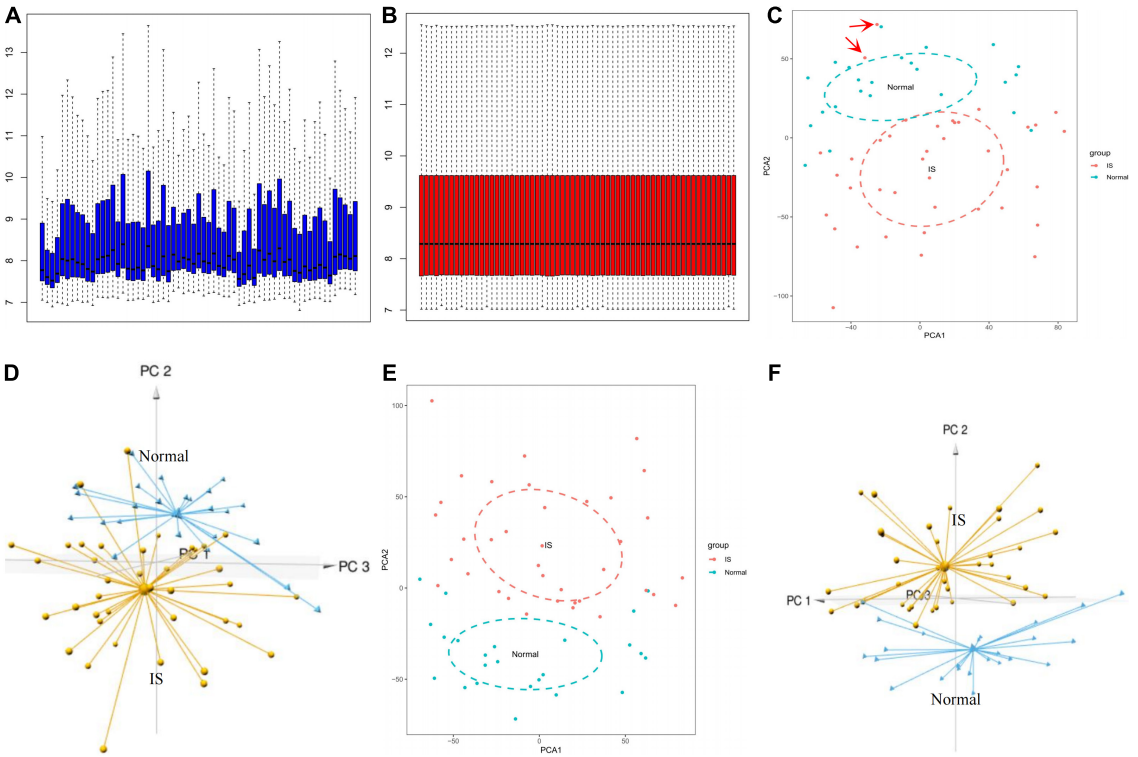

FIGURE 2
GSE16561 data set preprocessing. Box plot showing the gene expression level between different samples before **(A)** and after **(B)** normalization. 2D and 3D PCA plots demonstrated the distribution of samples before **(C,D)** and after **(E,F)** pretreatment. PCA, principal components analysis.

IS and normal samples was determined with PCA. Metascape (20) was used for functional analysis. Cut-off value: $P < 0.05$.

Construct a diagnostic model of 21 ischemic stroke-associated ferroptosis genes

A diagnostic model was constructed by the least absolute shrinkage and selection operator (Lasso) analysis to analyze and identify the redundancy factors. Finally, receiver operating feature (ROC) scores were used to evaluate the diagnostic performance of the model.

Bioinformatics analysis of 21 ferroptosis-related differentially expressed genes

In this study, Wilcoxon rank sum tests were used to examine the association between ferroptosis-related DEGs and age and gender of IS patients. *Via* the Enrichr platform[9] (21), the transcription factors, upstream miRNAs, and small-molecular drugs of the 21 ferroptosis-related DEGs were predicted using the TRRUST, miRTarBase, and DSigDB databases, respectively. The ROC curve was generated using the "pROC" package[10] (22) and visualized with the "ggplot2" package[11] (23).

Based on the STRING database (24), PPI networks were constructed, in which the interactions with a score higher than 0.4 were considered statistically significant. The hub genes were selected with the plug-in CytoNCA (25) of the Cytoscape software V3.7.1 (26) and subjected to functional enrichment analysis using GeneMANIA[12] (27). FDR < 0.05 was used as a cutoff point.

Consensus clustering analysis

ConsensusClusterPlus (28) was used for cluster analysis. We used an agglomerative km clustering algorithm with one Pearson correlation distance and resampled 80% of the samples

9 http://amp.pharm.mssm.edu/Enrichr/

10 https://CRAN.R-project.org/package=pROC

11 https://CRAN.R-project.org/package=ggplot2

12 http://genemania.org/

10 times. Empirical cumulative distribution function plots were used to determine the optimal number of clusters.

Immune cell infiltration analysis

Single sample gene set enrichment analysis (ssGSEA) was used to analyze the infiltration levels of immune cells based on 29 immune-related markers' expression profiles. Also, CIBERSORT (29) was used to further analyze immune cell infiltration levels. Wilcoxon rank-sum tests were used to determine differences in immune cell proportions. The statistical significance threshold was set at $p < 0.05$.

Gene set variation analysis

The GSVA (30) approach was used to examine important pathways and molecular processes by obtaining the h.all.v7.4.symbols.gmt and c2.cp.kegg.v7.4.symbols.gmt subsets from the Molecular Signatures Database (31). The minimum gene set was set to 5 and the maximum gene set was set to 5,000, and the enrichment scores were calculated for each sample in each gene set. The final enrichment score matrix was obtained. The differences in GSVA scores between subtypes for each gene set were compared using the limma package. FDR < 0.05 was used as a cutoff point.

Identification of differentially expressed genes between different subtypes

Differentially expressed genes were screened for subtypes in the integrated dataset using the R package limma with $|$ Fold Change$|$ > 1.5 and FDR < 0.05. The differential genes were shown by volcano plot and heat map.

Functional enrichment analysis

Biological functions were analyzed using the ClusterProfiler package (32), which includes GO and KEGG. Use the Benjamini–Hochberg method to adjust the p-value for multiple tests. $P < 0.05$ was used as a cutoff point.

Statistical analysis

Statistical analysis was performed using R 4.1.0. Wilcoxon or Student's t-test compared the two groups. Pearson's or Spearman's test determined the variables' correlation. A Chi-square test was performed to compare two categorized variable groups. The statistical significance threshold was set at $p < 0.05$.

Results

Data preprocessing

GSE16561

The box plot of the raw data demonstrated that gene expression levels were unevenly distributed across different samples (**Figure 2A**), which was processed *via* quantile normalization (**Figure 2B**). According to the 2D and 3D PCA plots (**Figures 2C,D**), two abnormal IS samples in the normal controls were deleted. Further validation distinguished between the groups and illustrated good clustering of samples within the same group (**Figures 2E,F**).

GSE37587 test set

Normalized gene expression data exhibited a uniform distribution in the samples (**Supplementary Figure 1A**). Three abnormal IS samples were excluded from the normal control based on the 2D and 3D PCA plots (**Supplementary Figures 1B,C**). PCA was repeated and demonstrated excellent discrimination between the groups and good clustering of samples within the same group (**Supplementary Figures 1D,E**).

Ferroptosis-related differentially expressed genes differentiate ischemic stroke patients from normal controls

According to $|$ log2FC $| > 0.5$ and adjustment $p < 0.05$, 584 DEGs were obtained from the GSE16561 dataset, including 319 genes up-regulated and 265 genes down-regulated in IS samples (**Figure 3A**; **Supplementary Table 2**). The top 20 genes with the most significant upregulation or downregulation were selected to plot the Heat map (**Figure 3B**). To investigate the association between IS and ferroptosis, 21 intersecting genes were obtained between 584 DEGs and 388 ferroptosis-related genes (**Figure 3C**). Correlation analysis suggested correlations between the expression of those genes in the GSE16561 dataset (**Figure 3D**). Prominently, the 21 genes fully differentiated IS cases from normal controls in the GSE16561 dataset as analyzed by PCA (**Figures 3E,F**), which was verified in the test set GSE37587 (**Figures 3G,H**). Taken together, the 21 ferroptosis-related genes were highly heterogeneous between normal and IS tissues, and their expression changes may play a vital role in the initiation and development of IS.

Enrichment analysis

The box plot described the expression pattern of the 21 ferroptosis-related DEGs in IS and normal samples. In the two datasets, most of the 21 genes exhibited upregulation in IS tissues vs. normal tissues, except for LPIN1, RPL8,

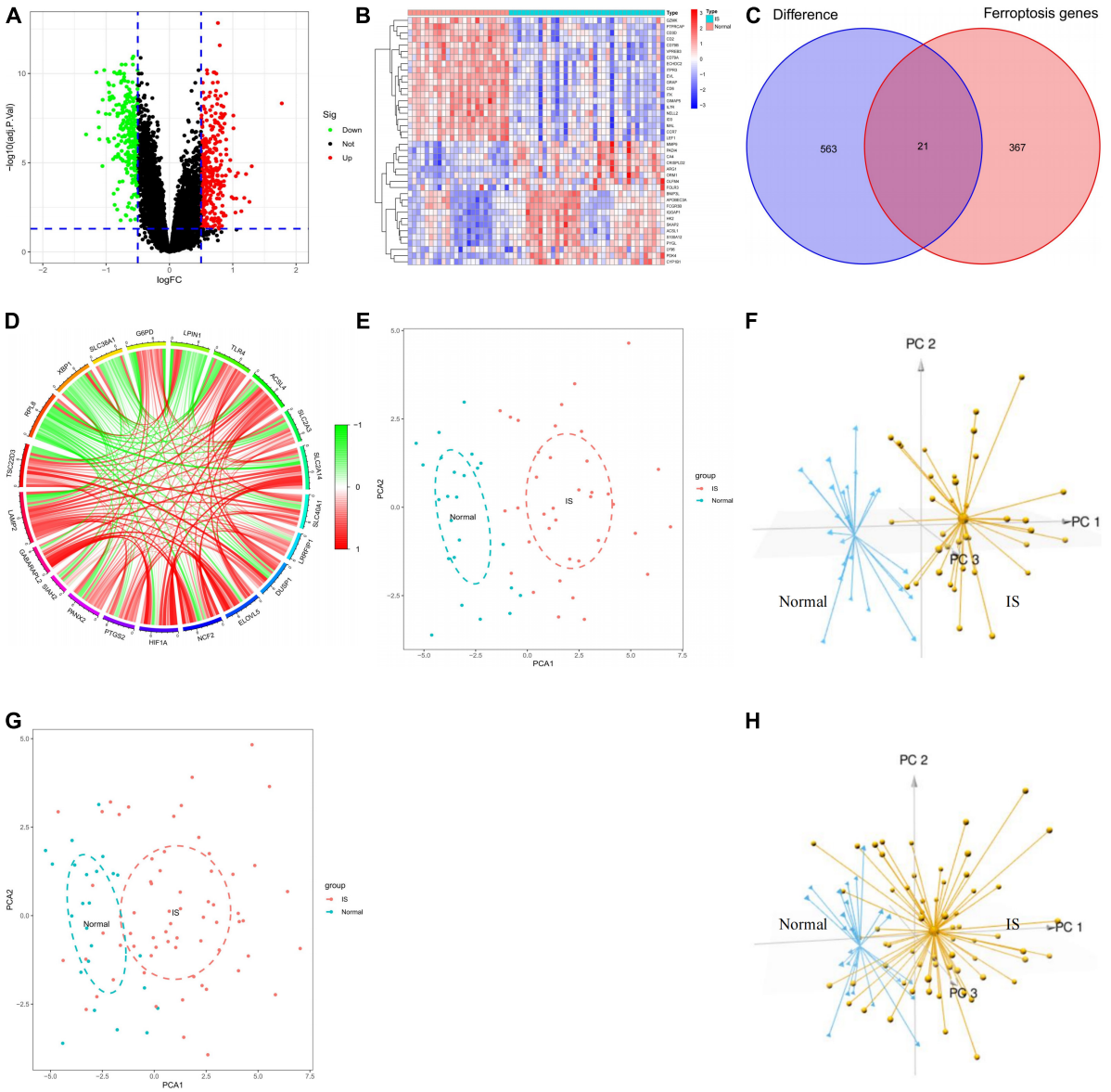

FIGURE 3
Analysis of differentially expressed genes. **(A)** Screening of DEGs shown by volcano plot. **(B)** Heatmap of the 40 genes expressed differently in IS samples compared to normal samples. **(C)** Venn diagram showing 21 ferroptosis-related DEGs. **(D)** Circle diagram showing the correlation of 21 ferroptosis-related DEGs. **(E,F)** 2D and 3D PCA plots showing PCA analysis based on 21 ferroptosis-related genes in GSE16561. **(G,H)** 2D and 3D PCA plots showing PCA analysis based on 21 ferroptosis-related genes in GSE37587. DEGs, differentially expressed genes; IS, ischemic stroke; PCA, principal components analysis.

SLC38A1, and XBP1, which showed down-regulated expression (Figures 4A,B). Among 21 genes examined by enrichment analysis, monocarboxylic acid metabolism, iron ion responses, and ferroptosis were primarily enriched (Figure 4C).

Clinical correlation analysis

The expression levels of HIF1A, GABARAPL2, LAMP2, SLC2A14, NCF2, ELOVL5, ACSL4, and XBP1 in patients ≥78 years old were significantly higher than those in patients < 78 years old in both the GSE16561 and GSE37587 datasets (Figures 5A,B). In the GSE16561 cohort, as compared to female patients, male patients displayed remarkably higher expression of HIF1A, GABARAPL2, SLC2A1, NCF2, and ELOVL5, while lower expression of TLR4 and SLC40A1 (Figure 5C). In the test set GSE37587, higher expression of HIF1A, GABARAPL2, SLC2A14, and NCF2 was also observed in male patients vs. female patients

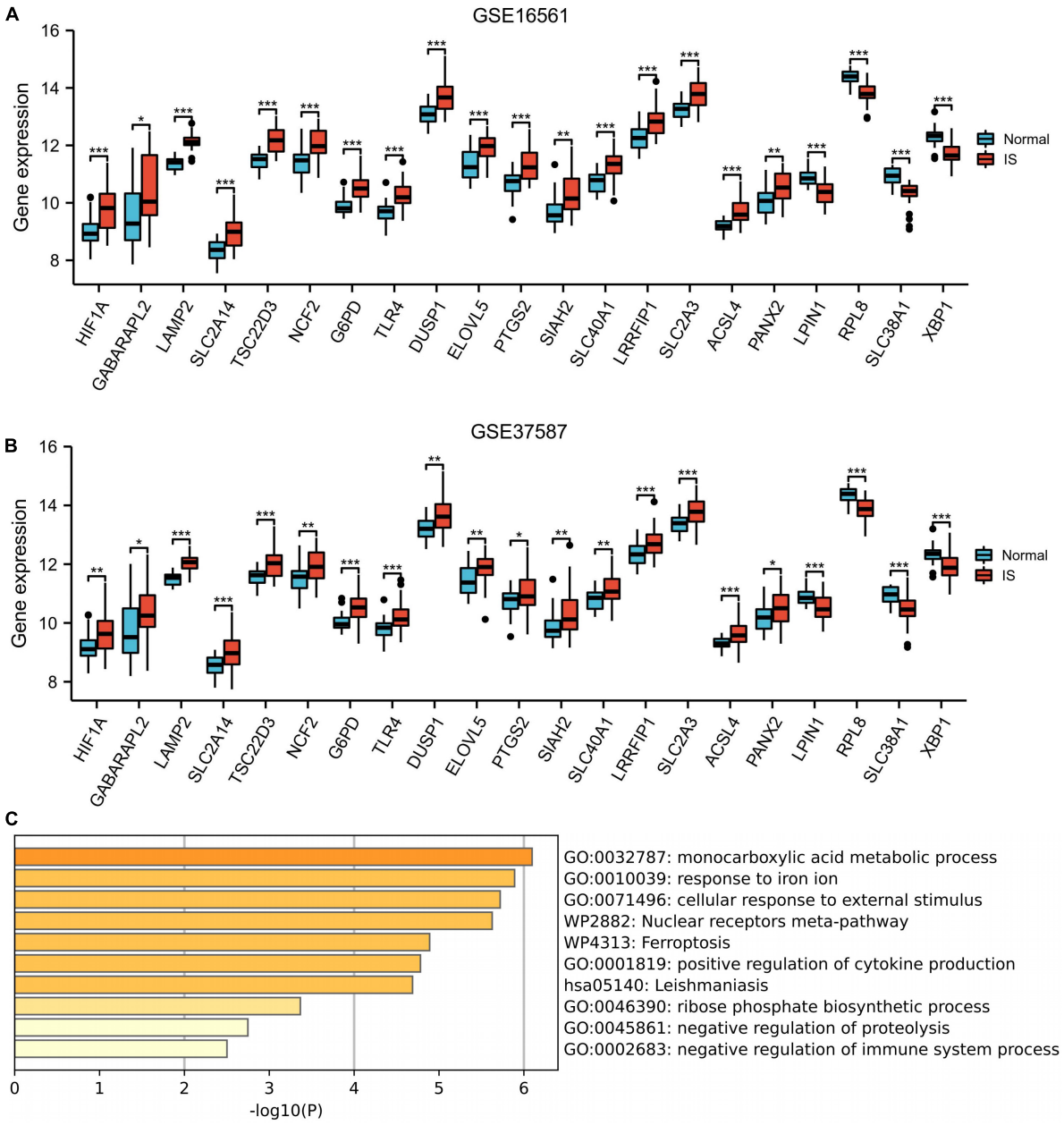

FIGURE 4
Enrichment analysis. Box plot described the expression pattern of the 21 ferroptosis-related genes between IS and normal samples in GSE16561 **(A)** and GSE37587 **(B)**. **(C)** 21 ferroptosis-related genes enrichment analysis. IS, ischemic stroke. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

(Figure 5D). Therefore, the expression of these ferroptosis-related genes was interrelated with the age and gender of IS patients.

Receiver operating feature analysis

The ROC curves of the GSE16561 dataset revealed excellent accuracy of LAMP2 (AUC = 0.98), TSC22D3 (AUC = 0.90), SLC38A1 (AUC = 0.89), and RPL8 (AUC = 0.89) in distinguishing between the outcomes of normal and IS groups (Figures 6A–C). The ROC curves of the test set GSE37587 manifested moderate accuracy of LAMP2 (AUC = 0.92), RPL8 (AUC = 0.85), SLC38A1 (AUC = 0.80), and TSC22D3 (AUC = 0.78) in terms of differentiating between the outcomes of normal and IS groups (Figures 6D–F). In the GSE16561 dataset, based on lasso regression, we constructed a model composed of seven genes (LAMP2,

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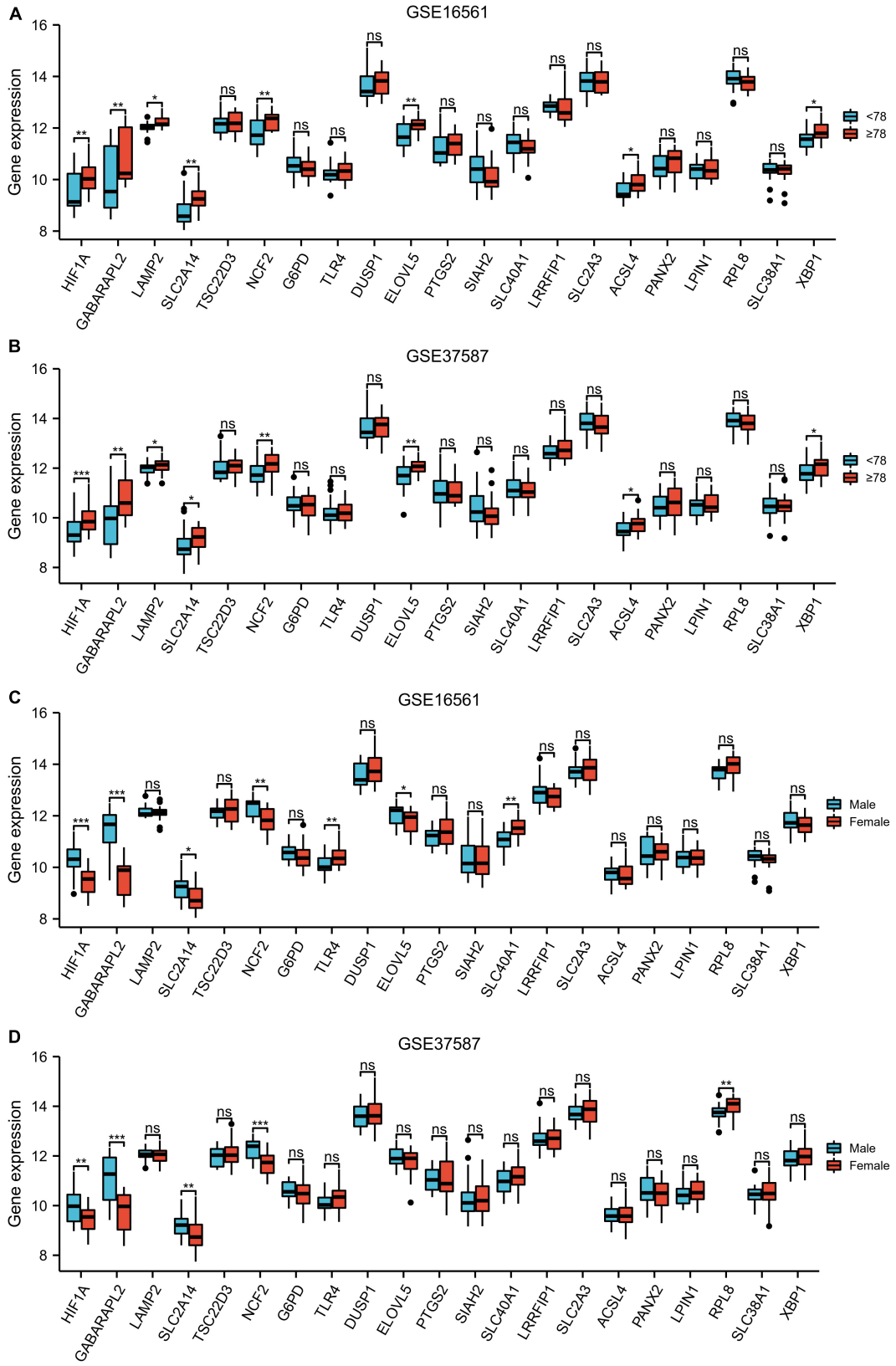

FIGURE 5
Clinical correlation analysis. Box plot showing the expression pattern of the 21 ferroptosis-related genes between <78 and ≥78 patients in GSE16561 **(A)** and GSE37587 **(B)**. Box plot showing the expression pattern of the 21 ferroptosis-related genes between male and female patients in GSE16561 **(C)** and GSE37587 **(D)**. ns: $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

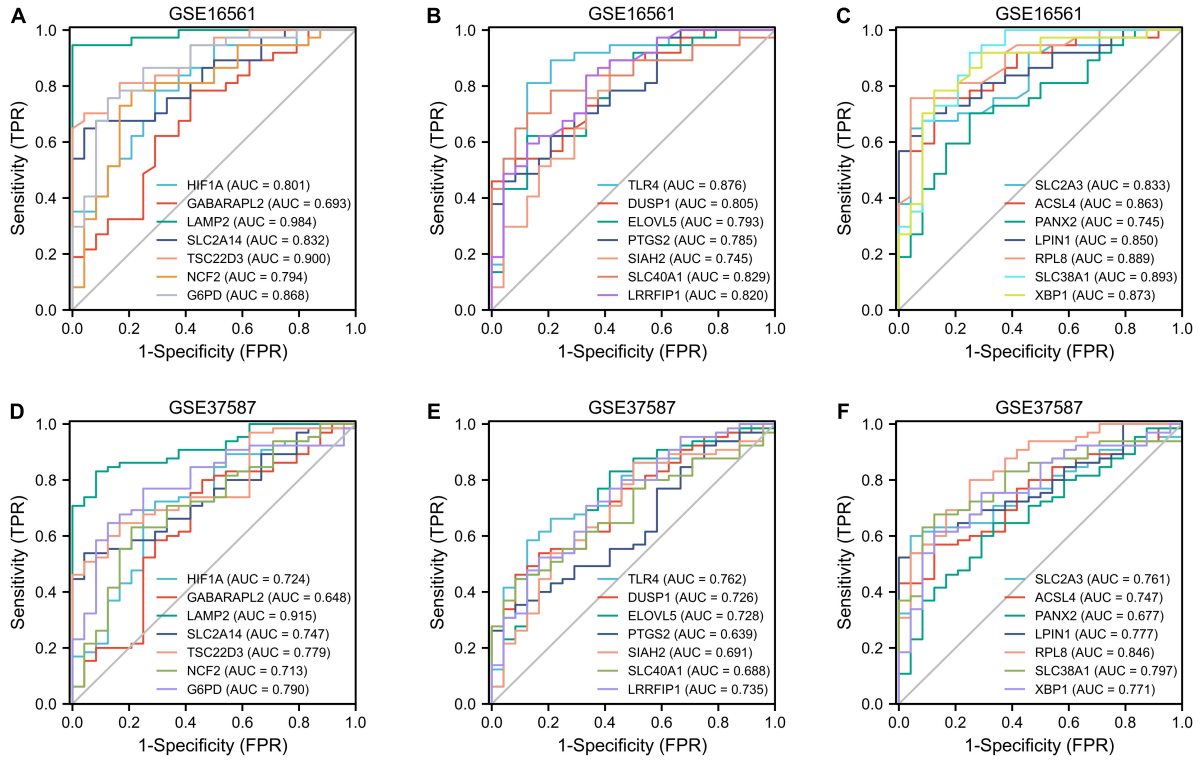

FIGURE 6
ROC analysis. Diagnostic ROC analysis of 21 ferroptosis-related genes in GSE16561 **(A–C)** and GSE37587 **(D–F)**. ROC, receiver operating characteristic; AUC, area under curve; FPR, false positive rate; TPR, true positive rate.

TABLE 1 Transcriptional factor targets of 21 ferroptosis-related genes in IS.

Term	*P-value*	Odds ratio	Combined score
IRF8	5.74E-05	233.5672515	2280.803907
HMGA1	1.41E-04	140.0982456	1241.768431
STAT6	6.97E-06	100.7373737	1196.206221
CREM	3.10E-04	91.33180778	737.7313505
PGR	3.10E-04	91.33180778	737.7313505
ATF2	5.11E-04	69.99649123	530.5117162
USF2	0.001103204	46.62923977	317.5235245
CEBPB	0.001791576	36.15426497	228.6634283
SPI1	0.001911633	34.94561404	218.7524601
EGR1	0.003806749	24.34883721	135.6468757

IS, ischemic stroke.

TABLE 2 MicroRNA targets of 21 ferroptosis-related genes in IS.

Term	*P-value*	Odds ratio	Combined score
hsa-miR-548ag	5.92E-05	47.40238095	461.4566536
hsa-miR-625-3p	7.60E-04	56.73399716	407.4703927
hsa-miR-329-5p	1.65E-04	33.13166667	288.5803973
hsa-miR-606	0.001973063	34.37100949	214.0684295
hsa-miR-548ba	0.002429044	30.82198142	185.5562669
hsa-miR-548ai	2.50E-03	30.37376049	182.0091651
hsa-miR-570-5p	2.57E-03	29.93834586	178.5755424
hsa-miR-8076	0.003081354	27.20710868	157.3220116
hsa-miR-4798-3p	2.59E-02	41.57291667	151.8276652
hsa-miR-4499	3.32E-03	26.18289474	149.4851799

IS, ischemic stroke.

LPIN1, TLR4, SLC2A3, LRRFIP1, PANX2, and GABARAPL2) to distinguish healthy subjects from IS patients (**Supplementary Figures 2A,B**). The AUC of GSE16561 was 1.000 in the training set, and the values of GSE37587 and GSE58294 AUC in the verification set were 0.961 and 0.730, respectively (**Supplementary Figures 2C–E**). Overall, these ferroptosis-related genes had remarkable diagnostic significance for IS patients.

Transcript factor, upstream miRNA, and drug prediction

The transcription factors, upstream miRNAs, and related drugs of the 21 ferroptosis-related genes were predicted *via* the Enrichr platform. STAT6, IRF8, and HMGA1 were the main transcription factors retrieved from the TRRUST

TABLE 3 Drug targets of 21 ferroptosis-related genes in IS.

Term	*P-value*	Odds ratio	Combined score
Vandetanib CTD 00004046	4.60E-08	151.4079696	2557.873945
FERRIC CITRATE CTD 00001186	6.89E-05	210.2	2014.429518
Flufenamic acid-(benzoic ring-13C6) TTD 00008058	9.48E-05	175.1491228	1622.485755
p-Phenylenediamine CTD 00001400	2.74E-07	93.78352941	1416.922104
CHLOROBENZENE CTD 00001495	1.25E-04	150.112782	1349.226508
Gossypol PC3 UP	3.67E-07	86.81917211	1286.427251
Tributyltin CTD 00000610	6.39E-06	103.890625	1242.619131
Dequalinium chloride HL60 DOWN	5.14E-07	79.44167498	1150.427383
Oligomycin CTD 00006434	1.59E-04	131.3355263	1148.713528
Mephentermine HL60 UP	7.87E-07	70.99108734	997.7694472

IS, ischemic stroke.

database (Table 1). Hsa-miR-548ag, hsa-miR-329-5p and hsa-miR-625-3p were the major upstream miRNAs according to the miRTarBase database (Table 2). Vandetanib, FERRIC CITRATE, etc., were the primary drugs predicted from the DSigDB database (Table 3). The identified transcription factors and miRNAs might be of prominent importance in the development of IS, and the predicted drugs can serve as potential drugs for IS.

Hub gene analysis

Based on the 21 ferroptosis-related genes, a PPI network was generated based on the STRING online database (Figure 7A). Hub genes were analyzed using the plug-in cytoNCA. The top 11 hub genes were identified by calculating the Betweenness, Closeness, and Degree (Figures 7B,C). Based on enrichment analysis, the top 11 hub genes were highly enriched in fatty acid metabolism, hypoxia response, and angiogenesis (Figure 7D).

Clustering analysis based on hub genes

We performed an unsupervised consistency clustering analysis on IS samples based on 11 hub genes (Supplementary Figures 3A–C). According to the average consistency evaluation within the cluster group, we choose the number of clusters as $K = 2$ (Figures 8A,B). We named these two subtypes C1 and C2, respectively. PCA analysis revealed significant differences between subtypes (Figure 8C). There was significant heterogeneity in the expression of 11 hub genes between

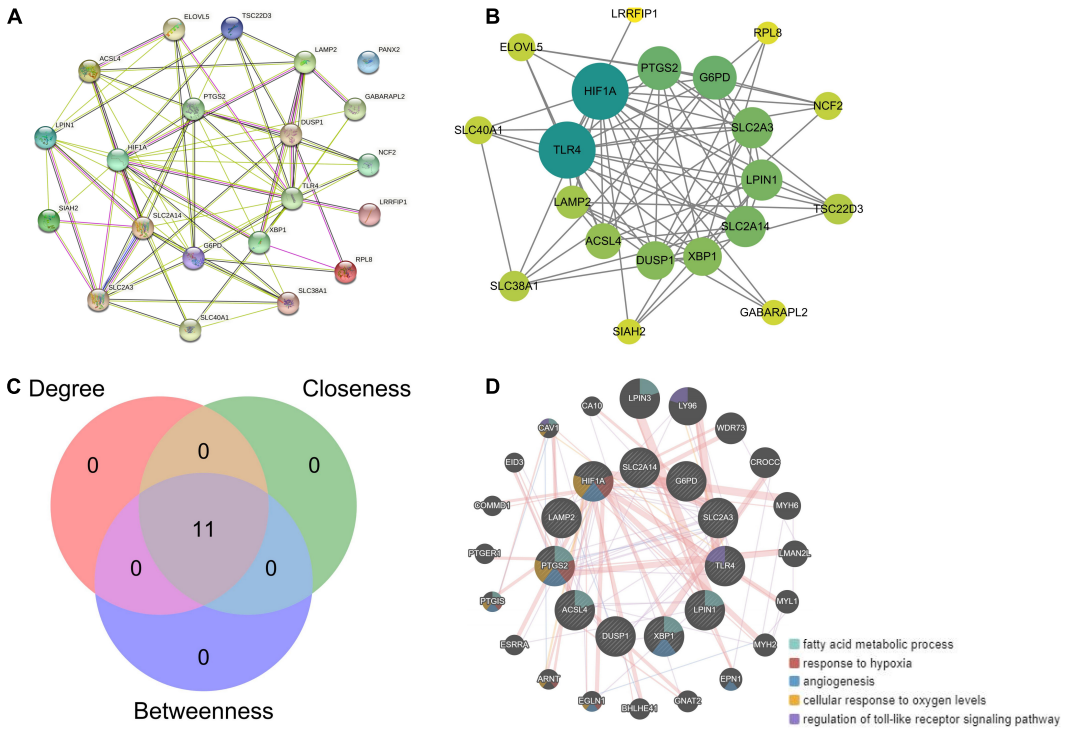

FIGURE 7
Hub gene analysis. **(A)** Based on the 21 ferroptosis-related genes, a PPI network was generated based on the STRING online database. **(B)** Hub genes were analyzed using the plug-in cytoNCA. **(C)** The top 11 hub genes were identified by calculating the betweenness, closeness, and degree. **(D)** Enrichment analysis of 11 hub genes by GeneMANIA online database.

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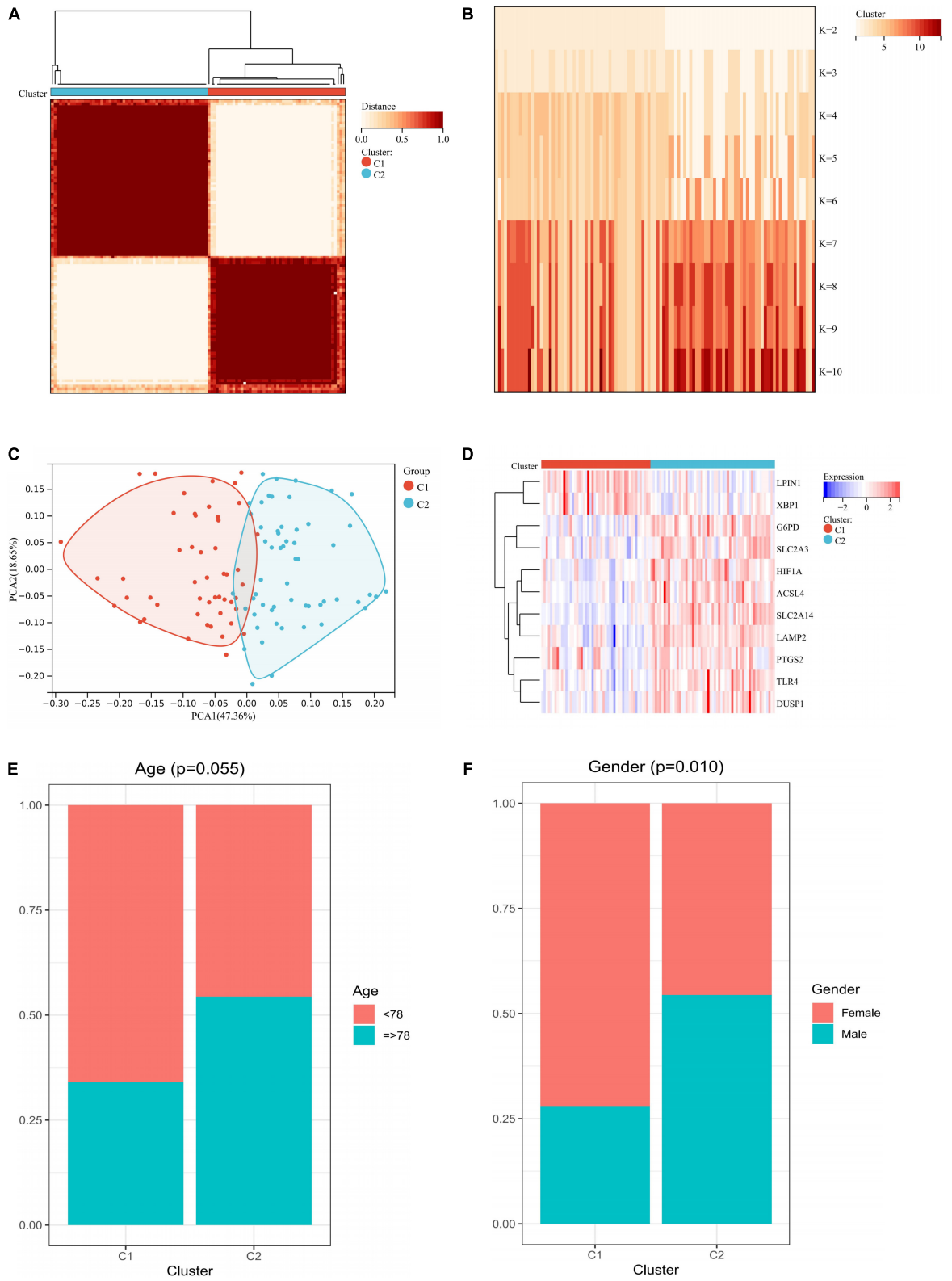

FIGURE 8
Unsupervised clustering of 11 hub genes. **(A)** Consensus matrix heatmap when k = 2. **(B)** Tracking plot showing the sample classification when k = 2–10. **(C)** PCA plots showing a remarkable difference in transcriptome between two subtypes. **(D)** Heatmap showing the expression of 11 hub genes in two subtypes. **(E)** Age ratio distribution in the two subtypes. **(F)** Gender ratio distribution in the two subtypes. PCA, principal components analysis.

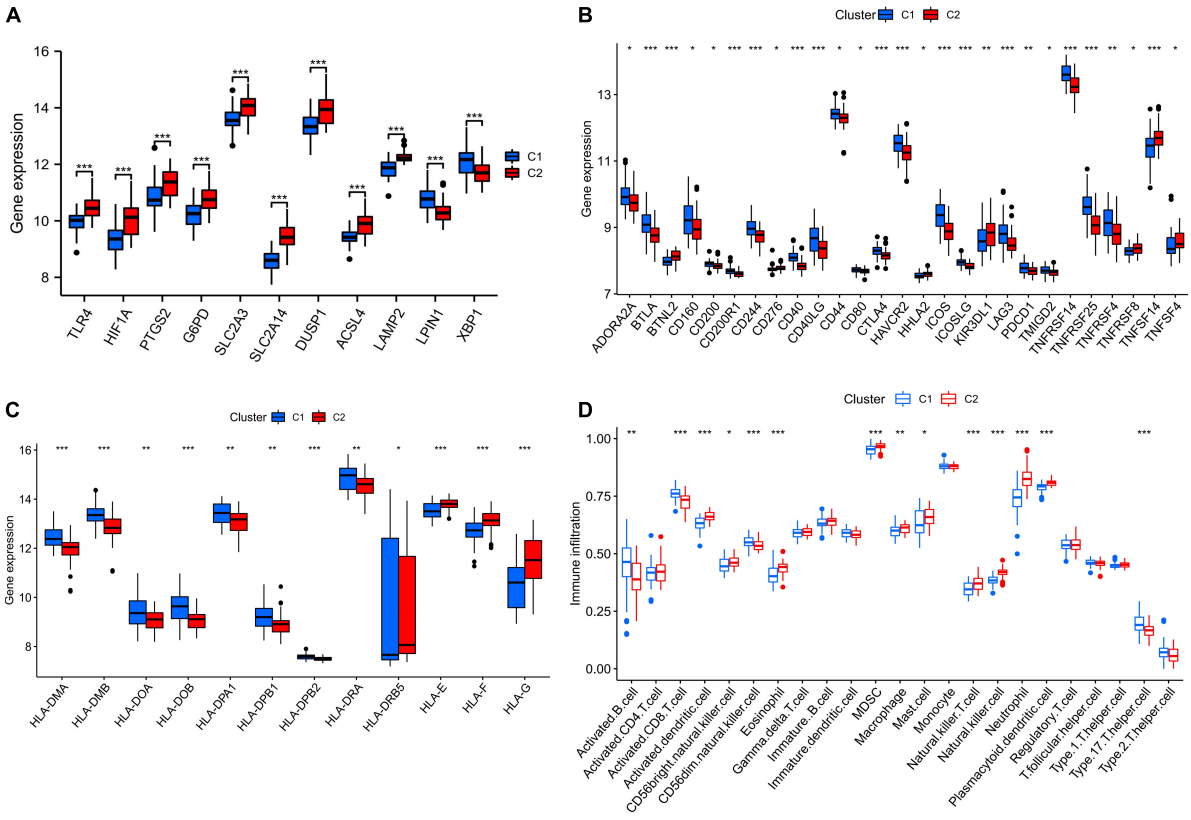

FIGURE 9
The features of the immunological microenvironment differ between subtypes. Box plots showing that there were differences in hub genes **(A)**, immune checkpoints **(B)**, HLA genes **(C)**, and immune cell infiltration **(D)** between the two subtypes. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

subtypes (Figure 8D), and there was some association between subtypes and the age and sex of IS patients (Figures 8E,F).

Characteristics of immune microenvironment in different subtypes

Figure 9A showed that most of the hub genes were expressed at higher levels in C1 subtype than in C2. Most of the immune checkpoints and HLA genes were significantly upregulated in C1 compared to C2 subtype (Figures 9B,C). The C2 subtype was more immunoactive (p53 pathway, complement, IL6-JAK-STAT3 signaling, TNFA signaling *via* NFKB, chemokine signaling pathway, etc.) than the C1 subtype, as shown in Supplementary Figure 4. Based on the results of the ssGSEA algorithm, most immune cell infiltration levels differed significantly between C1 and C2 subtypes (Figure 9D). And the CIBERSORT algorithm analysis revealed a significant difference between C1 and C2 subtypes in terms of T cell infiltration (Supplementary Figures 5A,B).

Functional enrichment analysis between different subtypes

Two hundred seventy-two DEGs were obtained from Cluster 1 and Cluster 2, of which 49 DEGs were upregulated in Cluster 1 and 223 DEGs were downregulated in Cluster 1 (Figure 10A). According to the heat map, two molecular subtypes could be distinguished by these DEGs (Figure 10B). Afterward, we analyzed DEGs among the two subtypes. As illustrated in Figures 10C,D, these DEGs were mainly associated with immune responses (immune system process, cell activation, leukocyte activation, etc.). Based on KEGG enrichment analysis, these DEGs were mainly associated with ribosomes, osteoclast differentiation, and autophagy (Figures 10E,F).

External dataset validation

At the same time, we use GSE58294 to verify our analysis results and get similar results. First, we normalized the GSE58294 data set (Supplementary Figures 6A–C). Most

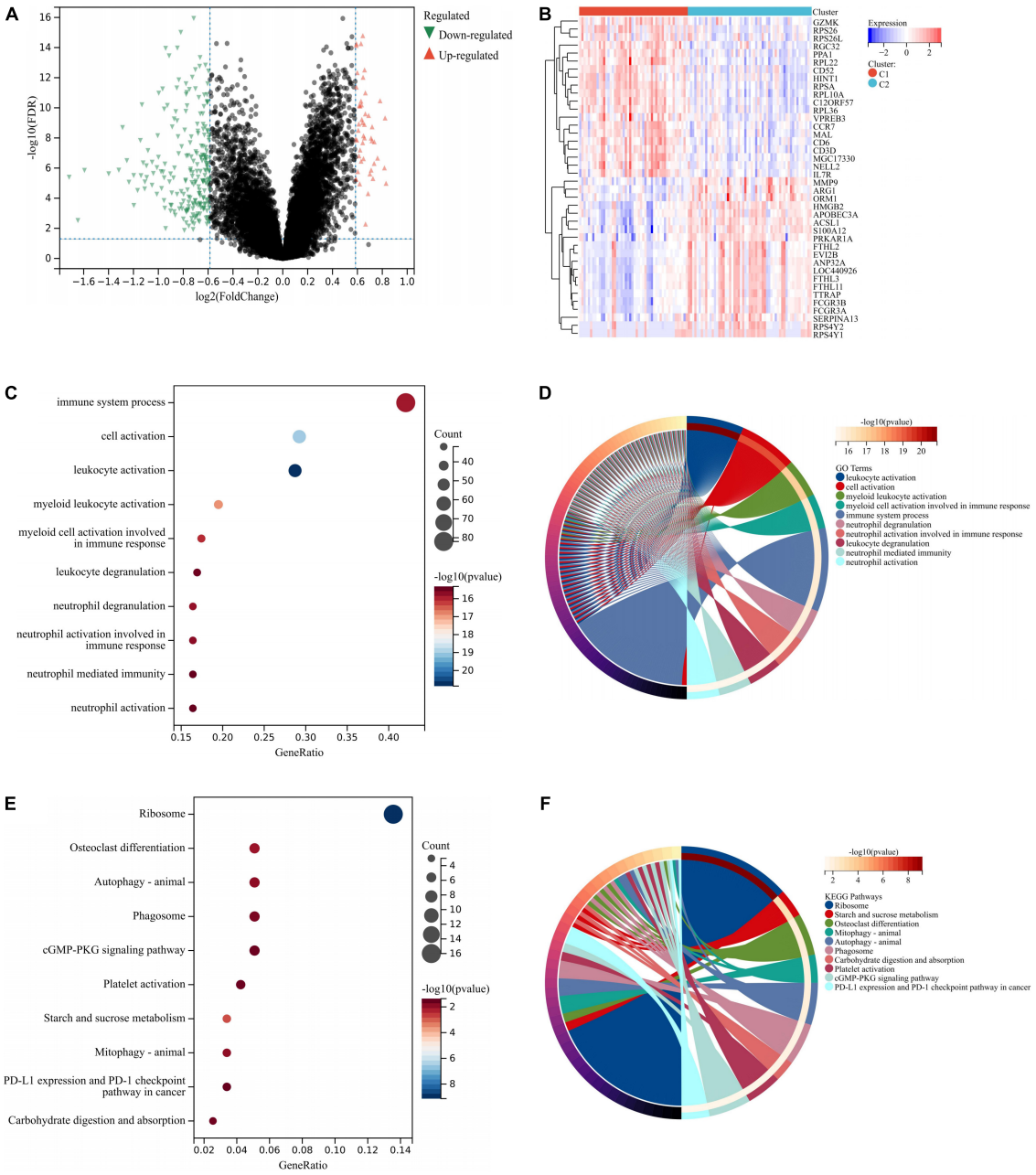

FIGURE 10
Functional analysis between two different subtypes. The DEGs were shown by volcano plot **(A)** and heat map **(B)** between two subtypes. **(C)** GO enrichment analysis was performed on the DEGs. GO terms are represented on the y-axis, gene ratios are shown on the x-axis, circle sizes refer to gene numbers, and colors represent *p*-values. **(D)** GO enrichment analysis of the DEGs. Different colors represent various significant GO terms and related enriched genes. **(E)** KEGG pathway analysis was performed on the DEGs. The y-axis represents different pathways, gene ratios enriched in relative pathways by the x-axis, circles represent gene numbers, and colors represent *p*-values. **(F)** KEGG pathway analysis of the DEGs. Different colors represent various significant pathways and related enriched genes. DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

21 genes exhibited upregulation in IS tissues vs. normal tissues (Supplementary Figure 6D). The ROC curves also revealed excellent accuracy of LAMP2 (AUC = 0.94), TSC22D3 (AUC = 0.80), and SLC38A1 (AUC = 0.82) in distinguishing between the outcomes of normal and IS groups (Supplementary Figures 6E–G). We performed an unsupervised consistency clustering analysis on IS samples based on 11 hub genes and divided IS samples into two subtypes, C1 and C2 (Supplementary Figure 7A). Most of the immune checkpoints and HLA genes were significantly upregulated in C1 compared

to C2 subtype (**Supplementary Figures 7B,C**). Most immune cell infiltration levels differed significantly between C1 and C2 subtypes (**Supplementary Figure 7D**). The C2 subtype was more immunoactive (complement, B cell receptor signaling pathway, VEGF signaling pathway, chemokine signaling pathway, etc.) than the C1 subtype (**Supplementary Figure 7E**).

Discussion

Ferroptosis is a unique type of programmed cell death involved in metabolism, redox biology, and various diseases (33), such as degenerative disorders, carcinogenesis, stroke, and traumatic brain injury. A recent study demonstrated that ferroptosis is critical for the progress of cerebral stroke (34). By understanding the association between ferroptosis and IS, new biomarkers and approaches to diagnosis and treatment can be developed.

In the present study, using GSE16561 dataset, 21 ferroptosis-related DEGs were identified. IS-related pathways like monocarboxylic acid metabolism, iron ion response, and ferroptosis were enriched. Monocarboxylic acids such as lactic acid (35), pyruvate (36), and ketone body (37) are closely related to IS. ACSL4 was reported to be a potential therapeutic target for IS, as it could aggravate IS by promoting ferroptosis (38). Therefore, the 21 ferroptosis-related DEGs identified in this study may contribute significantly to IS through these pathways.

Furthermore, clinical correlation analysis indicated that the expression of DEGs was related to patients' age and gender. It has been long recognized that stroke incidence is higher in men than in women globally (39). Moreover, men have a higher age-adjusted incidence of stroke than women (40).

Further analysis revealed that LAMP2, RPL8, and SLC38A exhibited excellent diagnostic performance for IS patients in both the GSE16561 and GSE37587 datasets. Tao et al. (41) revealed that miR-207 mediated the ischemic injury and spontaneous recovery by participating in the lysosome pathway *via* regulating LAMP2. After intracerebral hemorrhage, human brain RPL8 mRNA expression increased, suggesting it may be a therapeutic target (42). To conclude, the 21 ferroptosis-related DEGs might be critical to IS.

The transcription factors, upstream miRNA, and drugs that correspond to the 21 ferroptosis-related DEGs were also confirmed in this study. The transcription factors identified mainly were STAT6, IRF8, and HMGA1. STAT6/Arg1 promoted microglia/macrophage efferocytosis and inflammation resolution in stroke mice (43); IRF8 protected against cerebral ischemic-reperfusion injury (44); has-miR-196a alleviated ischemic brain injury in mice by directly targeting HMGA1 (45). The three miRNAs identified were hsa-miR-548ag, hsa-miR-329-5p, and hsa-miR-625-3p. The expression of hsa-miR-625-3p was correlated with cholesterol levels (46) and hsa-miR-625-3p was reported to be interrelated with cerebral infarction (47). Given these findings, the identified transcription factors and miRNAs were essential to the ferroptosis dysfunction in IS. Among the drugs, vandetanib could be used to treat thyroid and non-small cell lung cancer (48), and it might serve as a potential drug for IS.

Protein-protein interaction analysis identified 11 hub genes out of the 21 ferroptosis-related genes, which were majorly enriched in fatty acid metabolic process, response to hypoxia, and angiogenesis. Research showed that the fatty acid metabolic process was closely correlated with stroke (49). Hypoxia could induce IS (50), while angiogenesis-associated factors could act as biomarkers for IS patients (51). Therefore, the 11 hub genes are presumably of vital importance in IS.

In this study, using consistent clustering, we identified two subtypes (C1 and C2) in IS samples based on 11 ferroptosis-related genes. C1 contained 50 samples, and C2 contained 57 samples. Significant heterogeneity between the two subgroups was confirmed by immunoassay and enrichment analysis. At the same time, we use GSE58294 to verify our analysis results and get similar results. According to many studies, ferroptosis plays a vital role in immunity (52, 53). It is thought that ferroptotic cells activate innate immunity and release pro-inflammatory factors in various diseases, attracting many different immune cells to the area (54). In IS, BBB breaks down, allowing immune cells to flood into the central nervous system. Our findings suggested that NK and mast cells infiltrated less in C1 than in C2. Kong et al. (55) reported that the number of NK cells was reduced in IS patients. The mast cells contributed to the development of IS by speeding up BBB disruption and magnifying neuroinflammation by releasing cytokines (56). Two limitations in this study warrant mention. The ferroptosis-related DEGs with significance in IS might not be comprehensively included. Moreover, validations are required in further *in vivo* and *in vitro* experiments.

Conclusion

The current study identified 21 ferroptosis-related DEGs in IS, which were pertinent to the age and gender of IS patients and had an excellent diagnostic performance. Vandetanib, FERRIC CITRATE, etc., were identified as potential drugs for IS. In addition, we proposed a molecular classification based on ferroptosis-related genes, namely C1 and C2 subtypes in IS. In conclusion, our findings may help to design immunotherapies for IS 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**.

Author contributions

YJZ, BL, and ZP designed the study, performed data analysis, and wrote the manuscript. YSZ, RY, XH, LZ, and XZ gathered clinical and expression data. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

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SUPPLEMENTARY FIGURE 1
GSE37587 data set preprocessing. **(A)** Box plots showing gene expression levels between different samples after normalization. 2D and 3D PCA plots demonstrated the distribution of samples before **(B,C)** and after **(D,E)** pretreatment. PCA, principal components analysis.

SUPPLEMENTARY FIGURE 2
Construct a diagnostic model of 21 IS-associated ferroptosis genes. **(A)** Least absolute shrinkage and selection operator (LASSO) coefficient profiles of 21 IS-related ferroptosis genes. **(B)** Plots of the 10-fold cross-validation error rates. **(C–E)** The model's discrimination ability for healthy and IS samples was analyzed by ROC curve and evaluated by AUC value. IS, ischemic stroke; ROC, receiver operating characteristic; AUC, area under curve; FPR, false positive rate; TPR, true positive rate.

SUPPLEMENTARY FIGURE 3
Cluster parameter analysis. **(A)** Cumulative distribution curve when k = 2–10. **(B)** Relative alterations in the area under CDF curve. **(C)** Sample clustering consistency when k = 2–10.

SUPPLEMENTARY FIGURE 4
Two subtypes differ in biological function. Heatmap showing the enrichment levels of Hallmark **(A)** and KEGG **(B)** gene sets in two subtypes.

SUPPLEMENTARY FIGURE 5
CIBERSORT to assess immune cell infiltration. **(A)** Bar plot showing the proportion of 22 immunocytes in two subtypes. **(B)** Violin plot showing the ratio of immune cells between two subtypes.

SUPPLEMENTARY FIGURE 6
The expression level and diagnostic value of 21 IS-associated ferroptosis genes were verified in the GSE58294 data set. **(A)** Box plot showing the gene expression level between different samples after normalization. **(B,C)** 2D and 3D PCA plots demonstrated the distribution of samples after pretreatment. **(D)** Box plot described the expression pattern of the 21 ferroptosis-related genes between IS and normal samples. **(E–G)** Diagnostic ROC analysis of 21 ferroptosis-related genes in GSE58294. PCA, principal components analysis; IS, ischemic stroke; ROC, receiver operating characteristic; AUC, area under curve; FPR, false positive rate; TPR, true positive rate. Ns: $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

SUPPLEMENTARY FIGURE 7
The unsupervised cluster analysis of 11 hub genes was verified in the GSE58294 data set. **(A)** Based on 11 hub gene expression levels, IS samples were divided into two subtypes, C1 and C2. Box plots showing that there were differences in immune checkpoints **(B)**, HLA genes **(C)**, and immune cell infiltration **(D)** between the two subtypes. **(E)** Heatmap showing the enrichment levels of KEGG gene sets in two subtypes. KEGG, Kyoto Encyclopedia of Genes and Genomes. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

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The association of dietary glutamine supplementation with the development of high salt-induced hypertension in rats

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Glutamine supplementation has been reported to affect blood pressure (BP). However, its role in the progression of hypertension induced by high salt diet (HSD) has not been elucidated. Male normotensive Wistar rats were exposed to high salt diet and treated with different doses of glutamine supplementation. Rats aged 6 weeks were assigned to five groups: (1) Normal-salt diet (0.3% NaCl, NSD); (2) High-salt diet (8% NaCl, HSD); (3) High-salt + low-dose diet (8% NaCl, 0.5 g of L-glutamine/kg body weight, HSLGD); (4) High-salt + middle-dose diet (8% NaCl, 1.5 g of L-glutamine/kg body weight, HSMGD); and (5) High-salt + high-dose diet (8% NaCl, 2.5 g of L-glutamine/kg body weight, HSHGD). After supplementing different doses of glutamine to male Wistar 6-week-old rats fed with HSD for 7 weeks, we found no difference in body weight among groups. Importantly, we showed that dietary L-glutamine supplementation could prevent the development of hypertension in a dose-dependent manner [dramatically lowering systolic blood pressure (SBP) and slightly reducing diastolic blood pressure (DBP) of hypertensive rats, while the differences of DBP between groups did not reach statistical significance]. Our data further elucidated that dietary glutamine supplementation mildly alleviated the degree of left ventricular hypertrophy, including interventricular septal thickness (IVST) and left ventricular posterior wall thickness (LVPWT) in hypertensive rats. Together, our results offer evidence that the dietary uptake of glutamine may be associated with attenuating the development of high salt-induced hypertension and slightly

alleviating the degree of left ventricular hypertrophy in hypertensive rats. Therefore, glutamine supplementation may act as a prospective dietary intervention for the treatment of hypertension.

KEYWORDS

L-glutamine, blood pressure, hypertension, high salt, rates

Introduction

It is well established that hypertension, one of the most common chronic diseases, is the leading risk factor for heart attack, stroke, congestive heart failure, and kidney disease (1). Hypertension and its complications globally account for 9.4 million deaths among the 17 million deaths owing to cardiovascular diseases each year, and thus has become one of the most serious public health issues across the world (2, 3). Accumulating evidence indicates high dietary salt to be an independent risk factor for chronic non-communicable diseases, especially hypertension, thereby triggering more than half of diet-related deaths around the world (4–6). Therefore, it is urgent to seek for effective dietary intervention strategies to alleviate the occurrence and development of hypertension caused by a high salt diet (HSD).

Previous work has demonstrated that, compared with individuals who had normal blood pressure (BP), patients with hypertension often have concurrent metabolic abnormalities, mainly affecting the metabolism of amino acids, fatty acids, carbohydrates, and the intestinal microbiota (7–11). Glutamine, the most plentiful free amino acid in human serum, attributes to cell survival and growth in a similar fashion to glucose (12). Glutamine is the major nitrogen source for non-essential amino acids, hexosamines, and nucleotides (13) and further plays a role in providing intermediates (like α-ketoglutarate) to the tricarboxylic acid cycle. Liu et al. (10) reported that the level of glutamine was increased in hypertensive patients by the use of ultrasonication-assisted extraction and derivatization. Conversely, another metabolomics study showed that systolic blood pressure (SBP) and pulse pressure are inversely correlated with glutamine in black adults (14). Similarly, our previous work identified that in children aged 6−11 years with elevated BP, the abundance of glutamine was lower than those with normal BP using a case-control design (15). L-citrulline, the metabolite of glutamine, can increase the synthesis of NO and inhibit arterial tension, which may be the way to regulate BP (16). However, the conflicting data means that it remains elusive whether or not glutamine is involved in the development of hypertension.

Therefore, in this experimental study, we treated those high-salt diet-induced hypertensive rats with three different doses of dietary glutamine to preliminary explore the relationship involved between long-term glutamine intake and hypertension induced by HSD in Wistar rats. In addition, we further and firstly evaluated the effect of glutamine intake on the cardiovascular structure [including carotid intima-media thickness (cIMT) and left ventricular hypertrophy] in rats.

Materials and methods

Animals and treatment

A total of 65 Wistar 4-week-old rats (140.2 ± 8.8 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. All rats were male, considering that the prevalence of hypertension is higher in men than in women (17). One rat was housed per cage in 370 × 260 × 170 mm^3 cages at constant temperature (18−24°C), humidity (45%), and regular 12 h light/dark cycles with lights on from 06:30 to 18:30 (light = 270 lux). Animals had free access to water and food, except where noted. All animal experimental procedures were approved by the Ethics Committee of the School of Public Health, Shandong University (No. 20160308) and conformed to the Helsinki Declaration.

After 14 days of adaptive feeding, male normotensive Wistar rats aged 6 weeks were randomized to five groups according to BP values and body weight (n = 13 per group): (1) normal-salt diet group (a standard normal diet with 0.3% NaCl, NSD); (2) high-salt diet group (a high-salt diet with 8% NaCl, HSD); (3) high-salt + low glutamine diet group (a high-salt diet with 0.5 g of L-glutamine/kg body weight, HSLGD); (4) high-salt + middle glutamine diet group (a high-salt diet with 1.5 g of L-glutamine/kg body weight, HSMGD); and (5) high-salt + high glutamine diet group (a high-salt diet with 2.5 g of L-glutamine/kg body weight, HSHGD) (18). All standard normal diets and high-salt diets were customized by Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd. They were all identical in composition except for NaCl content (control: 0.3% NaCl, hypertension: 8% NaCl) (19) and irradiated by Co-60 to meet SPF level. After fully mixing the glutamine and standard high-salt feed using the proportional multiplication method (HSHGD: 33.3 g

L-glutamine/1 kg high-salt feed; HSMGD: 20 g L-glutamine/1 kg high-salt diet; HSLGD: 6.67 g L-glutamine/1 kg high-salt diet), three different doses of high-salt feed containing three different doses of glutamine were remade. Oven drying (40°C) and UV sterilization (duration was set to 3 h) were sequentially carried out after feed production. Body weights were monitored every 3 days and pre-weighted food was administered accordingly. After 7 weeks of dietary treatment followed by a 12 h fast, all rats were sacrificed.

Blood pressure measurements of conscious rats and definitions

Blood pressure measurements were performed from 8:00 a.m. to 6:00 p.m. in a warm and quiet room once a week. A rat was selected randomly for BP measurement from group A, followed by B, C, D, and E, sequentially. After one round, repeat the abovementioned operations until all BP measurements are completed. Systolic blood pressure and diastolic blood pressure (DBP) of conscious rats were measured by the tail-cuff system (BP-2010A, Softron, China) as described previously (19). Five BP measurements were recorded consecutively for each rat. If the difference between any two of the five BP readings per individual rat exceeded 10 mmHg, a sixth BP measurement was conducted. The mean value was calculated for analysis. Hypertension was defined as a SBP $\geq$ 140 mmHg.

Ultrasound imaging of the carotid artery and left ventricular function

After 7 weeks of dietary treatment, six rats per group were randomly chosen to be anesthetized using isoflurane (mixed in 95% oxygen and 5% carbon dioxide oxygen) for ultrasonic measurement using small animal color Doppler ultrasound device (Vevo 2100, Visual sonics, Canada). Skin preparation was conducted from the mandible to the upper abdomen and depilatory cream (Nair^{TM}) was applied. Rats were positioned on a heated platform in supine position. For measurements of both echocardiogram and ultrasound vessel internal diameter imaging, a trained user of the Vevo 2100 Imaging system, blinded to the intervention of all rats (intervention and dose), analyzed heart and vessel diameters of each rat. All images were saved as cine loops for the subsequent measurement and analyses of vessel and cardiac parameters.

Ultrasound gel was applied to prepared skin. The transducer head was locked (40 MHz probe; MS550D) in the adjustable arm of the Vevo mechanical rail-system to allow hands-free precise positioning of the transducer during cIMT image collection in M-mode. The anterior and posterior walls of cIMT of the right and left were measured, and the mean cIMT was calculated for statistical analysis. Another transducer head (15 MHz probe; MS201) was locked to detect the structure of left ventricle. The parameters included left ventricular end-systolic diameter (LVSD), left ventricular end-diastolic diameter (LVDD), interventricular septum thickness (IVST), and left ventricular posterior wall thickness (LVPWT) by performing M-mode echocardiography. The left ventricular mass (LVM) was calculated according to the Devereux formula: LVM (g) = $0.8\{1.04[(LVDD + IVST + LVPWT)^3 - LVDD^3]\} + 0.6$ (20).

Statistical analysis

All data were presented as means ± SEM. Statistical analyses and Graphs were performed using Graphpad Prism software (v.7) (GraphPad Software, La Jolla, CA, United States). For the comparison of more than two groups, one-way ANOVA followed by Bonferroni's *post-hoc* test was used to determine the statistical significance of differences. A *P*-value less than 0.05 was considered to be statistically significant.

Results

Glutamine supplementation had no effect on body weight

The dietary intervention program and grouping of rats are displayed in **Figure 1A**. We supplemented different doses of glutamine to male Wistar 6-week-old rats fed with HSD for 7 weeks. We did not observe any significant difference in body weight among the five groups (**Figure 1B** and **Supplementary Table 1**), indicating that glutamine supplementation in diet did not induce significant changes in the body weight of HSD-induced rats.

Dietary glutamine prevented high salt diet-induced increases in blood pressure in rats

Blood pressure measurement data showed 6-week HSD increased both the SBP and DBP levels in rats. Intriguingly, we found that 6-week of high-dose dietary glutamine supplementation dramatically prevented the increase of SBP (152.6 mm Hg) (**Figures 2A,B** and **Supplementary Table 2A**) and slightly prevented of DBP (120.9 mm Hg) (**Figures 2C,D** and **Supplementary Table 2B**) in hypertensive rats compared with HSD-fed rats (SBP: 165.6 mm Hg, DBP: 125.1 mm Hg), while there was not obvious difference of BP for low- and middle-dose dietary glutamine supplementation.

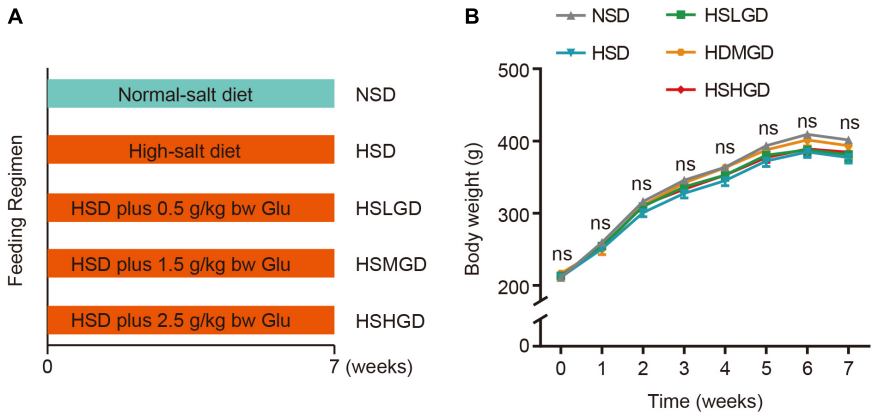

FIGURE 1
Effects of dietary glutamine on body weight in salt-induced hypertensive rats. **(A)** Schematic outlines of the feeding regimen for the five intervention groups. Male normotensive Wistar rats aged 6 weeks were assigned to five groups: (1) Normal-salt diet (0.3% NaCl, NSD); (2) High-salt diet (8% NaCl, HSD); (3) High-salt + low-dose diet (8% NaCl, 0.5 g of L-glutamine/kg body weight, HSLGD); (4) High-salt + middle-dose diet (8% NaCl, 1.5 g of L-glutamine/kg body weight, HSMGD); (5) High-salt + high-dose diet (8% NaCl, 2.5 g of L-glutamine/kg body weight, HSHGD). **(B)** Body weight of rats fed on NSD, HSD, HSLGD, HSMGD, or HSHGD for 7 weeks. n = 12 (NSD, HSLGD) or 13 (HSD, HSMGD, HSHGD) rats per group. Data are presented as means ± SEM. ns, no significance. One-way ANOVA followed by Bonferroni's *post-hoc* test **(B)**.

High glutamine delayed the development of left ventricular hypertrophy in hypertensive rats

We further sought to elucidate the role of a glutamine-rich diet on the function of both the carotid artery and left ventricle in high salt-induced hypertensive rats. Given that ultrasound imaging is a crucial tool for the assessment of the carotid artery and left ventricle structure, we performed ultrasound measurements of the carotid arteries and left ventricles after 7 weeks of feeding treatment. The data indicated that the IVST level was elevated in HSD-fed rats (2.649 mm), while supplemental high-dose glutamine markedly diminished this effect (2.095 mm) (**Figure 3B** and **Supplementary Table 3**). Similar results were observed on LVPWT (2.447 mm vs. 2.004 mm) (**Figure 3C** and **Supplementary Table 3**). However, no difference in LVM and carotid IMTs was found among different groups (**Figures 3A,D** and **Supplementary Table 3**). Two-dimensional echocardiography and M-mode echocardiography revealed that the thickness of the left ventricular wall and carotid intima-media were decreased in glutamine-supplemented groups (HSLGD, HSMGD, and HSHGD) compared with the HSD group (**Figure 4**).

Discussion

Accumulating evidence indicates that glutamine plays a crucial role in multiple physiological and pathological statuses. Here, we used an *in vivo* model to explore the role of dietary glutamine supplementation in the development of hypertension induced by HSD in Wistar rats. We found that glutamine supplementation might be inversely associated with elevated SBP and left ventricular hypertrophy in the hypertensive rat model. Our work highlights the pivotal *in vivo* effect of glutamine supplementation on the progression of salt-induced hypertension, paving the way for better therapeutic strategies.

Glutamine serves as an essential nutrient for the synthesis of a number of important molecules, including lipids, proteins, and DNA, and provides intermediates for the tricarboxylic acid cycle to generate ATP. Recently, the role of glutamine in the cardiovascular system has drawn considerable attention (21–23). Glutamine-cycling pathways might be prominently involved in the development of metabolic disorders. Prior studies have observed that serum glutamine levels were inversely linked to the development of obesity and other established risk factors for cardiometabolic disease (24–26). The inversely association of glutamine with metabolic disorders might be due to pancreatic β-cell insulin secretion, increased insulin sensitivity of adipose tissue, transcription of insulin-dependent enzymes, enhanced release of glucagon-like peptide 1, and externalization of glucose transporter type 4 (27–29). Cheng et al. (30) also found that glutamine was inversely related to insulin levels, SBP, and DBP, and positively associated with high-density lipoprotein levels based on two large community cohorts (the Malmo Diet and Cancer Study and the Framingham Heart Study). They further interrogated the regulation of administered glutamine on BP in C57BL/6 mice and found that SBP, DBP, and mean arterial pressure were significantly lower in glutamine-treated mice (intraperitoneal injection of glutamine plus saline) compared with controls (injection of saline alone), which is consistent with our results. Glutamine

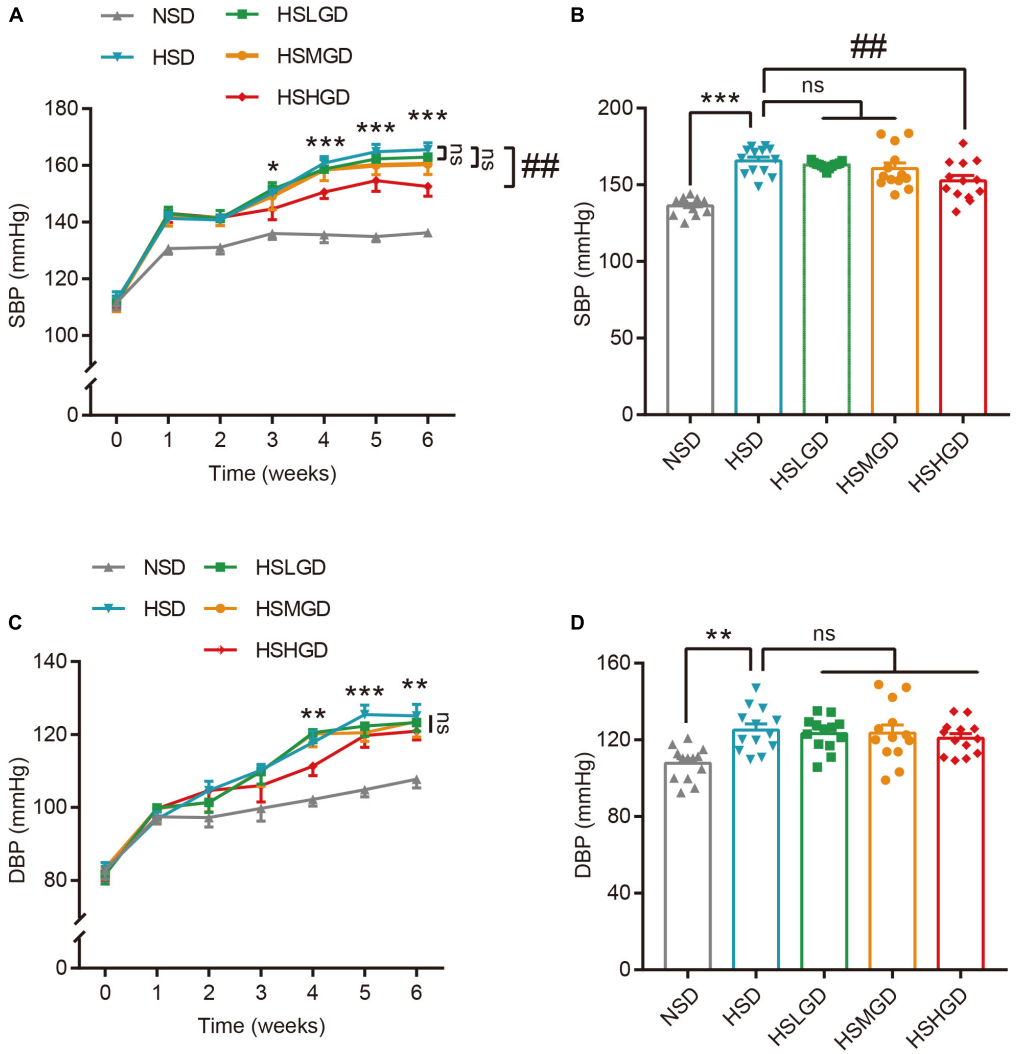

FIGURE 2
Effects of dietary glutamine on blood pressure (BP) in salt-induced hypertensive rats. **(A,B)** Rats were fed on a normal-salt diet (NSD), high-salt diet (HSD), high-salt + low-dose glutamine diet (HSLGD), high-salt + middle-dose glutamine diet (HSMGD), or high-salt + high-dose glutamine diet (HSHGD) for 7 weeks. Systolic blood pressure (SBP) was measured every week by the tail-cuff system. The changes of SBP from 1 to 6 weeks **(A)** and at 6-week feeding intervention **(B)**. **(C,D)** The diastolic blood pressure (DBP) of rats fed on NSD, HSD, HSLGD, HSMGD, or HSHGD from 1 to 6 weeks **(C)** and at 6-weeks of feeding intervention **(D)**. n = 13 rats per group. Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with NSD group; $\#\#P < 0.01$ compared with HSD group. ns: no significance. One-way ANOVA followed by Bonferroni's *post-hoc* test **(A–D)**.

generates L-citrulline, which is metabolized to L-arginine (nitric oxide synthase substrate) in the kidney through the synergistic action of arginosuccinate synthetase and arginine succinate lyase (31). The suppressive effect of glutamine on BP may be partly attributable to its role as a precursor of L-arginine, leading to the increase of NO synthesis (16), which regulates BP by the inhibition of arterial tone. It is noteworthy that in this animal study by Cheng et al. (30), the BP measurements were conducted every 12 min for 36 min following intraperitoneal injection of glutamine plus saline or saline alone, which reflected transient BP changes in response to the acute administration of glutamine. However, we observed a long-term antihypertensive effect of glutamine on HSD-induced hypertensive rats after a 7-week dietary glutamine intervention.

Current studies have identified the mitigation effects of glutamine on myocardial dysfunction. Clinical trials have observed that glutamine supplementation decreased the post-operative myocardial damage after coronary revascularization in cardiopulmonary bypass (32), increased the concentration of troponin at 24 h after operation, and improved myocardial function for patients with ischemic heart disease (33). In addition, glutamine administration improved contractile function of the left ventricle and protected from cardiac injury in diabetic rats induced by streptozotocin-nicotinamide, which

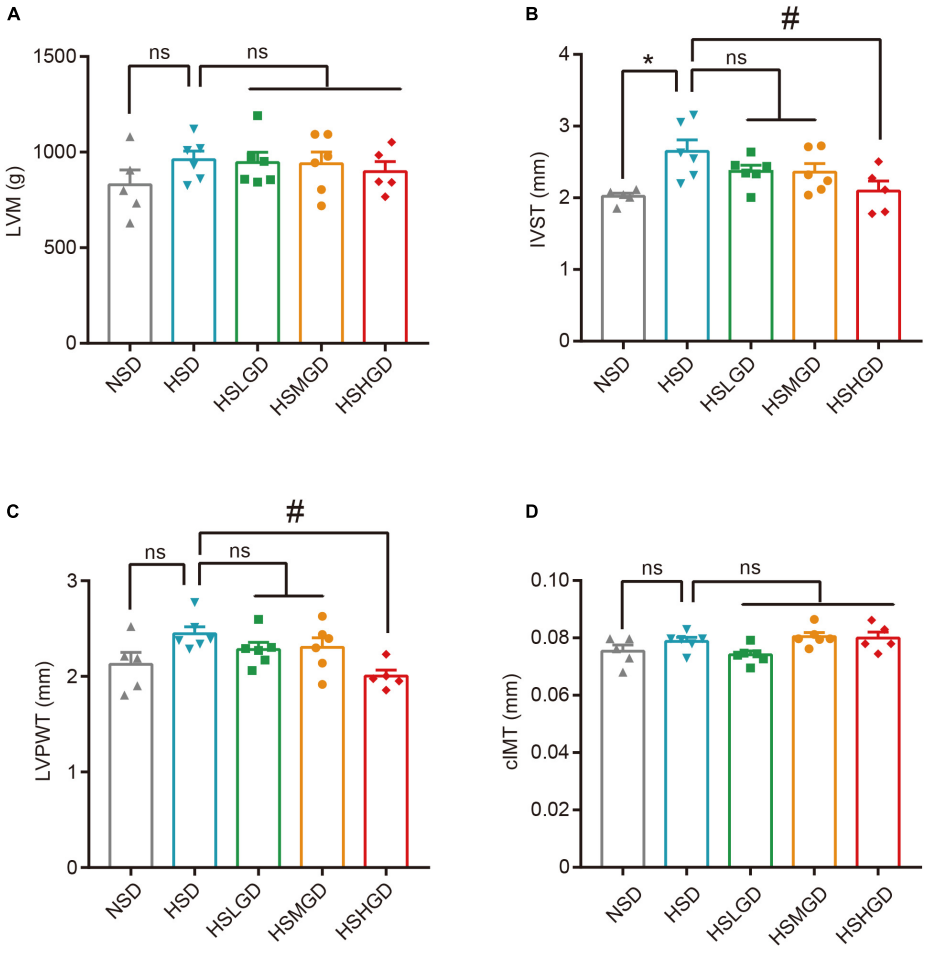

FIGURE 3
Effects of dietary glutamine on the carotid artery and heart in salt-induced hypertensive rats. **(A–D)** Rats were fed on a normal-salt diet (NSD), high-salt diet (HSD), high-salt + low-dose glutamine diet (HSLGD), high-salt + middle-dose glutamine diet (HSMGD), or high-salt + high-dose glutamine diet (HSHGD) for the 7-week rearing experiment. Data exhibit the indicated ultrasound parameters including LVM **(A)**, IVST **(B)**, LVPWT **(C)**, and cIMT **(D)** measured at 7 weeks. $n = 5$ (NSD, HSHGD) or 6 (HSD, HSLGD, HSMGD) rats per group. Data are presented as means ± SEM. $^*P < 0.05$ compared with NSD group; $\#P < 0.05$ compared with HSD group. ns, no significance. One-way ANOVA followed by Bonferroni's *post-hoc* test **(A–D)**. LVM, left ventricular mass; IVST, interventricular septal thickness; LVPWT, left ventricular posterior wall thickness; cIMT, carotid intima-media thickness.

acts through significantly reducing the levels of cardiac enzymes such as creatine kinase-isoenezyme, lactate dehydrogenase, and aspartate aminotransferase (34). Similar protective effects of glutamine on myocardial structure and function have also been found in severely burned rats (35). Our current work highlights that a high glutamine diet may exert alleviative effects on cardiac structural changes caused by HSD-induced hypertension in Wistar rat models. Further work is needed to unveil the mechanisms involving glutamine in the progression of myocardial dysfunction caused by hypertension.

This study also preliminarily explored the relationship between glutamine and carotid artery intima-media thickness. However, no difference in carotid IMTs was found among different groups. Addabbo et al. found that glutamine supplementation corrected endothelium-dependent relaxation in mice treated with L-Nω-methylarginine (36). Similarly, other experimental studies observed that glutamine guarded endothelial cells against inflammation and oxidative stress (37, 38). The protective mechanism of glutamine on blood vessels still requires investigation.

There were certain limitations should be acknowledged. First, considering the water solubility of glutamine, the way of supplementing glutamine is oral rather than gavage, which might lead to partial glutamine loss. Second, we measured only a limited indexes to represent the status of rat's hypertension to conduct a preliminary explore of the relationship between long-term glutamine intake and hypertension induced by HSD in Wistar rats. Further studies are needed to better understand the mechanisms underlying the effect of dietary glutamine supplementation on HSD-induced hypertension.

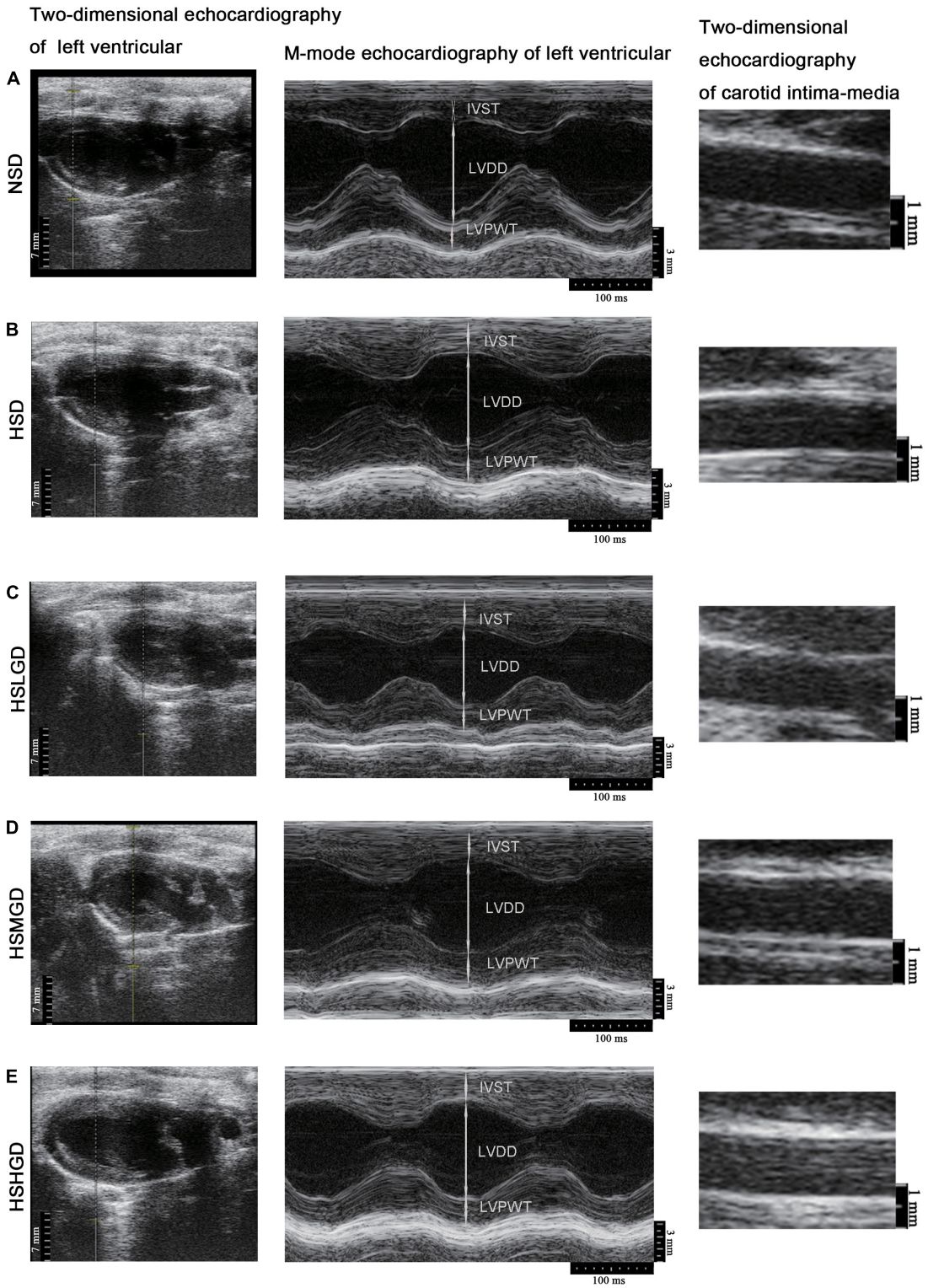

FIGURE 4
Representative echocardiography images of carotid arteries and cardiovasculature of rats fed on a normal-salt diet (NSD, **A**), high-salt diet (HSD, **B**), high-salt + low-dose glutamine diet (HSLGD, **C**), high-salt + middle-dose glutamine diet (HSMGD, **D**), or high-salt + high-dose glutamine diet (HSHGD, **E**) for the 7-week rearing experiment. Scale bars, 7 mm (Two-dimensional echocardiography of left ventricular), 3 mm (M-mode echocardiography of left ventricle), or 1 mm (Two-dimensional echocardiography of carotid intima-media). *n* = 5 (NSD, HSHGD) or 6 (HSD, HSLGD, HSMGD) rats per group.

Conclusion

In conclusion, our study revealed that a high glutamine diet might be inversely associated with the development of hypertension and left ventricular hypertrophy in HSD-induced hypertensive rats in a dose-dependent manner. Our work provides pivotal evidence that dietary glutamine supplementation may be used as a promising therapeutic tactic for the treatment of hypertension. The underlying molecular mechanisms of dietary glutamine supplementation on HSD-induced hypertension are worth further exploration both *in vivo* and *in vitro*.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Ethics Committee of the School of Public Health, Shandong University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LY and LX conducted the animal experiment, collected the data, and drafted the manuscript. JL analyzed the data, prepared the figures, and co-wrote the manuscript. HW and ZY assisted in animal experiments. XZ assisted in ultrasound information acquisition and reading. HW, JS, ZY, XZ, MZ, and BX critically revised the manuscript for important intellectual content. BX was the guarantor. MZ and BX conceived the study, designed the experiments, analyzed the data, co-wrote the manuscript, and attested that all the listed authors meet the authorship criteria and that no others meeting the criteria have been omitted. All authors have read and agreed with the final version of this manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1011739/full#supplementary-material

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Association between the geriatric nutritional risk index and the risk of stroke in elderly patients with hypertension: A longitudinal and cohort study

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Objective: We aimed to investigate the association between the GNRI and the risk of stroke in elderly patients with hypertension.

Methods: A total of 5312 elderly hypertensive patients free of history of stroke were included. Multivariate Cox models were used to calculate hazard ratios (HRs) and their 95% confidence intervals (CIs) for stroke and its subtypes.

Results: The average time of follow-up was 3.8 years, and the median time was 3.2 years. We identified 640 individuals with stroke, of whom 526 had an ischemic stroke (IS) and 114 had a hemorrhagic stroke (HS). After adjusting for confounding variables, compared with participants in the lowest quartile of the GNRI, those in the third and fourth quartiles were associated with a decreased risk of stroke (adjusted HR 0.72, 95% CI 0.58–0.90, and adjusted HR 0.58, 95% CI 0.46–0.74, respectively, P for trend < 0.001). Similar results were found for IS and HS. Moreover, there were L-shaped associations of GNRI with new-onset HS (P for non-linearity $= 0.034$). Multiple sensitivity analyses and stratified analyses did not materially change the results.

Conclusions: In summary, we found that a lower GNRI was associated with a higher risk of incident stroke in elderly hypertensive patients. Additional prospective data collection is required to confirm our findings.

KEYWORDS

geriatric nutritional risk index, stroke, elderly, hypertension, longitudinal cohort

Introduction

Stroke, including ischemic stroke (IS) and hemorrhagic stroke (HS), is the leading cause of the global burden of disease (1). China has the highest burden of strokes in the world (2). There are still 250 million new instances of stroke each year in China, and that number is rising, even though the incidence and frequency of stroke have decreased globally (3). Hypertension has now been identified as the primary variable risk factor for stroke (4). Several large epidemiological surveys in China have shown that more than 50% of people over 60 years of age have hypertension (5). Therefore, identifying the residual risk of stroke and early risk stratification in elderly patients with hypertension is essential to more effectively tailoring risk reduction strategies.

Malnutrition is associated with a poor clinical prognosis in patients with various diseases (6). According to studies, malnutrition is significantly linked to increased levels of inflammatory response, arterial calcification, and atherosclerosis progression, which raises the possibility that it plays a key role in the emergence of cardiovascular disease (7, 8). The geriatric nutritional risk index (GNRI) is a simple, well-established nutrition assessment tool that uses serum albumin and body mass index (BMI) (9). Recent studies have shown that GNRI is associated with the development of atherosclerosis and an increased risk of cardiovascular mortality in older patients (10, 11). However, studies on GNRI as a predictor of new-onset stroke are still limited. Until now, only one cohort study has reported lower GNRI in hemodialysis patients as an independent risk factor for cerebral infarction and hemorrhage, and it is unclear whether this effect can be extended to older patients with hypertension (10). Therefore, GNRI may have important clinical implications for stroke risk stratification in hypertensive patients. In addition, the status of the dose-response relationship between GNRI and the risk of stroke and its subtypes in elderly hypertensive patients is uncertain.

Therefore, the present study is based on a cohort study aiming to investigate the association between GNRI and the risk of stroke and its subtypes in elderly hypertensive patients and to characterize the nature of the dose-response relationship.

Materials and methods

Study population

We conducted a cohort study at the People's Hospital of Xinjiang Uygur Autonomous Region, Xinjiang, China. All patients were either older than 60 years of age and were recruited between January 1, 2010, and December 31, 2021. First, we excluded patients who had <6 months of follow-up or had the outcome at baseline. Second, we further excluded individuals with missing data on body height, body weight, or serum albumin level. Third, we excluded participants with severe wasting diseases (e.g., malignancy, autoimmune diseases, severe hepatic disease, and severe renal insufficiency). Finally, this left a final study population of 5,312 patients. Participant flow is shown in Figure 1. A comparison of baseline characteristics for in- and excluded participants are presented in Supplementary Table S1. This study was approved by the ethics committee of the People's Hospital of Xinjiang Uygur Autonomous Region (No. KY2021031901). A waiver of informed consent was granted due to the retrospective data collection. The study was reported as per the STROBE statement for observational cohort studies (12).

Covariate collection and definitions

Data were abstracted electronically from the patient's medical records, including demographic characteristics, diagnoses according to the International Classification of Diseases 10th Revision (ICD-10), prescribed medications, and laboratory reports. Weight, height, heart rate, and blood pressure (BP) were measured using standard protocols. The BMI (kg/m^2) was computed from the measured weight and height. Smoking status included categories of current smokers and non-smokers. Participants are classified as current drinkers and non-smokers. Blood samples were drawn after an overnight fast. The participants' prior medical histories were evaluated using ICD-10 codes. To ensure the accuracy of diagnoses, coronary heart disease (CHD) (I24 and I25), diabetes (E10-E14), atrial fibrillation (I48), and dyslipidemia (E78) were regarded as present if a participant was treated $\geq$ 2 times. To quantify the burden of comorbidities, the Charlson Comorbidity Index (CCI) was calculated as described previously (13). Prescription claims within the last year before the baseline defined concomitant medications. The list of concomitant medications included in the study is shown in Supplementary Table S2. The GNRI formula used was as follows: GNRI = (1.489 × albumin, g/l) + (41.7 × present/ideal body weight). Ideal weight was calculated using the Lorenz formulas: For males: height - 100 - [(height - 150)/4]. For females: height - 100 - [(height - 150)/2.5] (14).

Follow-up and outcome measures

The primary outcome was the first occurrence of stroke (ischemic or hemorrhagic), either nonfatal or fatal. Secondary outcomes included the first ischemic stroke and the first hemorrhagic stroke. Methods of determination of incident stroke are described in the Supplementary material. Outcomes of events since participants enrolled in the study were determined through medical records, patient and family interview, contact with local disease and death registries, or access to the database of basic medical insurance. These data

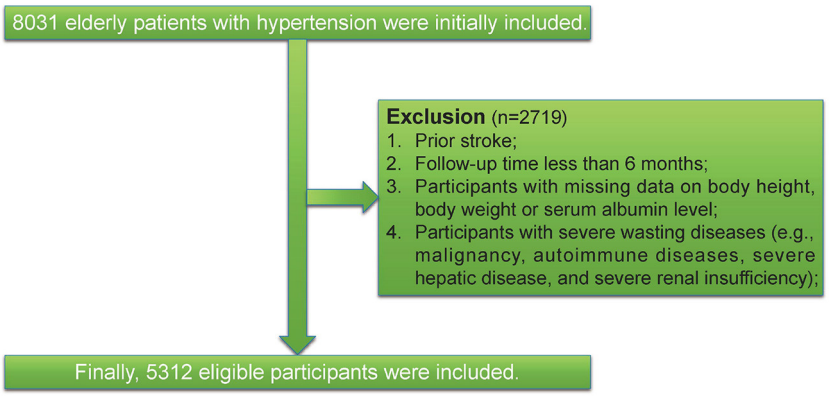

FIGURE 1
Study flowchart.

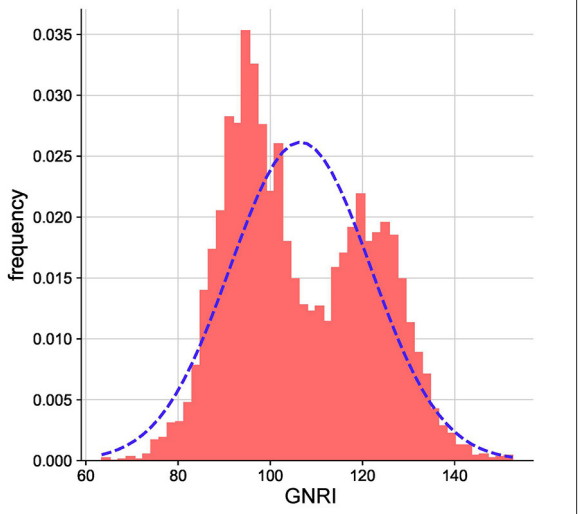

FIGURE 2
Distribution of GNRI among participants.

sources are linked using an individual national identification number assigned to each Chinese person for life. This number is replaced by a series number when provided for personal data analysis to anonymize the individual participant's data. Patients were followed from the date of enrollment to the end of the observation period, defined as the date of the last follow-up visit, the date of the first appearance of any study outcome, the date of death, or the end of the study period (December 31, 2021).

Statistical analysis

Details of the missing covariates are shown in Supplementary Table S3. Missing values of covariates (all covariates were missing in <6%) were imputed using multiple imputations by chained equations. Characteristics of study participants were expressed by GNRI quartiles. For differences in cumulative incidence between groups, we used Kaplan-Meier curves and the log-rank test. The multicollinearity test suggested that the variance inflation factors of all variables were less than five, confirming that the regression model was not affected by multicollinearity. The hazard ratio (HR) estimates and 95% confidence intervals (CI) were determined by the Cox regression models. Tests for non-linear associations were performed using restricted cubic spline regressions. We also performed subgroup analyses stratified by potential confounders. Sensitivity analyses were undertaken to evaluate the robustness of the results. First, to minimize the chance of reverse causation, we excluded events that occurred within 1 or 3 years after the baseline visit. Second, sensitivity analysis determined whether event risks remained stable after accounting for competing risks. Third, participants with CCI ≥2 were excluded to reduce confounding factors caused by associated comorbidity. Fourth, participants with atrial fibrillation were excluded. Lastly, to evaluate potential unmeasured confounding, we calculated E-values. Further analysis details are provided in the Supplementary material. Statistical analyses were performed using R software, version 4.1.1. Two-sided *P*-values <0.05 were considered statistically significant.

Results

Baseline characteristics

As illustrated in the flow chart (Figure 1), a total of 5312 participants were included in the current study. The average age of the study population was 66.5 years (SD 4.8). The GNRI was approximately normally distributed (Figure 2). Baseline characteristics of the study participants by GNRI quartiles are

TABLE 1 Baseline characteristics stratified across quartiles of GNRI.

Characteristics	GNRI quartiles				P-value
	Quartile 1 (≤94.33)	Quartile 2 (94.34–103.17)	Quartile 3 (103.18–119.61)	Quartile 4 (≥119.63)	
No. of participants	1327	1329	1328	1328	
Age, years	66.17 ± 4.70	66.67 ± 4.87	66.69 ± 4.82	66.43 ± 4.78	0.316
Male, *n* (%)	624 (47.02%)	732 (55.08%)	692 (52.11%)	696 (52.41%)	<0.001
Current smoker, *n* (%)	396 (29.84%)	423 (31.83%)	381 (28.69%)	385 (28.99%)	0.283
Current drinker, *n* (%)	339 (25.55%)	381 (28.67%)	329 (24.77%)	353 (26.58%)	0.119
Duration of hypertension, years					<0.001
<5	789 (59.46%)	1065 (80.14%)	972 (73.19%)	1044 (78.61%)	
5–9	167 (12.58%)	71 (5.34%)	185 (13.93%)	183 (13.78%)	
≥10	371 (27.96%)	193 (14.52%)	171 (12.88%)	101 (7.61%)	
Heart rate, bpm	80.37 ± 9.73	80.51 ± 10.07	80.37 ± 9.80	80.50 ± 9.73	0.970
SBP, mmHg	144.12 ± 20.62	143.28 ± 20.49	143.73 ± 20.52	143.92 ± 19.54	0.738
DBP, mmHg	89.02 ± 14.52	88.68 ± 13.92	88.89 ± 14.18	88.91 ± 14.21	0.940
BMI, kg/m^2	24.31 ± 3.30	24.41 ± 3.34	24.35 ± 3.32	24.19 ± 3.34	0.355
Comorbid conditions, *n* (%)					
Dyslipidemia	858 (64.66%)	807 (60.72%)	778 (58.58%)	787 (59.26%)	<0.001
Atrial fibrillation	26 (1.96%)	27 (2.03%)	33 (2.48%)	40 (3.01%)	0.255
Coronary heart disease	215 (16.20%)	233 (17.53%)	218 (16.42%)	225 (16.94%)	0.799
Diabetes	358 (26.98%)	371 (27.92%)	385 (28.99%)	362 (27.26%)	0.662
Charlson comorbidity index					0.139
0	617 (46.50%)	579 (43.57%)	631 (47.52%)	594 (44.73%)	
1	391 (29.46%)	380 (28.59%)	347 (26.13%)	387 (29.14%)	
≥2	319 (24.04%)	370 (27.84%)	350 (26.36%)	347 (26.13%)	
Laboratory tests					
ALT, U/L	24.17 (15.00–35.59)	24.42 (15.75–35.07)	24.41 (15.93–35.63)	24.62 (14.50–35.90)	0.685
AST, U/L	21.00 (16.00–28.27)	21.40 (16.00–27.71)	21.21 (16.00–28.45)	21.37 (16.00–28.17)	0.929
GGT, U/L	27.79 (17.04–41.51)	28.00 (17.89–40.40)	29.05 (18.08–42.49)	28.68 (17.45–41.48)	0.230
Cr, μmol/L	69.92 ± 22.48	69.08 ± 21.63	70.05 ± 22.25	69.62 ± 22.86	0.686
UA, μmol/L	334.05 ± 91.20	334.06 ± 90.17	333.41 ± 91.31	331.81 ± 90.97	0.910
BUN, mmol/L	5.26 ± 1.51	5.28 ± 1.51	5.28 ± 1.51	5.33 ± 1.54	0.712
TC, mmol/L	4.61 ± 0.96	4.58 ± 0.96	4.51 ± 0.96	4.47 ± 0.99	0.005
TG, mmol/L	1.69 (1.12–2.48)	1.60 (1.08–2.32)	1.64 (1.15–2.49)	1.56 (1.08–2.32)	0.016
HDL-C, mmol/L	1.04 ± 0.27	1.07 ± 0.26	1.15 ± 0.26	1.19 ± 0.27	0.006
LDL-C, mmol/L	2.88 ± 0.81	2.77 ± 0.80	2.73 ± 0.80	2.70 ± 0.83	0.009
HbA1c, %	6.21 ± 1.08	6.20 ± 1.06	6.13 ± 1.05	6.10 ± 1.03	0.014
FPG, mmol/L	5.20 ± 1.35	5.23 ± 1.37	5.27 ± 1.48	5.22 ± 1.42	0.621
Hcy, μmol/L	15.11 ± 6.36	15.15 ± 6.23	15.24 ± 6.28	15.12 ± 6.41	0.949
Concomitant medications, *n* (%)					
Statins	652 (49.13%)	626 (47.10%)	585 (44.05%)	559 (42.09%)	<0.001
Aspirin	955 (71.97%)	930 (69.98%)	903 (68.00%)	858 (64.61%)	<0.001
ACEI/ARB	928 (69.93%)	937 (70.50%)	963 (72.52%)	923 (69.50%)	0.333
Beta-blocker	461 (34.74%)	463 (34.84%)	490 (36.90%)	487 (36.67%)	0.509
Calcium channel blockers	1036 (78.07%)	1023 (76.98%)	1077 (81.10%)	1048 (78.92%)	0.064
Diuretics	296 (22.31%)	290 (21.82%)	331 (24.92%)	288 (21.69%)	0.156
Insulin	126 (9.50%)	134 (10.08%)	132 (9.94%)	125 (9.41%)	0.921
Oral antidiabetic agents	222 (16.73%)	229 (17.23%)	249 (18.75%)	235 (17.70%)	0.565

GNRI, metabolic score for insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; Hcy, homocysteine; UA, uric acid; Cr, blood creatinine; BUN, blood urea nitrogen.

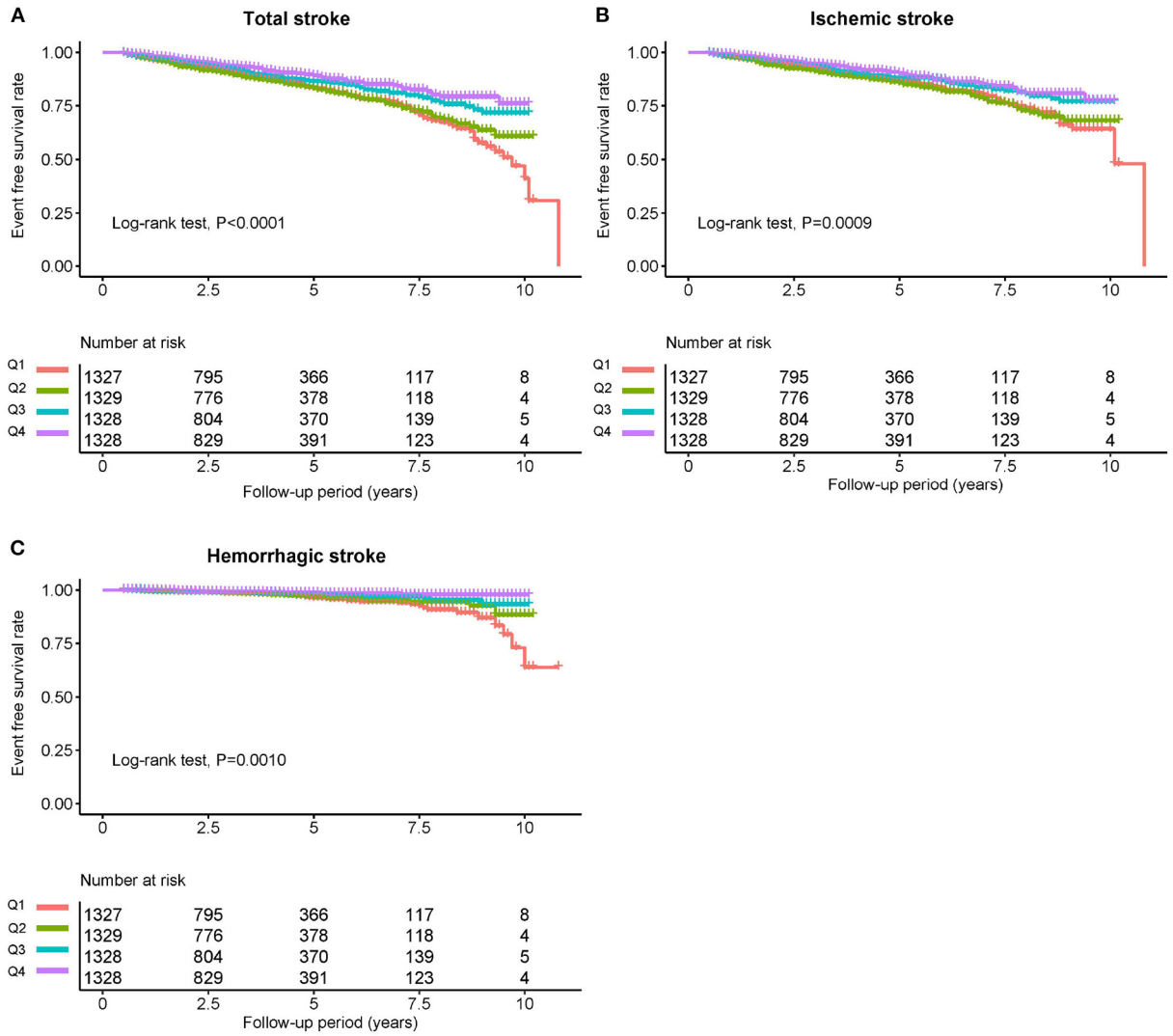

FIGURE 3
Kaplan-Meier survival curves for total stroke and individual outcomes based on GNRI quartiles. **(A)** Total stroke, **(B)** ischemic stroke, and **(C)** hemorrhagic stroke.

shown in Table 1. Participants with a lower GNRI tended to be women, have a higher duration of hypertension, a higher prevalence of dyslipidemia, take more statins and aspirin, have higher HbA1c, TC, TG, and LDL-C levels, and have lower HDL-C compared with participants in the quartile 4 group.

Association between GNRI and total stroke and its subtypes

The average time of follow-up was 3.8 years, and the median time was 3.2 years. We identified 640 individuals with stroke, of which 526 had IS and 114 had HS. The Kaplan-Meier curve showed that participants in the quartile 1 group had a higher risk of total stroke, IS instead of HS than those in other groups (log-rank test, $P < 0.001$, Figure 3A; $P = 0.001$, Figure 3B; $P = 0.001$, Figure 3C). Overall, there was a significant inverse association of GNRI with the risk of first total stroke (Figure 4A) (per SD increment; full adjusted HR: 0.80; 95% CI: 0.73, 0.87). Consistently, when GNRI was assessed as quartiles, the full adjusted HRs of first stroke for participants in quartile 2, quartile 3, and quartile 4 were 0.94 (95% CI: 0.77, 1.15), 0.72 (95% CI: 0.58, 0.90), and 0.58 (95% CI: 0.46, 0.74) respectively, compared with those in quartile 1 (P for trend <0.001) (Table 2). Similarly, a significant inverse association between GNRI and both IS and HS (Figure 4). Moreover, there were L-shaped associations of GNRI with new-onset HS (P for non-linearity = 0.034). Sensitivity analyses were conducted to verify the

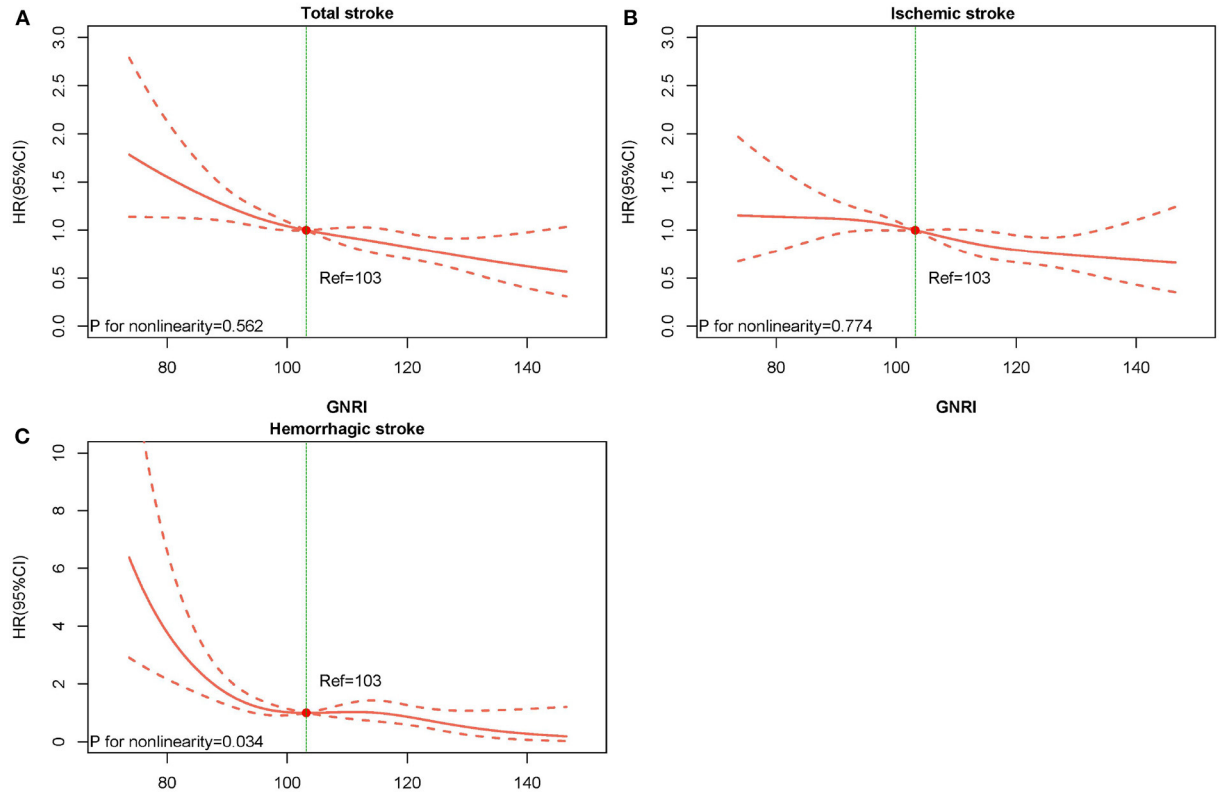

FIGURE 4
Dose-response association between GNRI and risk of stroke events. **(A)** Total stroke, **(B)** ischemic stroke, and **(C)** hemorrhagic stroke.

robustness of the reported findings. The results of sensitivity analyses were similar to those observed in the main analysis (Supplementary Tables S4–S9 and Supplementary Figure S1 in the Supplement).

Stratified analyses

Stratified analyses were performed to assess the association of GNRI (per SD increment) with total stroke and its subtypes, as provided in Figure 5. No interaction was found between subgroup variables and the association of GNRI with the risk of total stroke. Similar results were found for IS and HS.

Incremental predictive value of GNRI

As illustrated in Table 3, according to C-statistic, risk prediction was improved by adding the GNRI to established risk factors (C-statistic increased from 0.613 to 0.648, $P < 0.001$). Moreover, according to continuous NRI and IDI, the GNRI significantly improved risk discrimination for total stroke [continuous NRI (95% CI): 0.118 (0.066–0.172), $P < 0.001$; IDI (95% CI): 0.017 (0.009–0.029), $P < 0.001$]. Furthermore, the DCA for the different models is shown in Supplementary Figure S2. The decision curves show that using a combination of GNRI features to predict total stroke increases the net benefit more than using established risk factors alone. Similar results were observed in IS and HS.

Discussion

This study investigated the relationship between GNRI and incident stroke in elderly hypertensive patients. The findings revealed that the risk of stroke was significantly associated with baseline GNRI after adjusting for multiple confounders. In addition, a significant L-shaped dose-response relationship between GNRI and the risk of incident HS was observed, indicating a rapid increase in the risk of HS when GNRI was below 103. These findings were reliable in subgroup and multiple sensitivity analyses. Overall, the present study revealed that low CNRI was associated with a higher risk of incident stroke. To our knowledge, this is the first study to show an association between GNRI and the risk of incident stroke in a large retrospective cohort.

TABLE 2 Association between GNRI and the incidence of outcomes.

Exposure	Unadjusted	Model 1	Model 2	Model 3
	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Total stroke				
Per SD increment	0.81 (0.74, 0.87)	0.79 (0.73, 0.86)	0.80 (0.73, 0.87)	0.80 (0.73, 0.87)
Quartiles				
Q1	Ref	Ref	Ref	Ref
Q2	1.00 (0.82, 1.22)	0.95 (0.78, 1.16)	0.94 (0.77, 1.16)	0.94 (0.77, 1.15)
Q3	0.74 (0.60, 0.92)	0.71 (0.57, 0.88)	0.72 (0.58, 0.90)	0.72 (0.58, 0.90)
Q4	0.60 (0.48, 0.76)	0.58 (0.46, 0.73)	0.58 (0.46, 0.74)	0.58 (0.46, 0.74)
P for trend	<0.001	<0.001	<0.001	<0.001
Ischemic stroke				
Per SD increment	0.85 (0.78, 0.93)	0.84 (0.77, 0.92)	0.84 (0.77, 0.92)	0.84 (0.76, 0.91)
Quartiles				
Q1	Ref	Ref	Ref	Ref
Q2	1.07 (0.86, 1.34)	1.02 (0.81, 1.27)	1.00 (0.80, 1.26)	1.00 (0.80, 1.25)
Q3	0.79 (0.62, 1.00)	0.75 (0.59, 0.96)	0.76 (0.59, 0.96)	0.75 (0.59, 0.96)
Q4	0.69 (0.54, 0.88)	0.66 (0.51, 0.84)	0.65 (0.51, 0.84)	0.65 (0.50, 0.84)
P for trend	<0.001	<0.001	<0.001	<0.001
Hemorrhagic stroke				
Per SD increment	0.61 (0.50, 0.75)	0.61 (0.49, 0.75)	0.64 (0.52, 0.79)	0.64 (0.52, 0.79)
Quartiles				
Q1	Ref	Ref	Ref	Ref
Q2	0.76 (0.48, 1.20)	0.75 (0.47, 1.19)	0.78 (0.49, 1.24)	0.77 (0.48, 1.22)
Q3	0.54 (0.33, 0.89)	0.54 (0.33, 0.89)	0.58 (0.35, 0.96)	0.57 (0.35, 0.95)
Q4	0.34 (0.19, 0.61)	0.33 (0.19, 0.60)	0.38 (0.21, 0.69)	0.38 (0.21, 0.69)
P for trend	<0.001	<0.001	0.001	0.001

Model 1: adjusted for age, sex, BMI, heart rate, SBP, DBP, duration of hypertension, smoking, and drinking status.
Model 2: model 1 plus comorbid conditions.
Model 3: model 2 plus laboratory tests and concomitant medications.
SD, standard deviation; HR, hazard ratio; CI, confidence interval. Other abbreviations as presented in Table 1.

Aging is a condition that affects all people. One of the most susceptible demographics and one that is more likely to experience nutritional issues is the elderly (15). Similarly, malnutrition is an important independent risk factor for stroke, as is hypertension (16–19). In contrast to other clinical variables, nutritional status is a modifiable risk factor that physicians can act on. Therefore, appropriate tools are needed to assess the nutritional status of elderly patients with hypertension and to identify patients at risk to reduce their risk of stroke. Among multiple proxies of nutritional status, serum albumin levels and BMI are often used to evaluate nutritional status (20, 21). Hypoalbuminemia has been linked to acute and chronic inflammation, low BMI may be a sign of malnutrition, and both conditions may be a result of the loss of muscle and adipose tissue (22). In the general population, hypoalbuminemia and higher BMI have been reported as independent risk factors for stroke (23–26). Systemic edema, hepatic failure, and inflammation all have a negative influence on serum albumin levels. The status of body fluids also impacts body weight (27, 28). Consequently, assessing nutritional risk and prognosis solely based on albumin or BMI may not be sufficient. Simple hematological data (serum albumin) and anthropometric data can be used to compute the GNRI, a simplified form of the nutritional risk index (including height and weight) (14, 15). These indicators are readily available and can reduce information bias. Because of its objective nature, GNRI overcomes the problems of traditional nutrition indicators, including subjective issues such as mini-nutritional assessments (29). And GNRI correlates well with malnutrition-inflammation scores and has been regarded as one of the gold standards for nutritional assessment of elderly patients with chronic diseases (10, 30). There is evidence that patients with chronic illnesses, including chronic hemodialysis and peripheral vascular disease, have a lower GNRI (31–35). The results of Xiong et al. demonstrated that low GNRI levels were a strong predictor of cardiovascular and cerebrovascular events in patients with CKD (36). GNRI has also been reported to predict cardiovascular events, including cardiovascular disease mortality, in patients

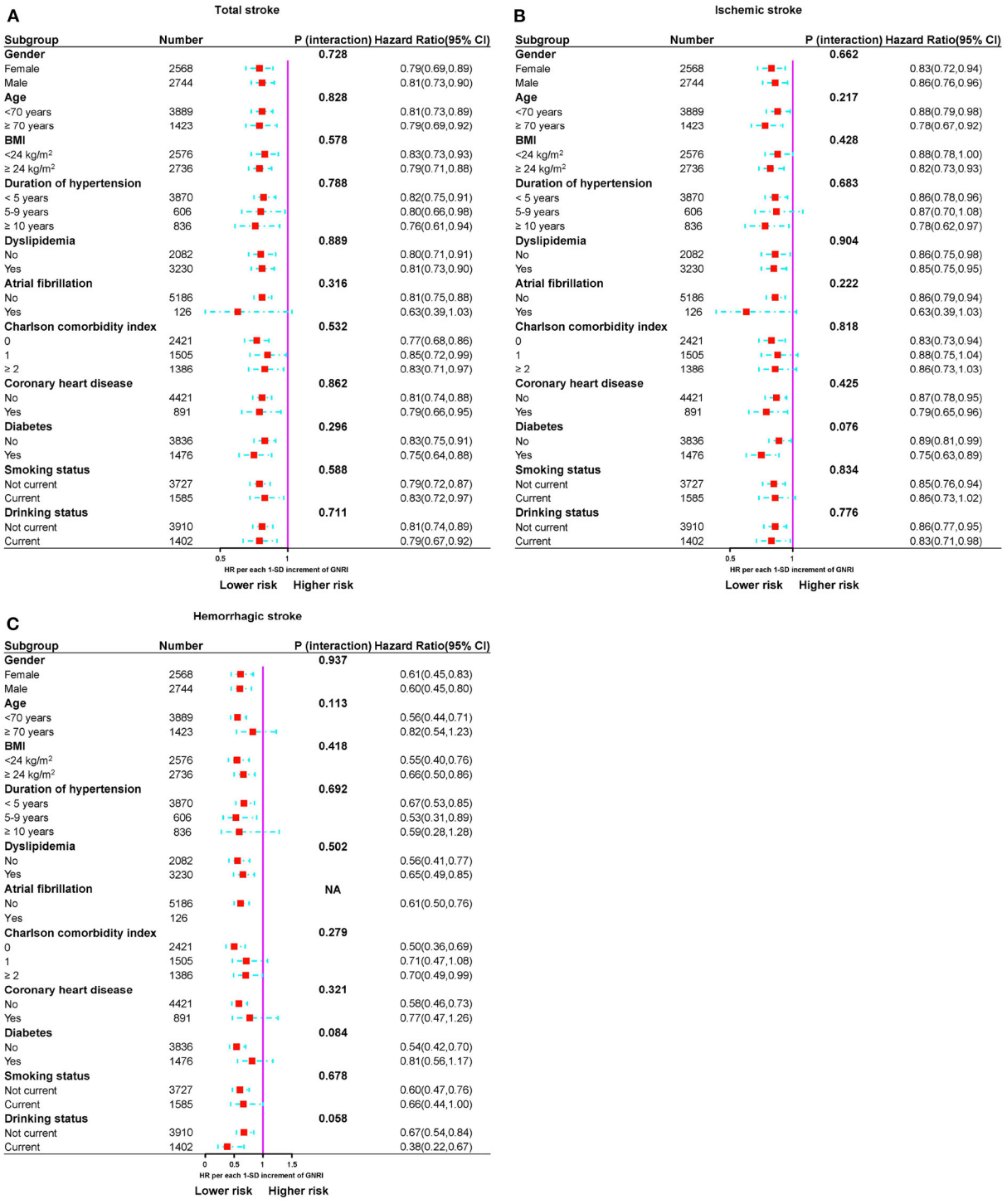

FIGURE 5
Subgroup analyses of the relationship between GNRI and risk of stroke events. **(A)** Total stroke, **(B)** ischemic stroke, and **(C)** hemorrhagic stroke.

with heart failure (37). Furthermore, a study by Anzaki et al. (38) showed that low GNRI levels were associated with all-cause mortality and major adverse cardiovascular events after elective percutaneous coronary intervention (PCI). Cheng et al. (11) shown that in patients with chronic coronary artery occlusion (CTO) following PCI, the GNRI score at admission was a reliable predictor of adverse cardiovascular events. The prediction of cardiovascular events following PCI in patients with CTO was

TABLE 3 Incremental predictive value of GNRI.

	C-Statistic	P-value	cNRI	P-value	IDI	P-value
	Estimate (95% CI)		Estimate (95% CI)		Estimate (95% CI)	
Total stroke						
Established risk factors	0.613 (0.590–0.636)		Reference		Reference	
Established risk factors + GNRI	0.648 (0.625–0.672)	<0.001	0.118 (0.066–0.172)	<0.001	0.017 (0.009–0.029)	<0.001
Ischemic stroke						
Established risk factors	0.638 (0.612–0.663)		Reference		Reference	
Established risk factors + GNRI	0.658 (0.632–0.683)	0.005	0.096 (0.044–0.160)	<0.001	0.012 (0.005–0.021)	0.033
Hemorrhagic stroke						
Established risk factors	0.673 (0.625–0.721)		Reference		Reference	
Established risk factors + GNRI	0.718 (0.670–0.766)	0.017	0.190 (0.057–0.311)	0.007	0.014 (0.005–0.052)	<0.001

The established risk factorsl included age, sex, heart rate, duration of hypertension, SBP, DBP, smoking status, drinking status, comorbid conditions, and laboratory tests.
DI, integrated discrimination improvement; cNRI, continuous net reclassification improvement. Other abbreviations as presented in Table 1.

greatly enhanced by including the GNRI score into existing risk prediction algorithms (11). Elderly patients with hypertension are prone to multiple chronic diseases and may focus more on the primary disease, but nutritional support is mostly neglected. Our findings suggest that physicians may incorporate the identification of nutritional status into their daily practice. From prior research and the findings of the current study, we suggest that modestly increasing calorie and protein intake in malnourished elderly patients with hypertension may reduce the risk of stroke (39, 40). Further prospective intervention trials are needed to establish causality.

Although the underlying mechanisms remain unclear, there are some possible explanations. Oxidative stress and inflammation play key roles in the pathogenesis of stroke (41). First, serum albumin is a multifunctional protein that exerts neuroprotective effects in ischemic strokes, such as resisting antioxidants and reducing erythrocyte pressure levels (42, 43). According to Dziedzic et al. (43) stroke patients with decreased serum albumin levels had worse prognoses. Low albumin levels significantly enhanced the probability of recurrence in stroke patients and were related with poor outcome in all stroke subtypes (43, 44). Second, another potential mechanism may be attributed to the inflammatory response. According to a number of studies, inflammation frequently fosters a catabolic state that increases protein breakdown and slows protein synthesis, resulting in malnutrition and a decrease in GNRI (45). Additionally, it has been proven in the past that malnutrition is associated with higher than normal levels of inflammatory markers (46). Lower albumin levels are a result of the catabolic cytokines, muscle catabolism, and hunger suppression that are linked to chronic inflammatory disorders (47, 48). As a result, inflammation may be a key relationship between dietary status and the risk of cardiovascular disease (49). Severe malnutrition is closely associated with high levels of inflammation, and inflammation can increase the burden of atherosclerosis (50). At the same time, the inflammatory response reduces albumin synthesis, further inducing malnutrition (51). There may be a positive feedback loop between inflammation, malnutrition, immune defense, and adverse events, resulting in a vicious cycle. Therefore, the link between these three entities is also described as the malnutrition-inflammation-atherosclerosis syndrome (52, 53).

The strengths of this study lie in the novelty, the long observational period, and the well-characterized participants. Despite the aforementioned merits, several limitations of the present study merit discussion. First, it was observational and cannot establish causation. Second, the time-dependent changes of GNRI during the follow-up period were not assessed. Third, we didn't examine the predictive value of GNRI against more thorough nutrition evaluations. Despite adjustment for major confounding factors, the risk of residual unmeasured confounding remains possible. Finally, this study is limited to China and needs to be replicated in other different populations. Given the limitations inherent in this study, these results should be interpreted with caution but warrant further investigation in subsequent studies.

Conclusion

In summary, we demonstrated that a lower GNRI was associated with a higher risk of incident stroke in elderly hypertensive patients. In addition, a significant L-shaped dose-response relationship between GNRI and the risk of incident HS was observed. Additional prospective data collection is required to confirm our findings.

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 People's Hospital of Xinjiang Uygur Autonomous Region. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

XC and JHu analyzed the data and wrote the manuscript. JHo, QZ, MW, YD, and WY helped with copyediting. XC and NL audited the data. SL, JHu, and XC conducted research. NL had primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1048206/full#supplementary-material

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The effects of saffron supplementation on cardiovascular risk factors in adults: A systematic review and dose-response meta-analysis

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Introduction: Cardiovascular disease (CVD) is one of the leading causes of death and disability in the world and is estimated to involve more people in the next years. It is said that alternative remedies such as herbs can be used to manage the complications of this disease. For this reason, we aimed to conduct this meta-analysis to systematically assess and summarize the effects of saffron supplementation as an important herb on cardiovascular risk factors in adults.

Methods: A systematic search was done in PubMed, Scopus, and Web of Science to find eligible articles up to September 2022. Randomized controlled trials (RCTs) that evaluated the effects of saffron on lipid profiles, glycemic control, blood pressure, anthropometric measures, and inflammatory markers were included. In the meta-analysis, 32 studies were taken into account (n = 1674).

Results: Consumption of saffron significantly decreased triglyceride (TG) (WMD = −8.81 mg/dl, 95%CI: −14.33, −3.28; P = 0.002), total cholesterol (TC) (WMD = −6.87 mg/dl, 95%CI: −11.19, −2.56; P = 0.002), low density lipoprotein (LDL) (WMD = −6.71 mg/dl, 95%CI: −10.51, −2.91; P = 0.001), (P = 0.660), fasting blood glucose (FBG) level (WMD = −7.59 mg/dl, 95%CI: −11.88, −3.30; P = 0.001), HbA1c (WMD = −0.18%, 95%CI: −0.21, −0.07; P < 0.001), homeostasis model assessment-insulin resistance (HOMA-IR) (WMD = −0.49, 95%CI: −0.89, −0.09; P = 0.016), systolic blood pressure (SBP) (WMD = −3.42 mmHg, 95%CI: −5.80, −1.04; P = 0.005), tumor necrosis factor α (TNF-α) (WMD = −2.54 pg/ml, 95%CI: −4.43, −0.65; P = 0.008), waist

circumference (WC) (WMD = −1.50 cm; 95%CI: −2.83, −0.18; *P* = 0.026), malondialdehyde (MDA) (WMD = −1.50 uM/L, 95%CI: −2.42, −0.57; *P* = 0.001), and alanine transferase (ALT) (WMD = −2.16 U/L, 95%CI: −4.10, −0.23; *P* = 0.028). Also, we observed that saffron had an increasing effect on total antioxidant capacity (TAC) (WMD = 0.07 mM/L, 95%CI: 0.01, 0.13; *P* = 0.032). There was linear regression between FBG and the duration of saffron intake. Additionally, the non-linear dose-response analysis has shown a significant association of saffron intervention with HDL (*P* = 0.049), HOMA-IR (*P* = 0.002), weight (*P* = 0.036), ALP (*P* = 0.016), FBG (*P* = 0.011), HbA1c (*P* = 0.002), and TNF-α (*P* = 0.042). A non-linear association between the length of the intervention and the level of HDL and DBP was also found.

Discussion: That seems saffron could effectively improve TG, TC, LDL, FBG, HbA1c, HOMA-IR, SBP, CRP, TNF-α, WC, MDA, TAC, and ALT.

KEYWORDS

saffron, cardiovascular risk factors, systematic review, meta-analysis, adult

Introduction

Cardiovascular disease (CVD) is known as one of the main causes of morbidity and mortality in societies (1). This complication which includes ischemic heart disease, stroke, heart failure, peripheral arterial disease, and other conditions (2), reduces the quality of life and life expectancy among patients and also leads to high medical care expenses on health systems and governments in different countries around the world (3, 4). Numbers show that the global prevalence of CVD almost doubled from 271 million in 1990 to 523 million in 2019 besides reaching a mortality rate from 12.1 to 18.6 million which was a third of all death globally (5). It is estimated that CVD would be the cause of more than 23 million deaths in 2030 around the world (6). Many risk factors such as gender, family history, high blood pressure, dyslipidemia, obesity, glucose abnormalities, insulin resistance, lifestyle risk factors (7, 8), and inflammation (9) are involved in the development of this disease. Accordingly, lifestyle modification especially nutritional interventions and alternative remedies like herbs can be applied to manage and treat CVD and related diseases (10, 11).

Saffron with the scientific name of "Crocus sativus Linn" (12) and bioactive compounds of crocetin, crocin, picrocrocin, and safranal (13), is a plant with medical properties (14) and is mainly cultivated in Asian and European countries (15). It has been shown that saffron could have positive impacts on hyperglycemia, insulin resistance (16), and hyperlipidemia (17) due to increasing glucose uptake and enhancing insulin sensitivity in cells (18) besides mitochondrial-β-oxidation (19). Furthermore, it is shown that this herb has anti-inflammatory and anti-oxidative benefits (18) by raising the glutathione reductase levels (20) and lowering the levels of pro-inflammatory enzymes (21). A meta-analysis conducted in 2018 on 11 RCTs showed that saffron consumption has no significant effect on improving lipid profile, fasting insulin, systolic blood pressure (SBP), and body mass index (BMI) but in subgroup analysis, a significant reduction in fasting plasma glucose levels was seen. Inflammatory factors were not examined in this study (22). Also, another meta-analysis was done in 2018 on 9 RCTs that had been conducted on diabetes and metabolic syndrome. In this study, only waist circumferences (WC), HbA1c, and fasting plasma glucose (FPG) were examined and they concluded that saffron can improve WC as well as FPG levels in sub-group analysis when intervention durations were more than 12 weeks. There was no significant effect on HbA1c levels (23). In a recent meta-analysis on 25 RCTs evaluating the effects of saffron on cardiometabolic indices in overweight and obese patients, a significant reduction in FPG was seen in participants with metabolic syndrome but there was

Abbreviations: CVD, cardiovascular disease; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1c, hemoglobin A1c; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor; TAC, total antioxidant capacity; BMI, body mass index; WC, waist circumference; FM, fat mass; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; MDA, malondialdehyde; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, confidence interval; WMD, weighted mean difference; MPO, myeloperoxidase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; NF-kB, nuclear factor kappa B; IFN-γ, Interferon gamma; PPARγ, peroxisome proliferator-activated receptor γ; IR, ischemia-reperfusion; ROS, reactive oxygen species; SOD, super oxide dismutase; CETP, cholesteryl ester transfer protein; FPG, fasting plasma glucose; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; MAPKs, mitogen-activated protein kinase; NO, nitric oxide; ICAM-1, intracellular adhesion molecule-1; GRADE, grading of recommendations assessment, development, and evaluation.

not any considerable effect on Hb1AC, weight, and BMI (24). Besides, Rahmani's meta-analysis containing 9 RCTs showed FPG reduction in interventions longer than 12 weeks without affecting HbA1C levels (23). Regarding lipid profile, in 2019 another meta-analysis on six RCTs showed an improvement in serum concentration of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein (HDL) following supplementation with saffron but no influence on serum FPG and low-density lipoprotein (LDL) concentrations was seen (25). In addition, a meta-analysis in 2019 demonstrated the positive impact of saffron on malondialdehyde (MDA) and total antioxidant capacity (TAC) in unhealthy patients (20). Based on a 2019 article, saffron supplementation did not affect inflammatory cytokines in adults (26).

Although some studies have been done in recent years, findings show contradictory impacts of saffron and its derivates on CVD risk factors. Due to this issue and because a comprehensive meta-analysis of all the risk factors related to CVD has not been performed on new findings since then, we conducted this meta-analysis on 32 RCTs and a wide range of related variables to systematically summarize the results and evaluate the effects of saffron supplementation on cardiovascular risk factors in adults.

Materials and methods

This systematic review and meta-analysis was performed under the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines (27). This study is registered at PROSPERO (CRD42022358721).

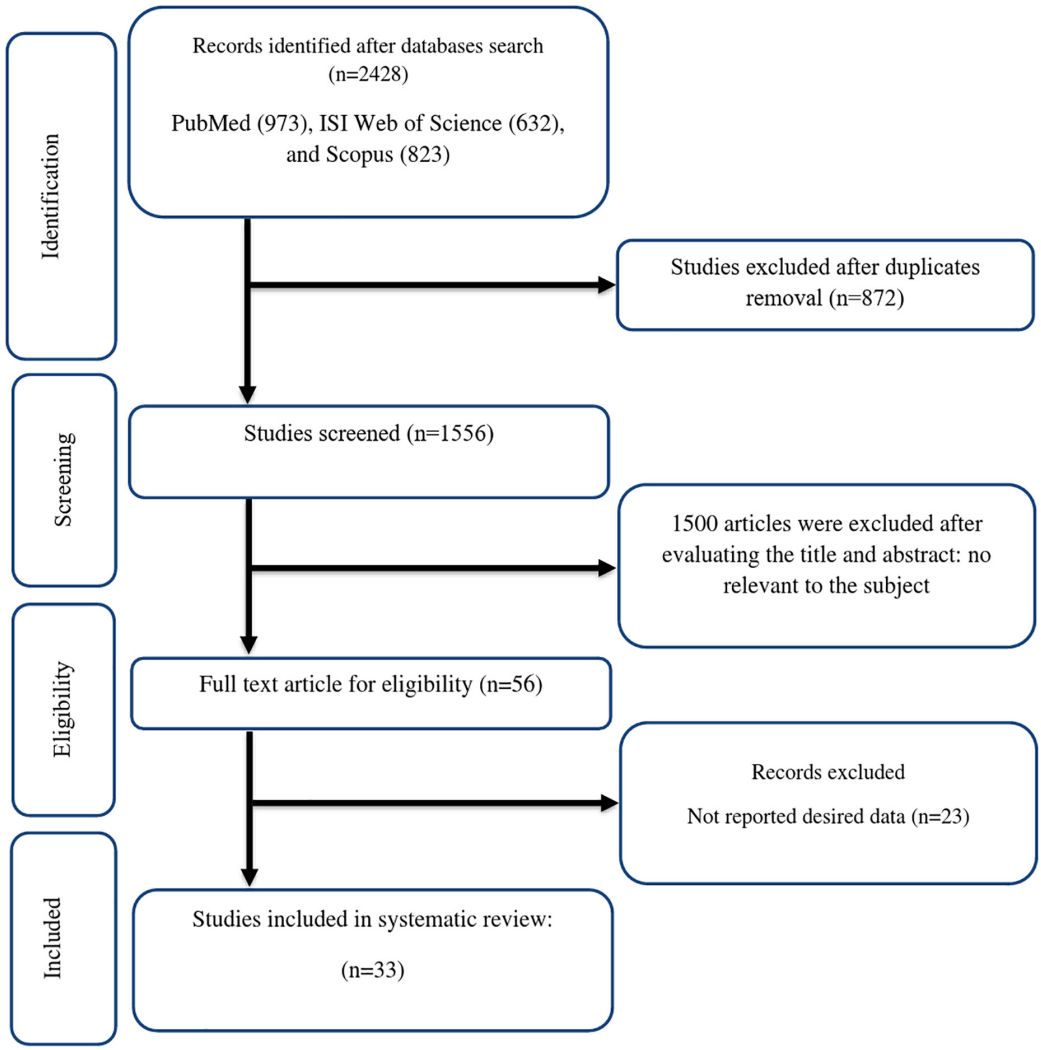

FIGURE 1
Flow chart of study selection for inclusion trials in the systematic review.

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

References	Country	Study design	Participant	Sample size and sex	Sample size		Trial duration (Week)	Means age		Means BMI		Intervention			Adverse events
					IG	CG		IG	CG	IG	CG	Type of intervention	Intervention (mg/d)	Control group	
Modaghegh et al. (40)	Iran	Parallel, R, PC, DB	healthy volunteers	M/F: 20	10	10	1	27 ± 6.5	28.7 ± 6.22	NR	NR	Saffron	200	Placebo	No major adverse events
Modaghegh et al. (40)	Iran	Parallel, R, PC, DB	healthy volunteers	M/F: 20	10	10	1	28.7 ± 5.5	28.7 ± 6.22	NR	NR	Saffron	400	Placebo	No major adverse events
Gout et al. (41)	France	Parallel, R, PC, DB	mildly overweight healthy women	F: 60	31	29	8	36.2 ± 5.5	35.9 ± 5.4	26.7 ± 1.2	26.9 ± 1.1	Satiereal	176.5	Placebo	Mild side effects
Mansoori et al. (42)	Iran	Parallel, R, PC, DB	patients with major depressive disorder	M/F: 20	10	10	4	35.3 ± 5.81	42.4 ± 8.44	NR	NR	saffron	30	Placebo	Dry mouth (n = 3), Restlessness (n = 2), Anxiety (n = 2), Daily drowsiness (n = 1), Morning drowsiness
Mohamadpour et al. (43)	Iran	Parallel, R, PC, DB	Healthy Volunteers	M/F: 44	22	22	4	31.1 ± 13	31.1 ± 13	24.9 ± 7.1	24.9 ± 7.1	Crocin	20	Placebo	No major adverse events
Fadai et al. (45)	Iran	Parallel, R, PC, TB	Patients with Schizophrenia	M/F: 44	22	22	12	48.1 ± 7.7	48.1 ± 6.1	NR	NR	Crocin	30	Placebo	No serious adverse effects
Fadai et al. (45)	Iran	Parallel, R, PC, TB	Patients with Schizophrenia	M/F: 44	22	22	12	49.3 ± 7.1	48.1 ± 6.1	NR	NR	Saffron Aqueous Extract	30	Placebo	No serious adverse effects
Azimi et al. (44)	Iran	Parallel, R, PC, SB	Type 2 diabetes	M/F: 81	42	39	8	57.02 ± 6.5	53.64 ± 7.9	28.86 ± 1.6	28.4 ± 1.3	saffron	1000	Control group	No adverse events
Mousavi et al. (46)	Iran	Parallel, R, PC, DB	patients with schizophrenia	M: 44	22	22	12	48.1 ± 7.7	48.1 ± 6.1	NR	NR	Crocin	30	Placebo	No serious adverse effects
Mousavi et al. (46)	Iran	Parallel, R, PC, DB	patients with schizophrenia	M: 44	22	22	12	49.3 ± 7.1	48.1 ± 6.1	NR	NR	Saffron Aqueous Extract	30	Placebo	No serious adverse effects
Nikbakht-Jam et al. (47)	Iran	Parallel, R, PC, DB	Subjects with Metabolic Syndrome	M/F: 60	30	30	8	38.97 ± 13.33	43.46 ± 12.77	NR	NR	Crocin	30	Control group	NR
Azimi et al. (48)	Iran	Parallel, R, PC, SB	Type 2 diabetes	M/F: 81	42	39	8	57.02 ± 6.8	53.64 ± 7.8	28.86 ± 1.5	28.4 ± 1.3	Saffron	1000	Control group	No adverse effects
Javandoost et al. (51)	Iran	Parallel, R, PC, DB	subjects with metabolic syndrome	M/F: 44	22	22	8	38.8 ± 12	40.45 ± 11.2	NR	NR	Crocin	30	Placebo	NR

(Continued)

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TABLE 1 (Continued)

References	Country	Study design	Participant	Sample size and sex	Sample size		Trial duration (Week)	Means age		Means BMI		Intervention			Adverse events
					IG	CG		IG	CG	IG	CG	Type of intervention	Intervention (mg/d)	Control group	
Abedimanesh et al. (49)	Iran	Parallel, R, PC, DB	patients with coronary artery disease	M/F: 50	25	25	8	53.36 ± 5.94	56.32 ± 5.91	27.92 ± 2.57	28.05 ± 2.89	Crocin	30	Placebo	No serious adverse effects
Abedimanesh et al. (49)	Iran	Parallel, R, PC, DB	patients with coronary artery disease	M/F: 50	25	25	8	56.04 ± 7.55	56.32 ± 5.91	28.64 ± 2.23	28.05 ± 2.89	Saffron Aqueous Extract	30	Placebo	No serious adverse effects
Kermani et al. (52)	Iran	Parallel, R, PC, DB	Subjects with Metabolic Syndrome	M/F: 44	22	22	12	43.64 ± 11.17	42.59 ± 8.44	31.02 ± 5.45	30.48 ± 6.26	saffron	100	Placebo	NR
Kermani et al. (53)	Iran	Parallel, R, PC, DB	Metabolic Syndrome	M/F: 48	24	24	6	53.8 ± 9.2	50.9 ± 8.8	29.9 ± 3.9	29.8 ± 5.3	saffron	100	Placebo	No adverse effects
Jafarnia et al. (50)	Iran	Parallel, R, PC, DB	Mild to Moderate Generalized Anxiety Disorder	M/F: 40	20	20	6	29.65 ± 8.45	32.4 ± 6.74	26.33 ± 5.12	25.49 ± 5.9	Saffron	450	Placebo	Constipation (n = 1), polydipsia (n = 1), headache (n = 2)
Milajerdi et al. (17)	Iran	Parallel, R, PC, TB	Type 2 diabetes	M/F: 54	27	27	8	54.57 ± 6.96	55.42 ± 7.58	23.84 ± 11.89	28.3 ± 3.24	Saffron	30	Placebo	Headache
Sepahi et al. (54)	Iran	Parallel, R, PC, DB	patients with refractory diabetic maculopathy	M/F: 68	34	34	12	54.31 ± 6.6	57.17 ± 2.9	NR	NR	Crocin	5	Placebo	Increased appetite, feet swelling, stomach ache, subconjunctival-hemorrhage, swelling, redness, and burning of the eyes
Sepahi et al. (54)	Iran	Parallel, R, PC, DB	patients with refractory diabetic maculopathy	M/F: 67	33	34	12	56.09 ± 4.3	57.17 ± 2.9	NR	NR	Crocin	15	Placebo	Increased appetite, feet swelling, stomach ache, subconjunctival-hemorrhage, swelling, redness, and burning of the eyes

(Continued)

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TABLE 1 (Continued)

References	Country	Study design	Participant	Sample size and sex	Sample size		Trial duration (Week)	Means age		Means BMI		Intervention			Adverse events
					IG	CG		IG	CG	IG	CG	Type of intervention	Intervention (mg/d)	Control group	
Zilaee et al. (55)	Iran	Parallel, R, PC, DB	patients with metabolic syndrome	M/F: 76	38	38	12	42.19 ± 11.52	43.6 ± 9.05	NR	NR	saffron	100	Placebo	NR
Moravej Aleali et al. (61)	Iran	Parallel, R, PC, DB	Type 2 diabetes	M/F: 64	32	32	12	53.5 ± 9.9	52.4 ± 13	28.8 ± 4	27.5 ± 4.2	Saffron	15	Placebo	NR
Ghaderi et al. (58)	Iran	Parallel, R, PC, DB	patients under methadone maintenance treatment	M/F: 53	26	27	8	44.5 ± 9.4	45.6 ± 9.9	24.5 ± 4.4	25.2 ± 4.2	Crocin	15	Placebo	No adverse effects
Ebrahimi et al. (56)	Iran	Parallel, R, PC, DB	Type 2 diabetes	M/F: 80	40	40	12	55.2 ± 7.3	53 ± 10.6	28.7 ± 4.15	29.91 ± 3.91	Saffron	100	Placebo	NR
Karimi-Nazari et al. (60)	Iran	Parallel, R, PC, DB	overweight/ obese prediabetic	M/F: 75	36	39	8	57.95 ± 8.12	57.9 ± 8.7	29.35 ± 1.5	28.78 ± 2.02	Saffron	15	Placebo	No adverse effects
Shahbazian et al. (62)	Iran	Parallel, R, PC, DB	Type 2 diabetes	M/F: 64	32	32	12	53.5 ± 9.9	52.4 ± 13	28.8 ± 4	27.5 ± 4.2	Saffron	15	Placebo	NR
Zilaee et al. (63)	Iran	Parallel, R, PC, DB	patients with mild and moderate persistent allergic asthma	M/F: 76	38	38	8	41.27 ± 9.77	40.77 ± 10.07	26.84 ± 1.9	26.84 ± 2.34	Saffron	100	Placebo	No serious adverse effects
Ghiasian et al. (59)	Iran	Parallel, R, PC, DB	multiple sclerosis patients	M/F: 40	20	20	4	29 ± 4.99	31.47 ± 5.31	NR	NR	Crocin	30	Placebo	NR
Ebrahimi et al. (57)	Iran	Parallel, R, PC, DB	Type 2 diabetes	M/F: 80	40	40	12	55.2 ± 7.3	53 ± 10.6	29.3 ± 4.9	30.5 ± 4.7	saffron	100	Placebo	NR
Behrouz et al. (64)	Iran	Parallel, R, PC, DB	Type 2 diabetes	M/F: 50	25	25	12	57.08 ± 7.41	59.86 ± 9.46	30.64 ± 4.79	30.85 ± 3.19	Crocin	30	Placebo	No serious adverse effects
Mobasseri et al. (66)	Iran	Parallel, R, PC, DB	Type 2 diabetes	M/F: 57	30	27	8	50.57 ± 9.88	51.63 ± 11.3	30.96 ± 4.23	31.02 ± 4.69	saffron	100	Placebo	NR
Parsi et al. (67)	Iran	Parallel, R, PC, DB	patients with non-alcoholic fatty liver disease	M/F: 60	30	30	8	33.08 ± 2.8	36.1 ± 5.47	29.84 ± 3.37	30.25 ± 3.31	Crocin	15	Placebo	NR

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10.3389/fnut.2022.1055517

TABLE 1 (Continued)

References	Country	Study design	Participant	Sample size and sex	Sample size		Trial duration (Week)	Means age		Means BMI		Intervention			Adverse events
					IG	CG		IG	CG	IG	CG	Type of intervention	Intervention (mg/d)	Control group	
Hamidi et al. (65)	Iran	Parallel, R, PC, DB	patients with active rheumatoid arthritis	F: 66	33	33	12	51.55 ± 8.26	51.8 ± 9.62	28.17 ± 3.74	28.39 ± 3.7	saffron	100	Placebo	Stomach pain.
Kavianipour et al. (68)	Iran	Parallel, R, PC, DB	patients with non-alcoholic fatty liver disease	M/F: 76	38	38	12	43.42 ± 10.62	42.05 ± 8.27	28.85 ± 5.45	29.6 ± 4.4	saffron	100	Placebo	No adverse effects
Tajaddini et al. (15)	Iran	Parallel, R, PC, DB	Type 2 diabetes	M/F: 70	35	35	8	50.5 ± 9.8	51.8 ± 10.9	30 ± 4.2	31.2 ± 4.6	Saffron	100	Placebo	No adverse effects
Tahvilian et al. (69)	Iran	Parallel, R, PC, DB	ulcerative colitis patients	M/F: 75	40	35	8	40.55 ± 12.71	40.97 ± 11.34	26.95 ± 10.68	24.8 ± 3.46		100	Placebo	NR

IG, intervention group; CG, control group; DB, double-blinded; SB, single-blinded; PC, placebo-controlled; CO, controlled; RA, randomized; NR, not reported; F, female; M, male; NR, not reported.
Age: mean age of participants; BMI: mean of body mass index.

Search strategy

To find relevant articles published up to September 2022, a systematic search was done in scientific databases including PubMed, Scopus, and Web of Science regardless of the length of studies and language. In addition, a manual search through the reference lists of relevant publications was performed to make sure we did not miss any potential studies. The PICO criteria (Participant, Intervention, Comparison/Control, Outcome) was used to search for items related to saffron supplementation and cardiovascular risk factors. (1) Participants: adults age≥18; (2) Intervention group (Saffron, Satiereal, Crocin); (3) Comparison/Control group (non-saffron supplementation), and (4) Outcome (all of the CVD risk factors that will be mentioned). The main terms and keywords we used to search the databases are as follow: ("Crocus sativus Linn" OR Safranal OR saffron OR crocin) AND (Intervention OR "Intervention Study" OR "Intervention Studies" OR "controlled trial" OR randomized OR randomized OR random OR randomly OR placebo OR "clinical trial" OR Trial OR "randomized controlled trial" OR "randomized clinical trial" OR RCT OR blinded OR "double blind" OR "double blinded" OR trial OR "clinical trial" OR trials OR "Pragmatic Clinical Trial" OR "Cross-Over Studies" OR "Cross-Over" OR "Cross-Over Study" OR parallel OR "parallel study" OR "parallel trial").

Study selection

Studies with the following criteria were included: (1) RCTs with either parallel or crossover design; (2) used oral supplementation of saffron; (3) investigated the effects of saffron on any of the cardiovascular risk factors and the desired variables such as triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), fasting blood glucose (FBG), hemoglobin A1c (HbA1c), insulin, serum insulin, homeostasis model assessment-insulin resistance (HOMA-IR), systolic blood pressure (SBP), diastolic blood pressure (DBP), C-reactive protein (CRP), interleukin-6, (IL-6), tumor necrosis factor (TNF-α), total antioxidant capacity (TAC), weight, waist circumference (WC), body mass index (BMI), fat mass% (FM), aspartate transaminase (AST), alanine transaminase (ALT), malondialdehyde (MDA), alkaline phosphatase (ALP), (4) were performed on the adult population (≥ 18 years old); (5) had an intervention duration of at least four days (RCTs with two or more eligible arms were considered as separate studies); (6) provided means and standard deviations (SDs) for data, or any other effect sizes from which the calculation of mean and SD was possible; (7) human studies. Two authors (OA, MZ) independently screened the titles, abstracts, and full texts, Checked the results, and assessed the eligibility of the selected studies. Any disagreement was resolved by discussion. Exclusion criteria included animal and

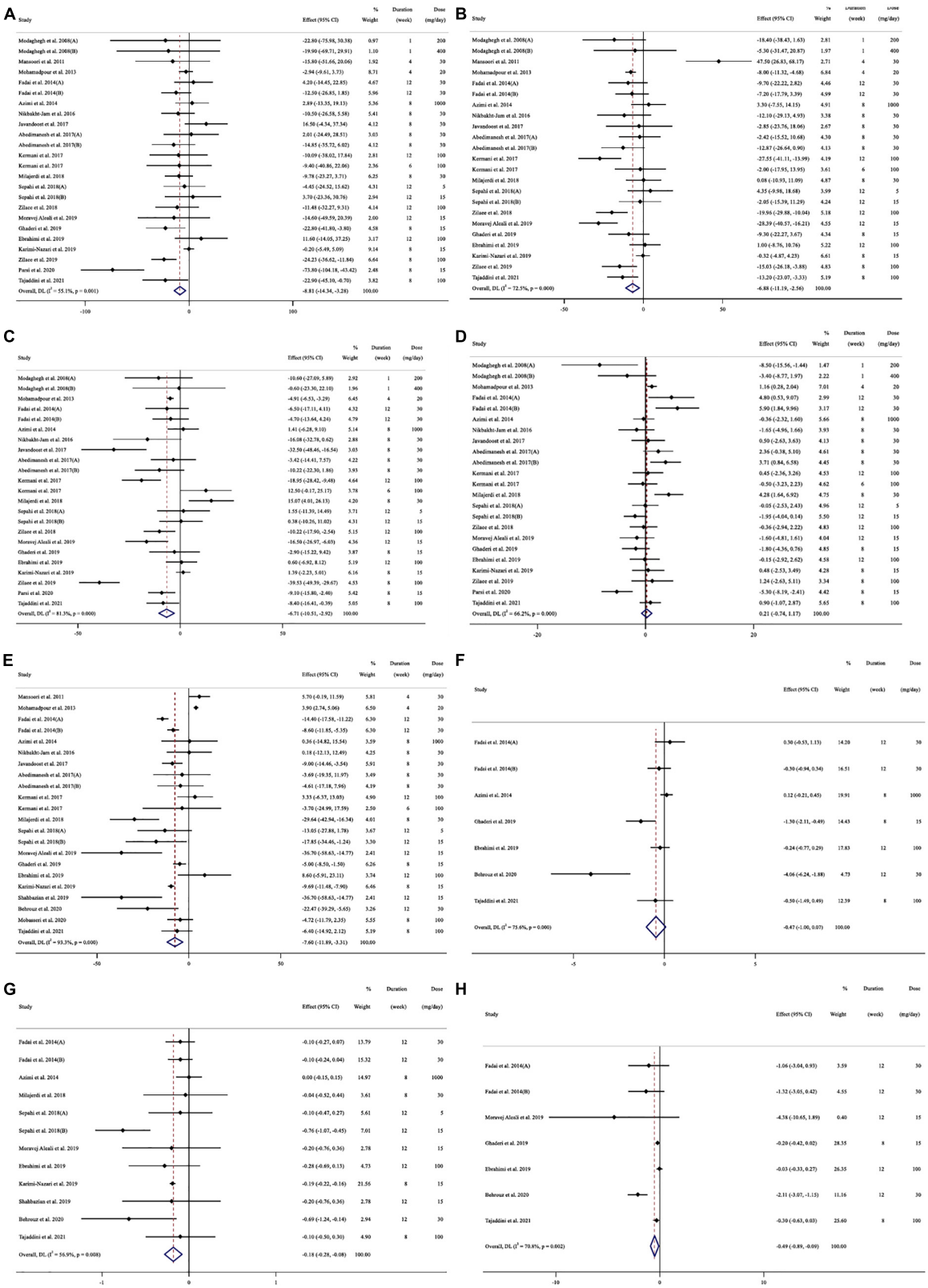

FIGURE 2
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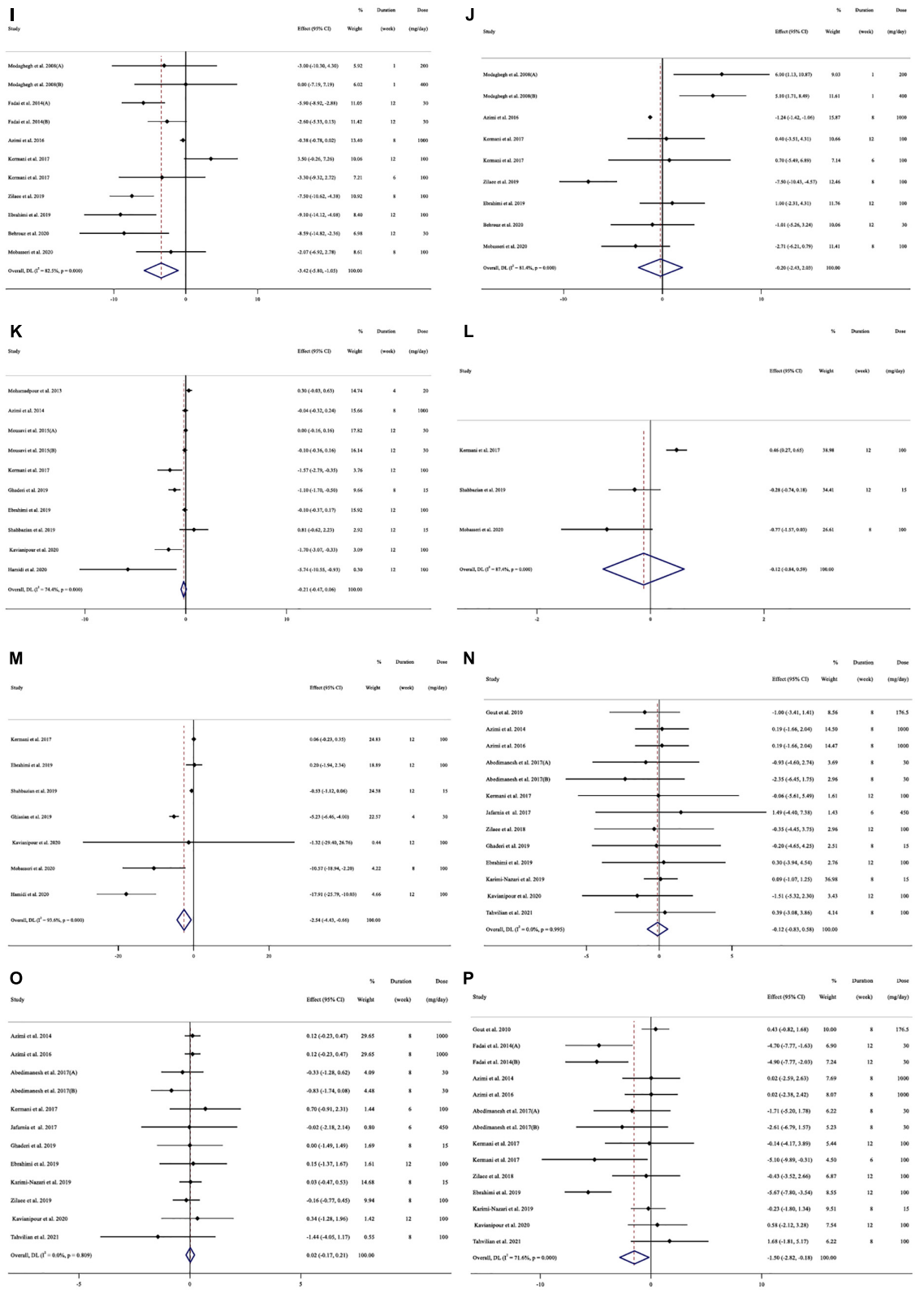

FIGURE 2
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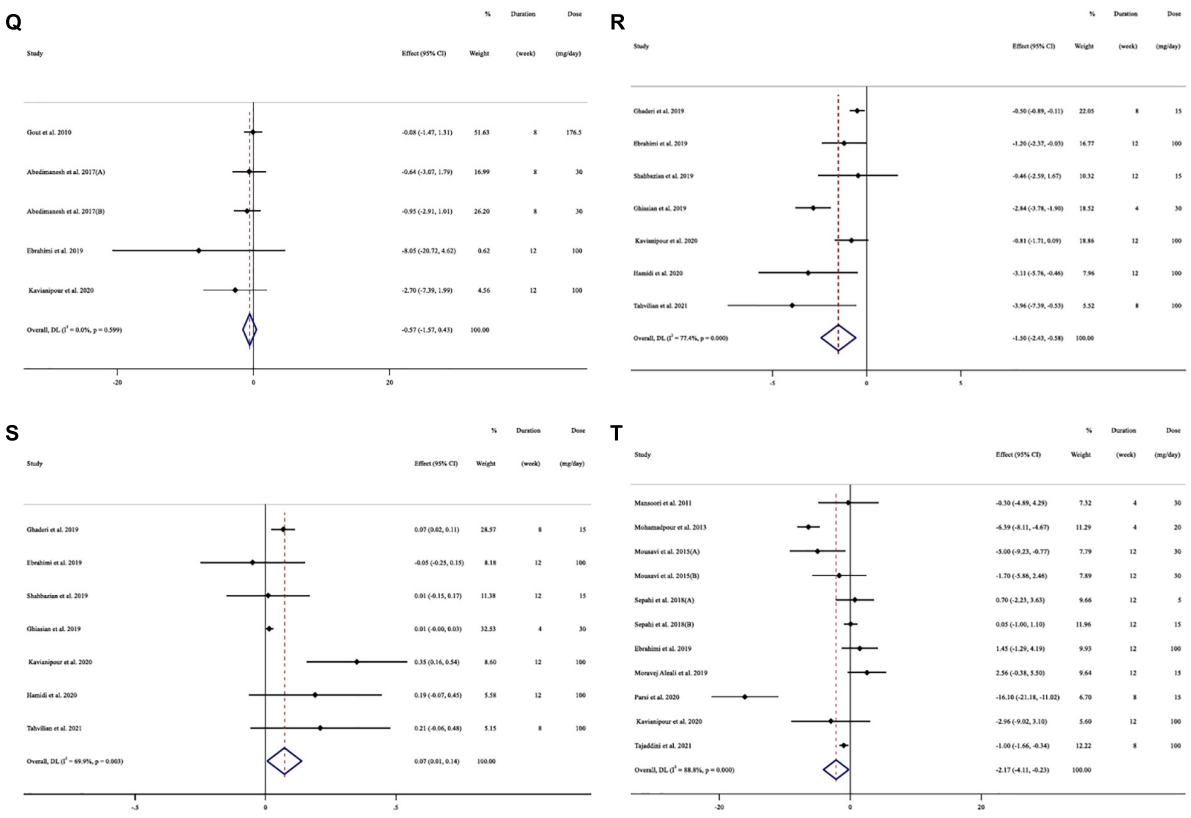

FIGURE 2
(Continued)

in vitro studies in addition to studies that examined the effect of another intervention along with saffron or done on children and adolescents. Moreover, studies with a non-RCT design, without a placebo group, unpublished documents, and gray literature like conference abstracts, editorial papers, and books were excluded.

Data extraction

The following required data were extracted from eligible studies by two independent authors (OA, MZ): The first author's name, country, publication year, type of clinical trial, participant characteristics (mean age, BMI, sex), health condition of participants, randomization, blinding, sample size, the number of participants in the intervention and control groups, the form and dose of supplemented saffron, study duration, and the desired variables. Furthermore, for both parallel and cross-over trials, means ± Standard Deviation (SD) of variables at the beginning and end of the study were collected. If this data was not available, the mean difference was calculated by subtracting the mean value at baseline from the mean value at the end of the study. If there were insufficient data in articles with pre-determined methods contact authors via email.

Quality assessment

To assess the quality of the studies, we benefited from the Cochrane Collaboration tool (28). All the studies were checked for the probability of bias. This included randomized sequence generation, allocation concealment, blindness (participants, staff, and outcome assessment), incomplete outcome data, selective outcome reporting, and other biases. Based on the recommendations of the Cochrane Handbook, three groups of high risk of bias, low risk of bias, and uncertain risk of bias were created. The quality of studies in which the number of high-risk biases was more than 2 was considered as bad and in the same way, those having 2 or less than 2 high-risk biases were considered fair and good, respectively. The quality of the work was checked by two authors (OA, MZ) and in case of any disagreement, the problem was resolved by discussion and consulting.

Statistical analysis

All statistical analyzes of eligible studies were performed using Stata software version 11.0 (Stata Corp, College Station,

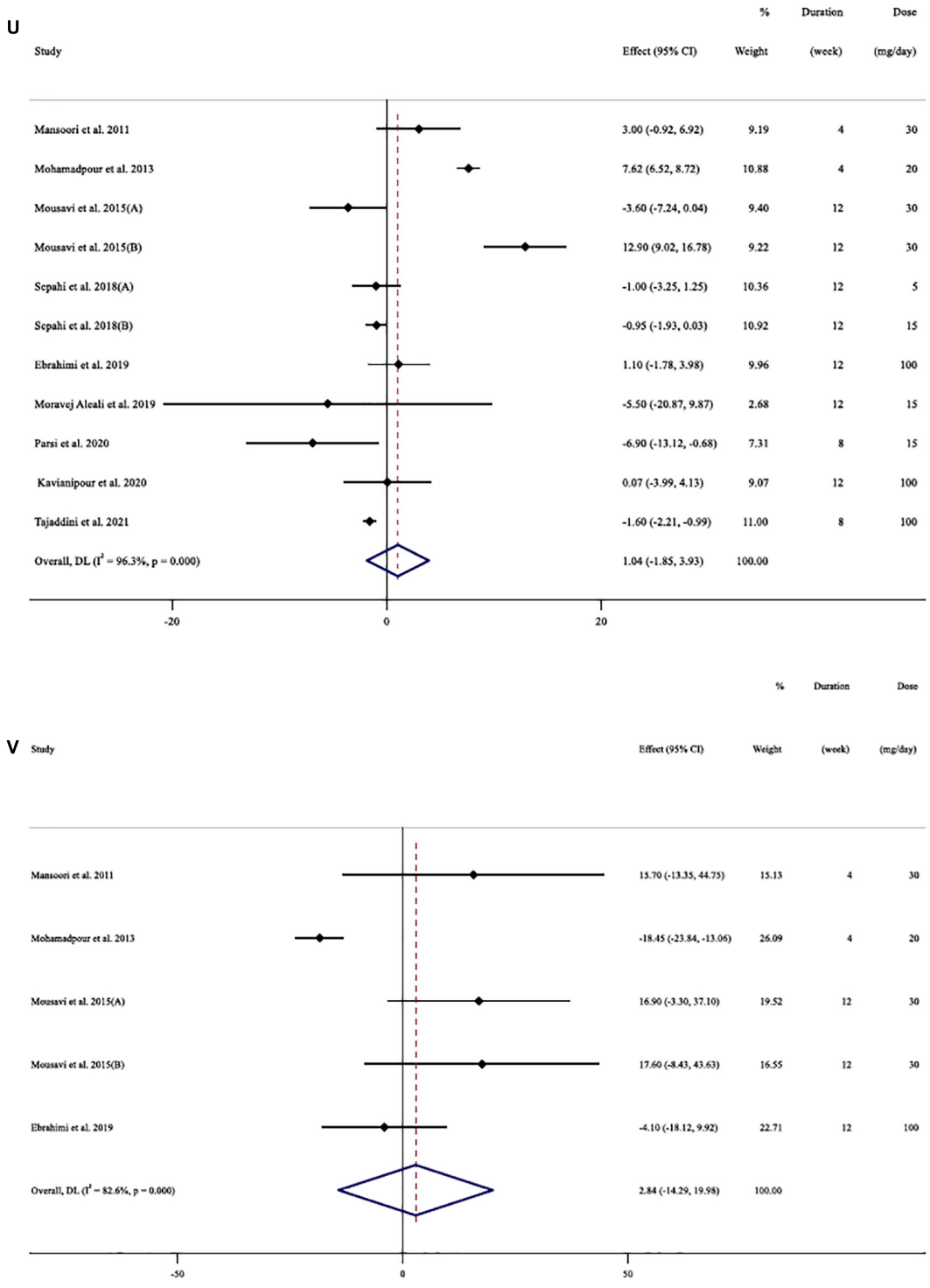

FIGURE 2
Forest plot detailing weighted mean difference and 95% confidence intervals (CIs) for the effect of saffron consumption on **(A)** TG (mg/dl); **(B)** TC (mg/dl); **(C)** LDL (mg/dl); **(D)** HDL (mg/dl); **(E)** FBG (mg/dl); **(F)** Insulin (miu/ml); **(G)** HbA1c (%); **(H)** HOMA-IR; **(I)** SBP (mmHg); **(J)** DBP (mmHg); **(K)** CRP (mg/l); **(L)**; IL-6 (pg/ml); **(M)** TNF-α (pg/ml); **(N)** weight (kg); **(O)** BMI (kg/m^2); **(P)** WC (cm); **(Q)** FM (%); **(R)** MDA (uM/L); **(S)** TAC (mM/L); **(T)** ALT (U/L); **(U)** AST (U/L) and **(V)** ALP (U/L). TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1C, hemoglobin A1C; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor; TAC, total antioxidant capacity; BMI, body mass index; WC, waist circumference; FM, fat mass; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; MDA, malondialdehyde; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, confidence interval; WMD, weighted mean difference.

TABLE 2 Quality assessment (A summary of the risk of bias according to Cochrane criteria).

References	Random sequence generation	Allocation concealment	Selective reporting	Other sources of bias	Blinding (participants and personnel)	Blinding (outcome assessment)	Incomplete outcome data	General risk of bias	Quality
Modaghegh et al. (40)	L	H	H	L	L	U	L	M	Fair
Gout et al. (41)	L	L	H	H	L	U	L	M	Fair
Mansoori et al. (42)	L	H	H	H	L	U	L	H	Bad
Mohamadpour et al. (43)	L	L	H	H	L	U	H	H	Bad
Fadai et al. (45)	L	L	H	H	L	L	L	M	Fair
Azimi et al. (44)	L	H	H	L	H	H	L	H	Bad
Mousavi et al. (46)	L	H	H	H	L	U	L	H	Bad
Nikbakht-Jam et al. (47)	L	L	H	H	L	U	L	M	Fair
Azimi et al. (48)	L	H	H	H	H	H	L	H	Bad
Javandoost et al. (51)	L	L	H	H	L	U	L	M	Fair
Abedimanesh et al. (49)	L	H	H	L	L	U	L	M	Fair
Kermani et al. (52)	L	H	H	H	L	U	L	H	Bad
Kermani et al. (53)	L	H	H	H	L	U	L	H	Bad
Jafarnia et al. (50)	L	L	H	L	L	U	L	L	Good
Milajerdi et al. (17)	L	L	H	L	L	L	L	L	Good
Sepahi et al. (54)	L	L	H	H	L	U	L	M	Fair
Zilaee et al. (55)	L	H	H	H	L	U	L	H	Bad
Moravej Aleali et al. (61)	L	L	H	L	L	U	L	L	Good
Ghaderi et al. (58)	L	L	H	H	L	U	L	M	Fair
Ebrahimi et al. (56)	L	L	H	L	L	U	L	L	Good
Karimi-Nazari et al. (60)	L	L	H	L	L	U	L	L	Good
Shahbazian et al. (62)	L	L	H	L	L	U	L	L	Good
Zilaee et al. (63)	L	L	H	L	L	U	L	L	Good
Ghiasian et al. (59)	L	H	H	H	L	U	L	H	Bad
Ebrahimi et al. (57)	L	L	H	L	L	U	L	L	Good
Behrouz et al. (64)	L	L	H	H	L	U	L	M	Fair
Mobasseri et al. (66)	L	L	H	H	L	U	L	M	Fair
Parsi et al. (67)	L	L	H	H	L	U	L	M	Fair
Hamidi et al. (65)	L	L	H	H	L	U	L	M	Fair
Kavianipour et al. (68)	L	L	H	L	L	U	L	L	Good
Tajaddini et al. (15)	L	L	H	L	L	U	L	L	Good
Tahvilian et al. (69)	L	L	H	L	L	U	L	L	Good

H, high risk of bias; L, low risk of bias; U, unclear risk of bias.
The Cochrane collaboration tool was used to assess the quality of studies.
Bad > 2 high risks; Good < 2 high risk; Fair = 2 high risk.

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TABLE 3 Subgroup analyses of saffron on CVD risk factors in adults.

	NO	WMD (95%CI)	*P*-value	Heterogeneity		
				P heterogeneity	I^2	P between sub-groups
Subgroup analyses of saffron on serum TG (mg/dl)						
Overall effect	24	−8.81 (−14.33, −3.28)	**0.002**	0.001	55.1%	
Baseline TG (mg/dl)						
<150	11	−4.65 (−8.88, −0.43)	**0.031**	0.430	1.2%	0.405
≥150	12	−9.95 (−21.67, 1.77)	0.096	<0.001	70.6%	
Trial duration (week)						
<12	16	−11.18 (−18.53, −3.84)	**0.003**	<0.001	67.7%	0.253
≥12	8	−5.04 (−12.60, 2.50)	0.190	0.686	0.0%	
Intervention dose (mg/day)						
<100	15	−7.80 (−14.44, −1.16)	**0.021**	0.001	60.6%	0.553
≥100	9	−11.29 (−20.69, −1.89)	**0.019**	0.178	30.0%	
Baseline BMI (kg/m^2)						
Normal (18.5−24.9)	3	−8.92 (−19.09, 1.24)	0.085	0.128	51.4%	0.679
Overweight (25−29.9)	9	−11.76 (−24.49, 0.96)	0.070	<0.001	77.9%	
Obese (>30)	2	17.93 (−35.31, −0.55)	**0.043**	0.482	0.0%	
Health status						
Diabetic	7	−5.08 (−12.80, 2.64)	0.197	0.403	3.0%	0.295
Non-diabetic	17	−10.66 (−17.70, −3.62)	**0.003**	<0.001	64.4%	
Intervention						
Saffron	13	−8.96 (−16.01, −1.93)	**0.013**	0.069	39.7%	0.882
Crocin	9	−7.94 (−19.55, 3.67)	0.180	<0.001	72.9%	
Subgroup analyses of saffron on serum TC (mg/dl)						
Overall effect	23	−6.87 (−11.19, −2.56)	**0.002**	<0.001	72.5%	
Baseline TC (mg/dl)						
<200	18	−7.39 (−13.16, −1.62)	**0.012**	<0.001	77.4%	0.223
≥200	4	−1.54 (−8.96, 5.87)	0.683	0.520	0.0%	
Trial duration (week)						
<12	15	−4.44 (−9.45, 0.56)	0.082	<0.001	69.0%	0.180
≥12	8	−11.21 (−19.74, −2.69)	**0.010**	<0.001	76.0%	
Intervention dose (mg/day)						
<100	14	−4.52 (−9.96, 0.92)	0.104	<0.001	74.9%	0.181
≥100	9	−10.76 (−18.12, −3.41)	**0.004**	0.002	66.5%	
Baseline BMI (kg/m^2)						
Normal (18.5−24.9)	3	−7.43 (10.52, −4.34)	<**0.001**	0.371	0.0%	
Overweight (25−29.9)	8	−6.65 (−13.77, 0.46)	0.067	<0.001	73.2%	0.236
Obese (>30)	2	−19.59 (−33.56, −5.61)	**0.006**	0.094	64.4%	
Health status						
Diabetic	7	−5.05 (−13.54, 3.43)	0.243	0.001	74.1%	0.594
Non-diabetic	16	−7.77 (−13.03, −2.50)	**0.004**	<0.001	73.4%	
Intervention						
Saffron	13	−6.88 (−14.66, 0.90)	0.083	<0.001	83.8%	0.947
Crocin	8	−7.15 (−9.98, −4.33)	<**0.001**	0.716	0.0%	
Subgroup analyses of saffron on serum LDL (mg/dl)						
Overall effect	23	−6.71 (−10.51, −2.91)	**0.001**	<0.001	81.3%	
Baseline LDL (mg/dl)						
<100	7	−4.49 (−11.88, 2.88)	0.233	0.002	71.6%	0.466
≥100	15	−8.10 (−14.38, −1.82)	**0.011**	<0.001	85.4%	

(Continued)

TABLE 3 (Continued)

	NO	WMD (95%CI)	*P*-value	Heterogeneity		
				P heterogeneity	I^2	P between sub-groups
Trial duration (week)						
<12	15	−6.68 (−11.83, −1.52)	**0.011**	<0.001	85.7%	0.944
≥12	8	−6.94 (−12.31, −1.58)	**0.011**	0.014	60.4%	
Intervention dose (mg/day)						
<100	14	−5.10 (−5.10, −1.23)	**0.010**	<0.001	71.5%	0.508
≥100	9	−8.55 (−18.00, 0.90)	0.076	<0.001	87.9%	
Baseline BMI (kg/m^2)						
Normal (18.5−24.9)	3	1.77 (−10.36, 13.92)	0.774	0.002	83.8%	0.177
Overweight (25−29.9)	9	−6.91 (−15.12, 1.30)	0.099	<0.001	89.6%	
Obese (>30)	2	−13.36 (−23.68, −3.03)	**0.011**	0.095	64.0%	
Health status						
Diabetic	7	−1.04 (−7.65, 5.55)	0.756	0.002	70.8%	0.044
Non-diabetic	16	−9.41 (−14.17, −4.65)	<**0.001**	<0.001	83.7%	
Intervention						
Saffron	12	−6.31 (−13.85, 1.21)	0.100	<0.001	88.9%	0.953
Crocin	9	−6.58 (−10.91, −2.25)	**0.003**	0.033	52.1%	
Subgroup analyses of saffron on serum HDL (mg/dl)						
Overall effect	23	0.21 (−0.73, 1.16)	0.660	<0.001	66.2%	
Baseline HDL (mg/dl)						
<40	4	−0.20 (−1.66, 1.25)	0.782	0.765	0.0%	0.668
≥40	18	0.22 (−1.07, 1.51)	0.738	<0.001	71.6%	
Trial duration (week)						
<12	15	0.07 (−1.14, 1.29)	0.902	<0.001	69.9%	0.726
≥12	8	0.43 (−1.17, 2.05)	0.595	0.015	59.9%	
Intervention dose (mg/day)						
<100	14	0.61 (−0.76, 2.00)	0.381	<0.001	76.1%	0.371
≥100	9	−0.15 (−1.13, 0.82)	0.755	0.371	7.8%	
Baseline BMI (kg/m^2)						
Normal (18.5−24.9)	3	1.19 (−1.45, 3.84)	0.378	0.005	81.0%	0.682
Overweight (25−29.9)	9	−0.03 (−1.65, 1.59)	0.969	0.002	66.5%	
Obese (>30)	2	0.75 (−0.86, 2.36)	0.362	0.797	0.0%	
Health status						
Diabetic	7	0.14 (−1.29, 1.58)	0.843	0.018	60.8%	0.484
Non-diabetic	16	0.23 (−1.05, 1.52)	0.723	<0.001	69.3%	
Intervention						
Saffron	12	0.09 (−1.03, 1.22)	0.864	0.051	43.8%	0.669
Crocin	9	−0.33 (−1.93, 1.28)	0.688	<0.001	75.5%	
Subgroup analyses of saffron on serum FBG (mg/dl)						
Overall effect	22	−7.59 (−11.88, −3.30)	**0.001**	<0.001	93.3%	
Baseline FBG (mg/dl)						
<100	5	−6.55 (−12.14, −0.96)	**0.022**	<0.001	89.8%	0.510
≥100	16	−9.00 (13.70, −4.31)	<**0.001**	<0.001	66.1%	
Trial duration (week)						
<12	13	−4.77 (−9.91, 0.36)	0.068	<0.001	94.0%	0.079
≥12	9	−12.02 (−18.28, −5.77)	<**0.001**	<0.001	77.1%	
Intervention dose (mg/day)						
<100	16	−10.05 (−15.17, −4.92)	<**0.001**	<0.001	95.1%	0.018
≥100	6	−2.03 (−6.26, 2.20)	0.348	0.426	0.0%	

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TABLE 3 (Continued)

	NO	WMD (95%CI)	*P*-value	Heterogeneity		
				P heterogeneity	I^2	P between sub-groups
Baseline BMI (kg/m^2)						
Normal (18.5−24.9)	3	−7.40 (−17.77, 2.97)	0.162	<0.001	95.6%	0.861
Overweight (25−29.9)	8	−8.59 (−16.57, −0.61)	**0.035**	0.004	66.4%	
Obese (>30)	4	5.57 (−13.03, 1.89)	0.144	0.070	57.6%	
Health status						
Diabetic	10	−14.08 (−22.38, −5.78)	**0.001**	<0.001	73.4%	0.047
Non-diabetic	12	−4.11 (−9.38, 1.15)	0.126	<0.001	95.8%	
Intervention						
Saffron	11	−7.49 (−13.98, −1.01)	**0.023**	<0.001	83.3%	0.897
Crocin	9	−8.13 (−15.41, −0.86)	**0.028**	<0.001	94.8%	
Subgroup analyses of saffron on serum Insulin (mIU/ml)						
Overall effect	7	−0.46 (−1.00, 0.06)	0.088	<0.001	75.6%	
Trial duration (week)						
<12	3	−0.50 (−1.43, 0.42)	0.285	0.005	81.5%	0.970
≥12	4	−0.53 (−1.39, 0.33)	0.229	0.004	77.7%	
Intervention dose (mg/day)						
<100	4	−0.96 (−2.10, 0.16)	0.094	<0.001	83.2%	0.121
≥100	3	−0.04 (−0.34, 0.26)	0.795	0.321	12.0%	
Baseline BMI (kg/m^2)						
Overweight (25−29.9)	2	0.00 (−0.33, 0.33)	**0.002**	0.254	23.0%	0.008
Obese (>30)	2	−2.14 (−5.62, 1.33)	0.996	0.004	88.2%	
Health status						
Diabetic	4	−0.55 (−1.35, 0.24)	0.175	0.002	80.4%	0.836
Non-diabetic	3	−0.43 (−1.28, 0.41)	0.319	0.023	73.6%	
Intervention						
Saffron	3	−0.04 (−0.34, 0.26)	0.795	0.321	12.0%	0.146
Crocin	3	−1.41 (−3.23, 0.41)	0.130	<0.001	88.1%	
Subgroup analyses of saffron on serum HbA1c (%)						
Overall effect	12	−0.18 (−0.21, −0.07)	<**0.001**	0.008	56.9%	
Trial duration (week)						
<12	4	−0.11 (−0.24, 0.02)	0.104	0.088	54.1%	0.163
≥12	8	−0.27 (−0.45, −0.08)	**0.004**	0.008	63.1%	
Intervention dose (mg/day)						
<100	9	−0.21 (−0.33, −0.09)	<**0.001**	0.014	58.2%	0.050
≥100	3	−0.03 (−0.17, 0.09)	0.557	0.431	0.0%	
Baseline BMI (kg/m^2)						
Overweight (25−29.9)	5	−0.14 (−0.25, −0.03)	**0.013**	0.178	36.5%	0.689
Obese (>30)	2	−0.36 (−0.94, 0.21)	0.214	0.088	65.7%	
Health status						
Diabetic	9	−0.25 (−0.46, −0.03)	**0.020**	0.003	65.1%	0.463
Non-diabetic	3	−0.17 (−0.22, −0.11)	<**0.001**	0.296	17.8%	
Intervention						
Saffron	7	−0.15 (−0.22, −0.08)	<**0.001**	0.342	11.5%	0.229
Crocin	4	−0.38 (−0.75, −0.01)	**0.042**	0.001	81.7%	
Subgroup analyses of saffron on HOMA−IR						
Overall effect	7	−0.49 (−0.89, −0.09)	**0.016**	0.002	70.8%	

(Continued)

TABLE 3 (Continued)

	NO	WMD (95%CI)	*P*-value	Heterogeneity		
				P heterogeneity	I^2	P between sub-groups
Trial duration (week)						
<12	2	−0.23 (−0.41, −0.04)	**0.013**	0.618	0.0%	0.147
≥12	5	−1.19 (−2.49, 0.09)	0.070	<0.001	80.3%	
Intervention dose (mg/day)						
<100	5	−1.22 (−2.42, −0.02)	**0.045**	0.001	77.7%	0.088
≥100	2	−0.15 (−0.42, 0.10)	0.246	0.234	29.4%	
Baseline BMI (kg/m^2)						
Overweight (25–29.9)	2	−1.03 (−4.62, 2.55)	0.071	0.174	45.9%	0.527
Obese (>30)	2	−1.14 (−2.91, 0.62)	0.573	<0.001	91.8%	
Health status						
Diabetic	4	−0.68 (−1.40, 0.04)	0.066	<0.001	83.6%	0.422
Non-diabetic	3	−0.32 (−0.79, 0.15)	0.180	0.326	10.8%	
Intervention						
Saffron	3	−0.17 (−0.49, 0.15)	0.305	0.206	36.8%	0.236
Crocin	3	−1.07 (−2.53, 0.38)	0.149	0.001	86.7%	
Subgroup analyses of saffron on SBP (mmHg)						
Overall effect	11	−3.42 (−5.80, −1.04)	**0.005**	<0.001	82.5%	
Baseline SBP (mmHg)						
<120	6	−2.83 (−6.29, 0.62)	0.108	<0.001	78.7%	0.602
≥120	5	−4.24 (−8.22, −0.25)	**0.037**	0.001	79.3%	
Trial duration (week)						
<12	6	−2.81 (−6.03, 0.41)	0.088	0.001	76.6%	0.601
≥12	5	−4.21 (−8.38, −0.05)	**0.047**	<0.001	83.0%	
Intervention dose (mg/day)						
<100	3	−4.97 (−8.06, −1.88)	**0.002**	0.114	53.9%	0.293
≥100	8	−2.67 (−5.64, 0.29)	0.078	<0.001	81.1%	
Baseline BMI (kg/m^2)						
Overweight (25–29.9)	4	−4.88 (−9.93, 0.16)	0.058	<0.001	90.5%	0.501
Obese (>30)	3	−2.01 (−8.67, 4.64)	0.553	0.004	82.2%	
Health status						
Diabetic	4	−4.54 (−9.34, 0.26)	0.064	<0.001	83.8%	0.576
Non-diabetic	7	−2.91 (−5.99, 0.17)	0.064	0.001	74.4%	
Intervention						
Saffron	8	−2.67 (5.64, 0.29)	0.078	<0.001	81.1%	0.069
Crocin	2	−6.41 (−9.12, −3.69)	<**0.001**	0.446	0.0%	
Subgroup analyses of saffron on DBP (mmHg)						
Overall effect	9	−0.19 (−2.42, 2.03)	0.862	<0.001	81.4%	
Baseline DBP (mmHg)						
<80	5	2.23 (−0.32, 4.79)	0.087	0.070	53.8%	0.017
≥80	4	−2.95 (−6.35, 0.43)	0.088	<0.001	83.8%	
Trial duration (week)						
<12	6	−0.25 (−3.52, 3.02)	0.880	<0.001	87.7%	0.787
≥12	3	0.29 (−1.88, 2.46)	0.793	0.764	0.0%	
Baseline BMI (kg/m^2)						
Overweight (25–29.9)	4	−2.02 (−5.44, 1.39)	0.246	<0.001	84.7%	0.706
Obese (>30)	3	−1.24 (−3.46, 0.98)	0.275	0.506	0.0%	

(Continued)

TABLE 3 (Continued)

	NO	WMD (95%CI)	*P*-value	Heterogeneity		
				P heterogeneity	I^2	P between sub-groups
Health status						
Diabetic	4	−1.23 (−1.41, −1.05)	<**0.001**	0.485	0.0%	0.471
Non-diabetic	5	0.83 (−4.79, 6.47)	0.771	<0.001	89.9%	
Subgroup analyses of saffron on serum CRP (mg/l)						
Overall effect	10	−0.20 (−0.46, 0.05)	0.127	<0.001	74.4%	
Trial duration (week)						
<12	3	−0.22 (−0.84, 0.39)	0.478	<0.001	87.7%	0.805
≥12	7	−0.31 (−0.65, 0.03)	0.074	0.001	71.1%	
Intervention dose (mg/day)						
<100	5	−0.08 (−0.42, 0.24)	0.603	0.001	77.8%	0.061
≥100	5	−0.72 (−1.30, −0.14)	**0.014**	0.001	76.0%	
Baseline BMI (kg/m^2)						
Normal (18.5−24.9)	2	−0.37 (−1.74, 0.99)	0.590	<0.001	93.9%	0.220
Overweight (25−29.9)	5	−0.40 (−0.94, 0.13)	0.144	0.004	71.2%	
Health status						
Diabetic	3	−0.05 (−0.24, 0.13)	0.572	0.466	0.0%	0.048
Non-diabetic	7	−0.52 (−0.94, −0.10)	**0.015**	<0.001	82.1%	
Intervention						
Saffron	6	−0.57 (−1.12, −0.02)	**0.040**	0.001	73.5%	0.327
Crocin	3	−0.19 (−0.72, 0.34)	0.489	<0.001	87.7%	
Subgroup analyses of saffron on serum IL−6 (pg/ml)						
Overall effect	3	−0.12 (−0.83, 0.59)	0.739	<0.001	87.4%	
Subgroup analyses of saffron on serum TNF-α (pg/ml)						
Overall effect	7	−2.54 (−4.43, −0.65)	**0.008**	<0.001	93.6%	
Trial duration (week)						
<12	2	−6.22 (−10.31, −2.14)	**0.003**	0.216	34.7%	0.009
≥12	5	−0.55 (−1.76, 0.66)	0.375	<0.001	78.1%	
Intervention dose (mg/day)						
<100	2	−2.84 (−7.45, 1.75)	0.226	<0.001	97.8%	0.704
≥100	5	−4.02 (−7.94, −0.10)	**0.044**	<0.001	80.9%	
Baseline BMI (kg/m^2)						
Overweight (25−29.9)	4	−2.95 (−6.81, 0.89)	0.133	0.001	79.1%	0.797
Obese (>30)	2	−4.39 (−14.67, 5.88)	0.402	0.013	83.8%	
Health status						
Diabetic	3	−0.91 (−3.21, 1.37)	0.433	0.050	66.5%	0.103
Non-diabetic	4	−5.44 (−10.38, −0.51)	**0.031**	<0.001	95.4%	
Subgroup analyses of saffron on Weight (Kg)						
Overall effect	13	−0.12 (−0.82, 0.58)	0.732	0.995	0.0%	
Trial duration (week)						
<12	9	−0.07 (−0.82, 0.67)	0.840	0.960	0.0%	0.512
≥12	4	−0.75 (−2.62, 1.12)	0.431	0.959	0.0%	
Intervention dose (mg/day)						
<100	4	−0.16 (−1.20, 0.87)	0.757	0.695	0.0%	0.989
≥100	9	−0.17 (−1.10, 0.75)	0.714	0.989	0.0%	
Health status						
Diabetic	3	0.20 (−1.05, 1.45)	0.755	0.999	0.0%	0.488
Non-diabetic	10	−0.33 (−1.16, 0.50)	0.434	0.978	0.0%	

(Continued)

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TABLE 3 (Continued)

	NO	WMD (95%CI)	*P*-value	Heterogeneity		
				P heterogeneity	I^2	P between sub-groups
Intervention						
Saffron	9	0.02 (−0.73, 0.78)	0.954	0.996	0.0%	0.661
Crocin	2	−0.63 (−3.46, 2.19)	0.661	0.804	0.0%	
Subgroup analyses of saffron on BMI (kg/m^2)						
Overall effect	12	0.01 (−0.17, 0.21)	0.853	0.809	0.0%	
Trial duration (week)						
<12	10	0.01 (−0.18, 0.20)	0.910	0.670	0.0%	0.574
≥12	2	0.27 (−0.62, 1.16)	0.548	0.981	0.0%	
Intervention dose (mg/day)						
<100	4	−0.18 (−0.57, 0.20)	0.346	0.428	0.0%	0.222
≥100	8	0.09 (−0.12, 0.31)	0.417	0.947	0.0%	
Health status						
Diabetic	3	0.12 (−0.12, 0.36)	0.338	0.999	0.0%	0.223
Non-diabetic	9	−0.12 (−0.42. 0.18)	0.428	0.783	0.0%	
Intervention						
Saffron	9	0.08 (−0.12, 0.28)	0.429	0.971	0.0%	0.456
Crocin	2	−0.23 (−1.03, 0.57)	0.569	0.714	0.0%	
Subgroup analyses of saffron on WC (cm)						
Overall effect	14	−1.50 (−2.83, −0.18)	**0.026**	<0.001	71.6%	
Trial duration (week)						
<12	8	−0.20 (−1.10, 0.70)	0.662	0.322	13.8%	0.110
≥12	6	−2.18 (−4.44, 0.07)	0.058	<0.001	77.2%	
Intervention dose (mg/day)						
<100	5	−2.68 (−4.88, −0.48)	**0.017**	0.018	66.3%	0.151
≥100	9	−0.70 (−2.25, 0.84)	0.370	<0.001	71.4%	
Health status						
Diabetic	3	−1.92 (−5.83, 1.98)	0.334	<0.001	87.7%	0.692
Non-diabetic	11	−1.09 (−2.32, 0.13)	0.080	0.006	58.0%	
Intervention						
Saffron	9	−0.80 (−2.33, 0.72)	0.304	0.001	68.6%	0.134
Crocin	2	−3.32 (−6.24, −0.40)	**0.026**	0.207	37.1%	
Subgroup analyses of saffron on FM (%)						
Overall effect	5	−0.57 (−1.57, 0.42)	0.262	0.599	0.0%	
Trial duration (week)						
<12	3	−0.42 (−1.44, 0.60)	0.422	0.762	0.0%	0.131
≥12	2	−3.05 (−6.31, 0.20)	0.066	0.726	0.0%	
Intervention dose (mg/day)						
<100	2	−0.82 (−2.35, 0.69)	0.287	0.845	0.0%	0.982
≥100	3	−0.85 (−2.57, 0.87)	0.332	0.341	10.4%	
Subgroup analyses of saffron on MDA (uM/L)						
Overall effect	7	−1.50 (−2.42, −0.57)	**0.001**	<0.001	77.4%	
Trial duration (week)						
<12	3	−2.08 (−4.17, −0.01)	0.050	<0.001	91.5%	0.304
≥12	4	−0.96 (−1.48, −0.43)	<**0.001**	0.537	0.0%	
Intervention dose (mg/day)						
<100	3	−1.32 (−3.10, 0.45)	0.145	<0.001	90.2%	0.881
≥100	4	−1.17 (−1.88, −0.47)	**0.001**	0.222	29.9%	

(Continued)

TABLE 3 (Continued)

	NO	WMD (95%CI)	*P*-value	Heterogeneity		
				P heterogeneity	I^2	P between sub-groups
Health status						
Diabetic	2	−1.02 (−2.05, −0.01)	**0.049**	0.549	0.0%	0.484
Non-diabetic	5	−1.52 (−2.48, −0.57)	**0.002**	<0.001	80.9%	
Intervention						
Saffron	5	−1.08 (−1.69, −0.46)	**0.001**	0.306	16.7%	0.650
Crocin	2	−1.62 (−3.91, 0.66)	0.163	<0.001	95.1%	
Subgroup analyses of saffron on TAC (mM/L)						
Overall effect	7	0.07 (0.01, 0.13)	**0.032**	0.003	69.9%	
Trial duration (week)						
<12	3	0.04 (−0.01, 0.10)	0.121	0.035	70.2%	0.180
≥12	4	0.16 (−0.00, 0.33)	0.056	0.005	73.5%	
Intervention dose (mg/day)						
<100	3	0.03 (−0.01, 0.07)	0.132	0.091	58.2%	0.033
≥100	4	0.21 (0.05, 0.37)	**0.009**	0.033	61.8%	
Health status						
Diabetic	2	−0.01 (−0.13, 0.11)	0.836	0.645	0.0%	0.044
Non-diabetic	5	0.14 (0.05, 0.23)	**0.001**	<0.001	83.5%	
Intervention						
Saffron	5	0.17 (0.02, 0.31)	**0.021**	0.009	67.2%	0.087
Crocin	2	0.03 (−0.01, 0.08)	0.173	0.029	79.0%	
Subgroup analyses of saffron on ALT (U/L)						
Overall effect	11	−2.16 (−4.10, −0.23)	**0.028**	<0.001	88.8%	
Trial duration (week)						
<12	4	−5.58 (−10.42, −0.75)	**0.024**	<0.001	95.3%	0.036
≥12	7	−0.17 (−1.61, 1.26)	0.811	0.099	41.8%	
Intervention dose (mg/day)						
<100	8	−3.01 (−6.20, 0.19)	0.065	<0.001	91.6%	0.197
≥100	3	−0.71 (−2.09, 0.66)	0.310	0.285	20.9%	
Health status						
Diabetic	5	0.19 (−0.95, 1.34)	0.738	0.041	59.9%	0.003
Non-diabetic	6	−5.10 (−8.41, −1.78)	**0.003**	<0.001	78.0%	
Intervention						
Saffron	5	−0.05 (−1.68, 1.57)	0.944	0.112	44.1%	0.043
Crocin	5	−4.94 (−9.38, −0.50)	**0.029**	<0.001	94.6%	
Subgroup analyses of saffron on AST(U/L)						
Overall effect	11	1.03 (−1.85, 3.92)	0.482	<0.001	96.3%	
Trial duration (week)						
<12	4	0.86 (−5.49, 7.22)	0.789	<0.001	98.6%	0.995
≥12	7	0.88 (−1.95, 3.73)	0.541	<0.001	86.3%	
Intervention dose (mg/day)						
<100	8	1.40 (−2.82, 5.64)	0.514	<0.001	96.3%	0.338
≥100	3	−0.77 (−2.18, 0.64)	0.285	0.229	30.6%	
Health status						
Diabetic	5	−1.26 (−1.85, −0.66)	<**0.001**	0.349	10.0%	0.155
Non-diabetic	6	2.46 (−2.63, 7.56)	0.342	<0.001	93.2%	

(Continued)

TABLE 3 (Continued)

	NO	WMD (95%CI)	*P*-value	Heterogeneity		
				P heterogeneity	I^2	P between sub-groups
Intervention						
Saffron	5	−0.05 (−1.82, 1.71)	0.950	0.094	46.8%	0.841
Crocin	5	−0.60 (−5.64, 4.43)	0.814	<0.001	97.5%	
Subgroup analyses of saffron on ALP(U/L)						
Overall effect	5	2.84 (−14.29, 19.97)	0.745	0.544	82.6%	
Trial duration (week)						
<12	2	−4.48 (−37.38, 28.42)	0.790	0.023	80.5%	0.510
≥12	3	7.75 (−7.86, 23.37)	0.330	0.146	48.1%	

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CI, confidence interval; CRP, c-reactive protein; FBG, fasting blood glucose; FM, fat mass; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; LDL, low-density lipoprotein; DBP, diastolic blood pressure; MDA, malondialdehyde; SBP, systolic blood pressure; TAC, total antioxidant capacity; TC, total cholesterol, TG, triglyceride; WC, waist circumference; WMD, weighted mean differences; IL-6, interleukin 6.
Subgroup analyses have been done.
$P < 0.05$ was considered a significance. Bold means significant *p*-value ($P < 0.05$).

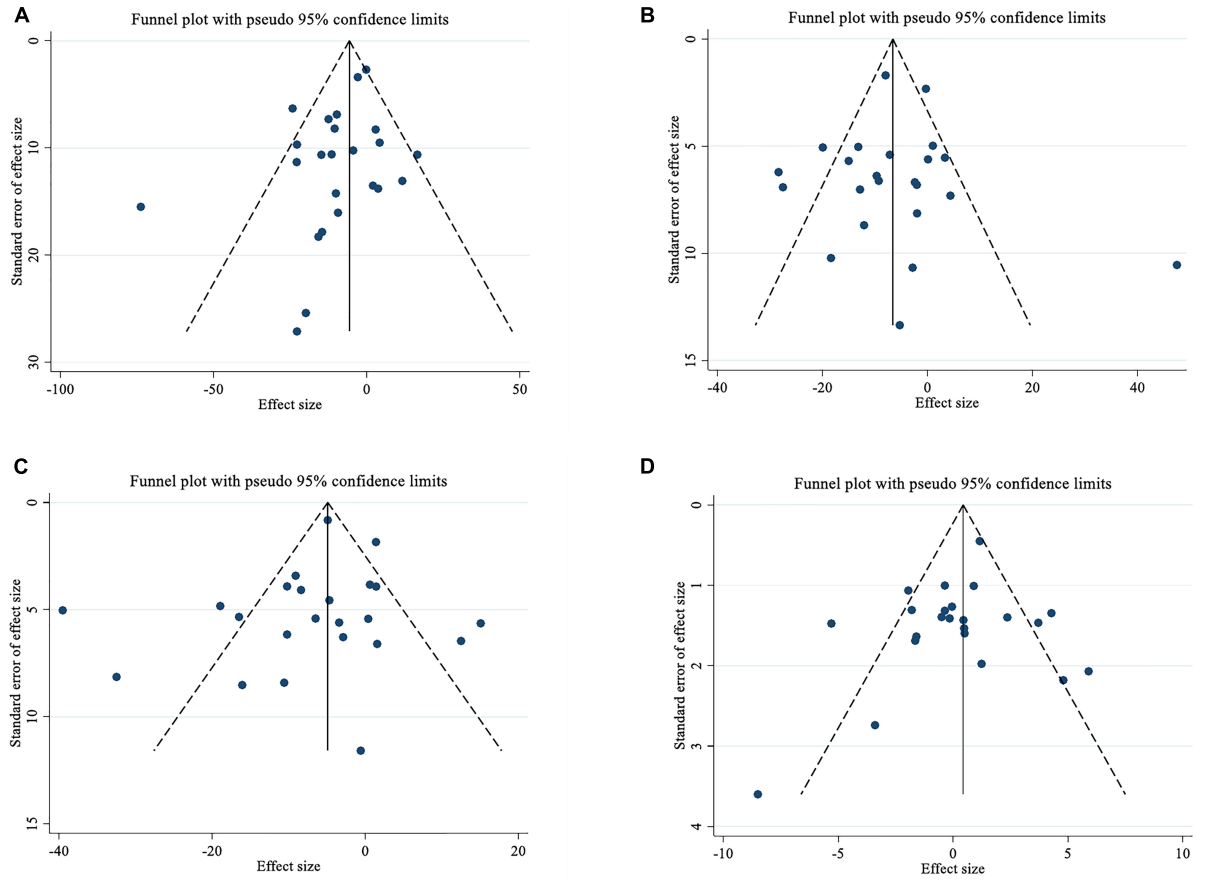
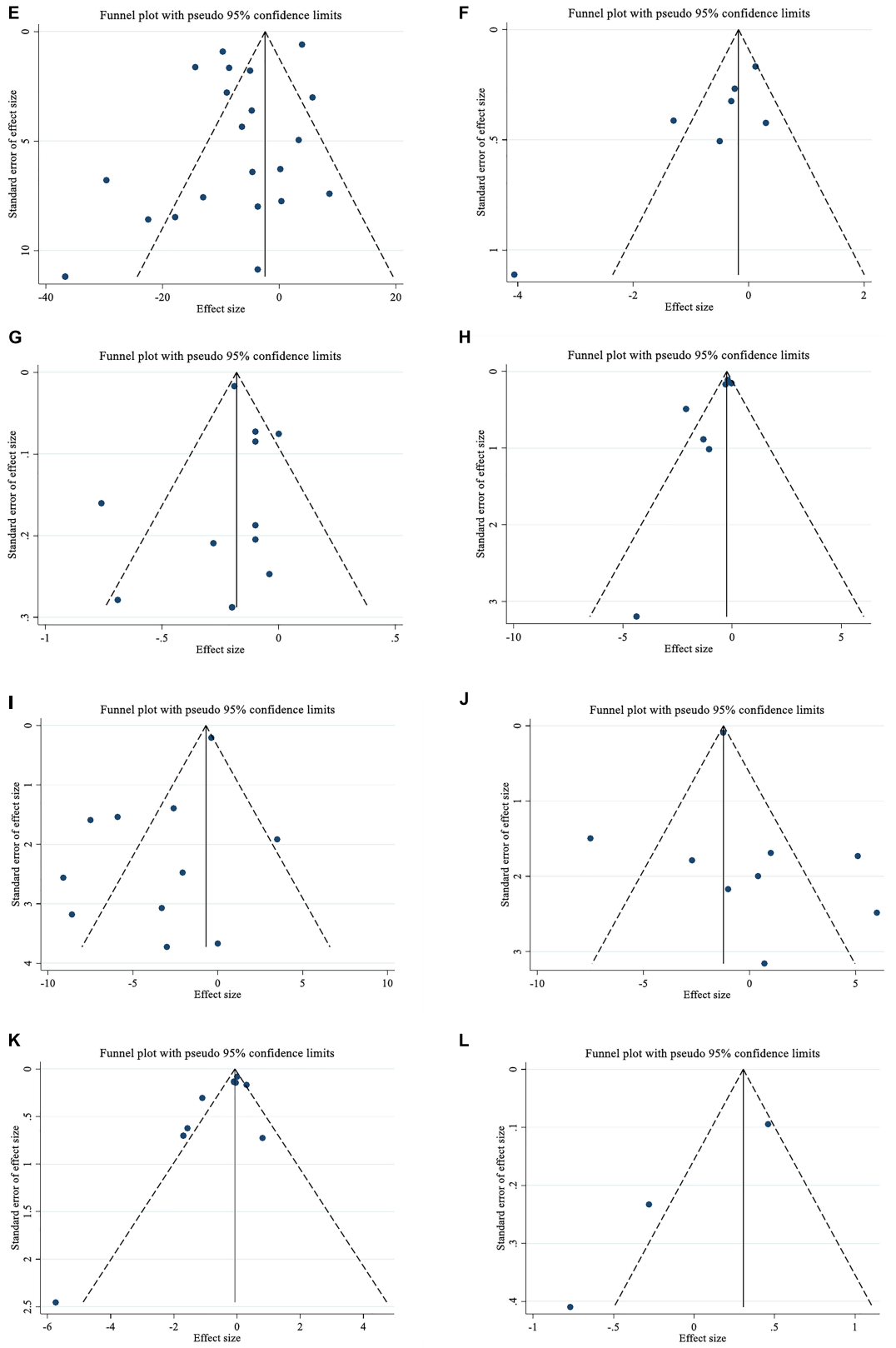


FIGURE 3
(Continued)

TX). All tests were two-tailed, and $p < 0.05$ were considered statistically significant. The pooled weighted mean difference (WMD) was calculated by a random-effects model to consider the existing heterogeneity (29) and also the Interstudy heterogeneity was performed using I-square (I^2) test (30), with values greater than 40% indicating strong heterogeneity (31).



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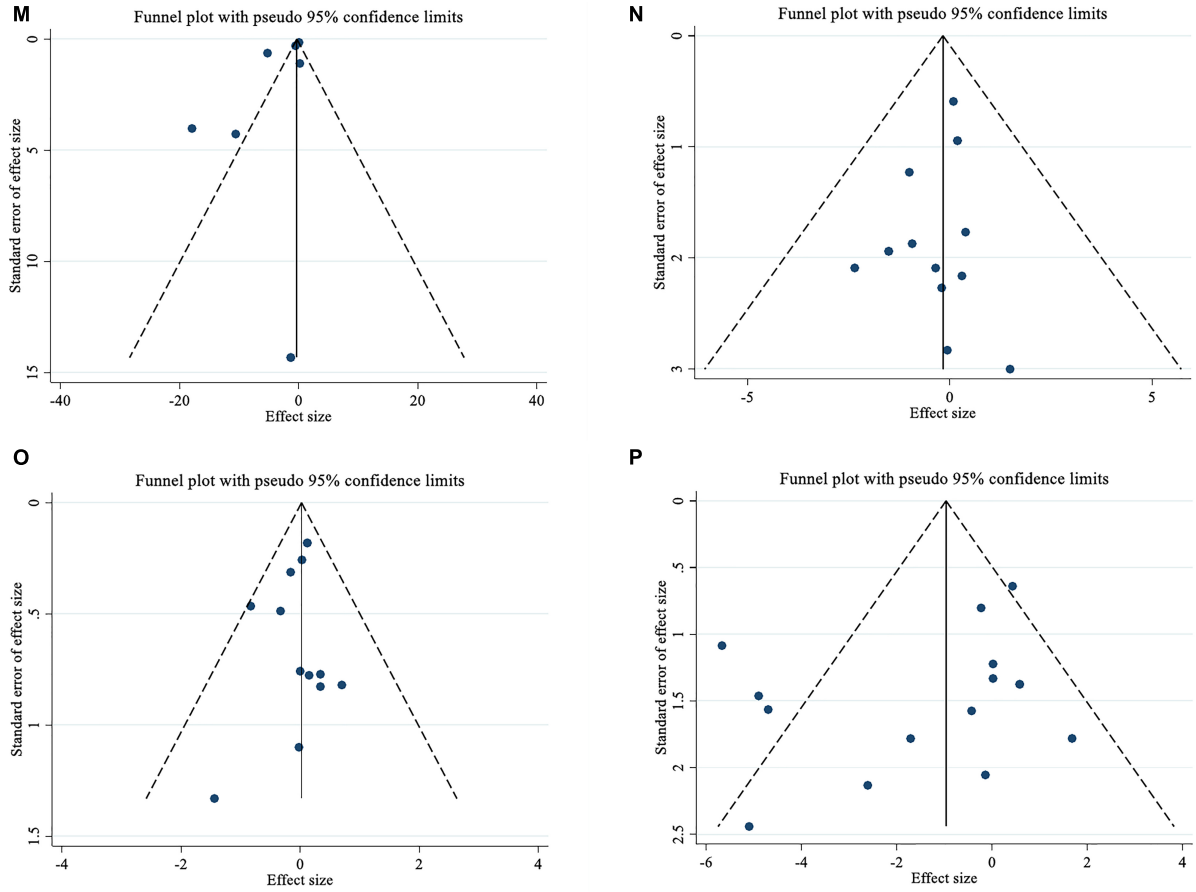

FIGURE 3
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The mean differences of required variables in both intervention and control groups at the beginning and end of the study were calculated and also the SD of these mean differences was computed using the following formula: SD = square root [(SD at baseline)^2 + (SD at the end of study)^2 − (2r × SD at baseline × SD at the end of study)] (32). All standard errors (SEs), 95 percent confidence intervals (CIs), and interquartile ranges (IQRs) to SDs which had been reported in studies, converted to SD using a method introduced by Hozo et al. and this formula: SD = SE × $\sqrt{n}$ (n = the number of individuals in each group) (33). We applied a correlation coefficient of 0.8 for r (28). To define the source of heterogeneity, a subgroup analysis was done. Subgroups were selected based on the required minimum number of studies according to the established criteria, where there should be at least 6 to 10 studies for continuous and a minimum of 4 studies for categorical subgroup variables (34, 35). The analysis of baseline TG (<150 mg/dl, ≥150 mg/dl), TC (<200 mg/dl, ≥200 mg/dl), LDL (<100 mg/dl, ≥100 mg/dl), HDL (<40 mg/dl, ≥40 mg/dl), FBG (<100 mg/dl, ≥100 mg/dl), SBP (<120 mmHg, ≥120 mmHg), DBP (<80 mmHg, ≥80 mmHg), Intervention duration (≤12 weeks, >12 weeks), and dosage of saffron (<100 mg/day, ≥100 mg/day) were based on the median values of the included studies. Other subgroup analyses were performed according to health status (diabetic, non-diabetic), and baseline BMI [normal (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obese (≥30 kg/m^2)].

The potential publication bias was reviewed by a funnel plot test (36, 37). Sensitivity analyses were conducted to explore the impact of each study on the pooled effect size. We used the trim-and-fill method to detect and adjust the publication bias's impact (38). Meta-regression was performed to evaluate the potential effects of saffron (mg/d) dosage and duration on the variables. Furthermore, we used non-linear regression for dose-response analysis between saffron supplementation and our variables.

Certainty assessment

The overall quality of evidence in all studies was assessed and summarized using the GRADE (Grading of Recommendations Assessment, Development, and Evaluation) approach (39).

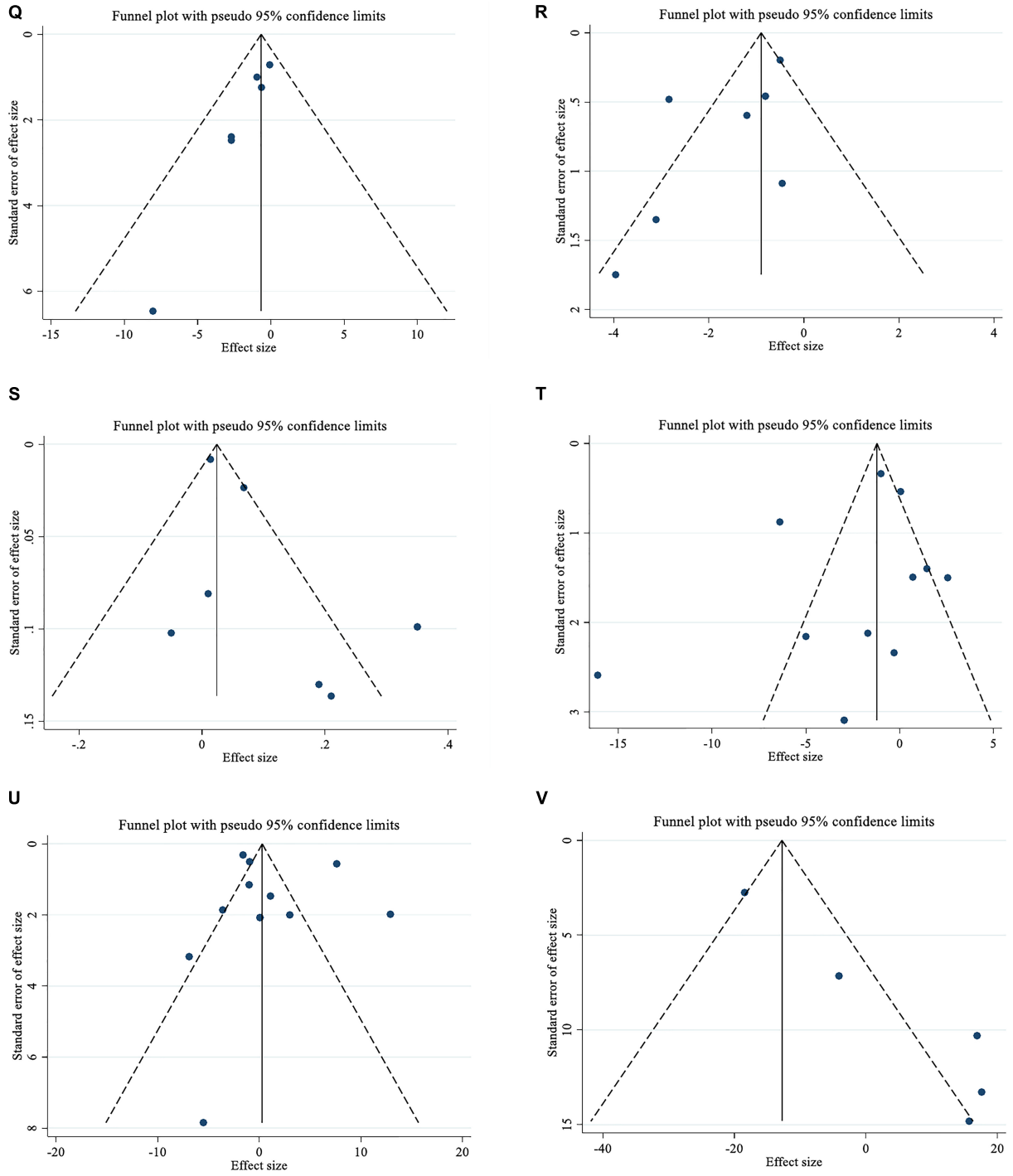

FIGURE 3
Funnel plots for the effect of saffron consumption on **(A)** TG (mg/dl); **(B)** TC (mg/dl); **(C)** LDL (mg/dl); **(D)** HDL (mg/dl); **(E)** FBG (mg/dl); **(F)** Insulin (miu/ml); **(G)** HbA1c (%); **(H)** HOMA-IR; **(I)** SBP (mmHg); **(J)** DBP (mmHg); **(K)** CRP (mg/l); **(L)**; IL-6 (pg/ml); **(M)** TNF-α (pg/ml); **(N)** weight (kg); **(O)** BMI (kg/m^2); **(P)** WC (cm); **(Q)** FM (%); **(R)** MDA (uM/L); **(S)** TAC (mM/L); **(T)** ALT (U/L); **(U)** AST (U/L) and **(V)** ALP (U/L). TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1c, hemoglobin A1c; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor; TAC, total antioxidant capacity; BMI, body mass index; WC, waist circumference; FM, fat mass; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; MDA, malondialdehyde; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, confidence interval; WMD, weighted mean difference.

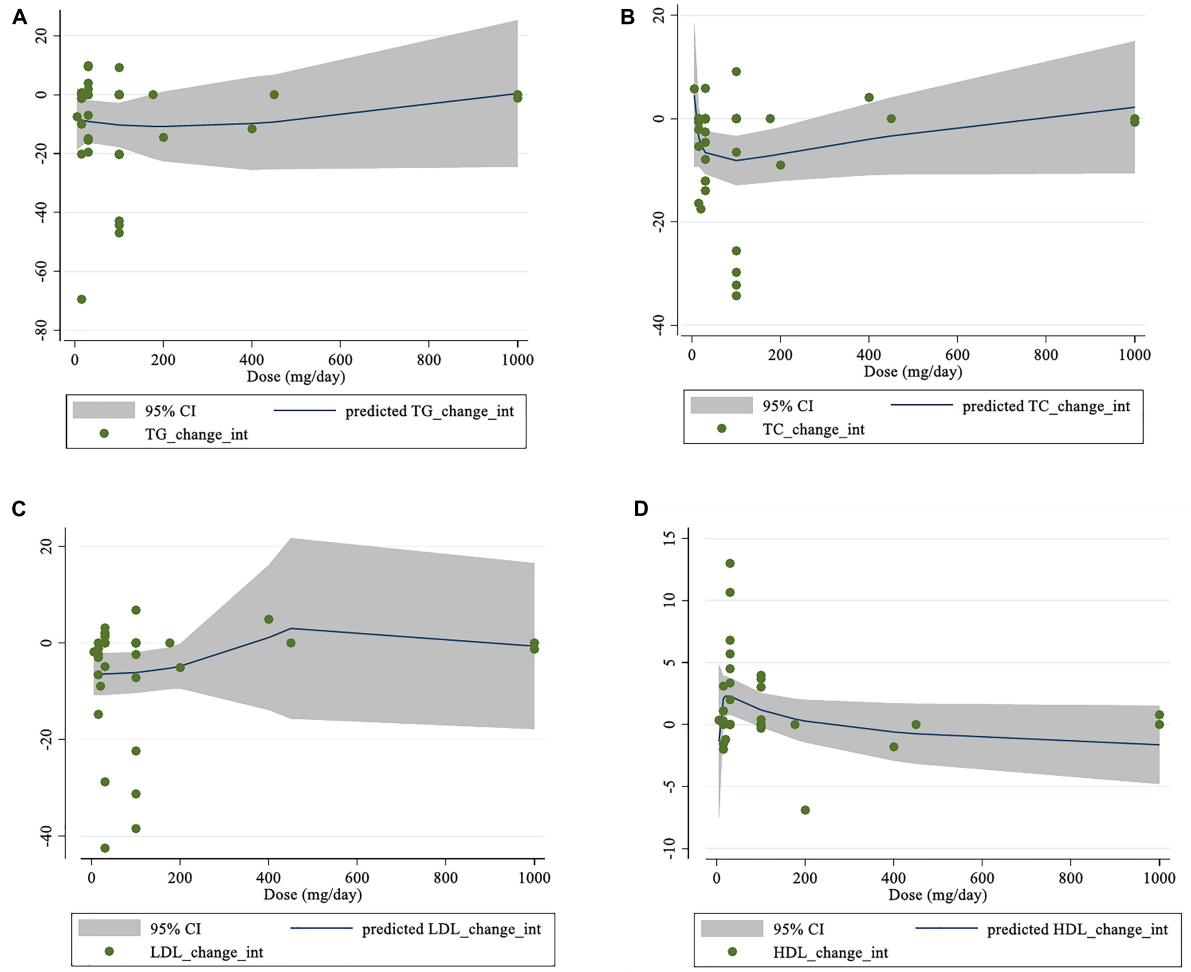

FIGURE 4
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Result

The flow of study selection

Initially, 2428 potentially eligible records were found in the literature using an electronic search [PubMed (973), ISI Web of Science (632), and Scopus (823)]. After duplicates were eliminated (n = 873) and title/abstract screening, 1500 articles were excluded because they had no relevance to the topic. As a result, 55 full-text papers were collected for a thorough evaluation. 23 of these studies had papers with no useful data (**Figure 1**). Finally, 32 trials (15, 17, 40–69), were considered eligible for the systematic review. The meta-analysis was conducted on 24 effect sizes for TG (15, 17, 40, 42, 44, 45, 47, 49, 51–55, 57, 58, 60, 61, 63, 67), 23 for TC (15, 17, 40, 42, 44, 45, 47, 49, 51–55, 57, 58, 60, 61, 63), 23 for LDL (15, 17, 40, 44, 45, 47, 49, 51–55, 57, 58, 60, 61, 63, 67), 23 for HDL (15, 17, 40, 44, 45, 47, 49, 51–55, 57, 58, 60, 61, 63, 67), 22 for FBG (15, 17, 42, 44, 45, 47, 49, 51–54, 57, 58, 60–62, 64, 66, 67), 7 for insulin (15, 44, 45, 57, 58, 64), 12 for HbA1c (15, 17, 44, 45, 54, 57, 60–62, 64), 7 for HOMA-IR (15, 45, 57, 58, 61, 64), 11 for SBP (40, 45, 48, 52, 53, 57, 63, 64, 66), 9 for DBP (40, 48, 52, 53, 57, 63, 64, 66), 10 for CRP (44, 46, 52, 57, 58, 62, 65, 68), 3 for IL-6 (53, 62, 66), 7 for TNF-α (53, 57, 59, 62, 65, 66, 68), 13 for weight (41, 44, 48–50, 52, 55, 57, 58, 60, 68, 69), 12 for BMI (44, 48–50, 52, 57, 58, 60, 63, 68, 69), 14 for WC (41, 44, 45, 48, 49, 52, 53, 55, 57, 60, 68, 69), 5 for FM% (41, 49, 57, 68), 7 for MDA (57–59, 62, 65, 68, 69), 7 for TAC (57–59, 62, 65, 68, 69), 11 for ALT (15, 42, 46, 54, 57, 61, 67, 68), 11 for AST (15, 42, 46, 54, 57, 61, 67, 68), and 5 for ALP (42, 46, 57, 61).

Study characteristics

Table 1 lists the characteristics of the trials that included a total of 1674 participants who were enrolled in the studies,

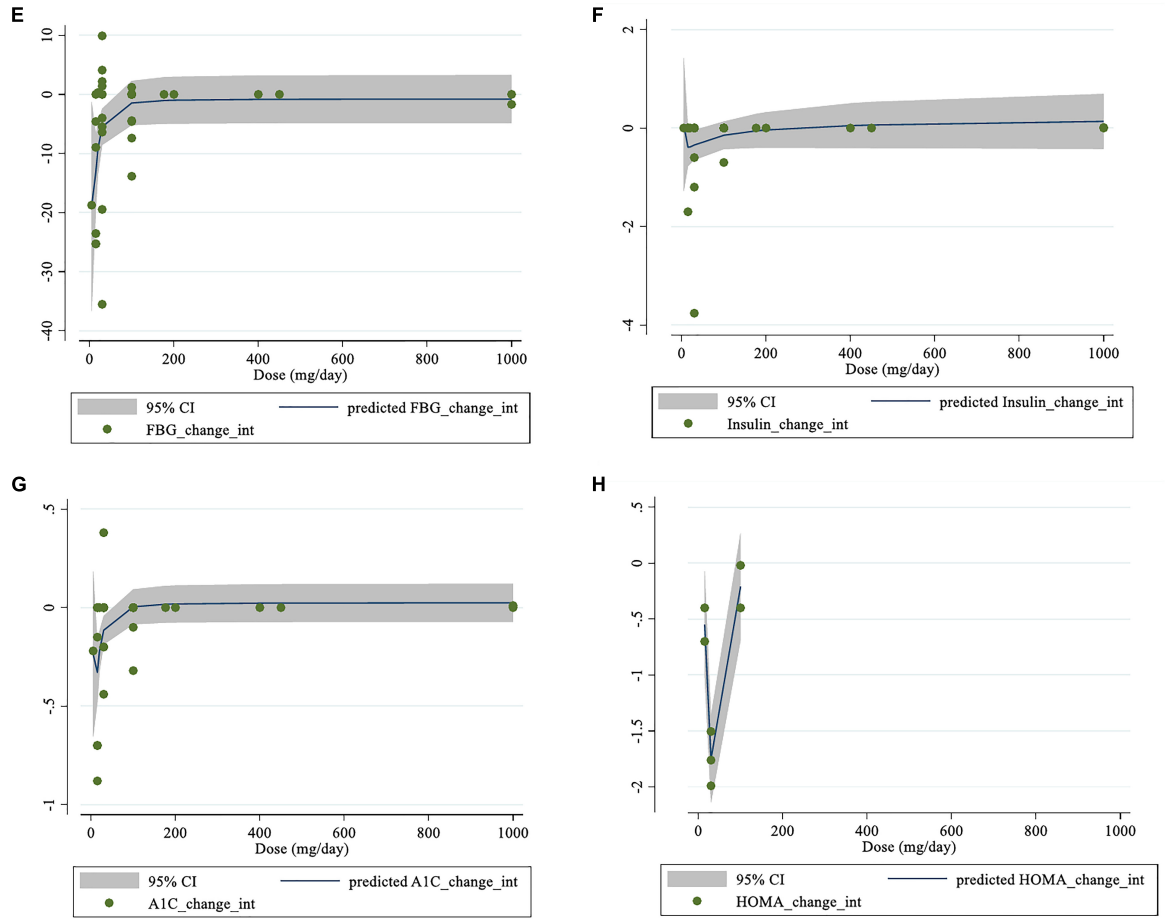

FIGURE 4
(Continued)

842 of them were assigned to the intervention group and 832 to the control group. All publications that were included in the present systematic review were randomized controlled clinical trials in design, and a parallel research design was used in all studies (15, 17, 40–69). All of these investigations were conducted in France (41) and Iran (15, 17, 40, 42–69), and were published between 2008 and 2021. The participants' average ages ranged from 27 to 57.95, while their average baseline BMIs ranged from 23.84 to 31.02 kg/m^2. The follow-up period ranged from 1 to 12 weeks. Daily supplementation dosage of saffron varied between 5 (54) and 1000 (44, 48) mg/day in these studies. In two studies (40, 54), data were reported for two different doses, hence four effect sizes were calculated. Six effect sizes were estimated as a result of the three studies (45, 46, 49) data on two varieties of saffron being provided. Only one (46) of the included studies had a male-only population, two (41, 65) had a female-only population, and the remaining trials (15, 17, 40, 42–45, 47–64, 66–69) involved mixed-gender populations.

Subjects with a variety of health conditions were all included in the study: type 2 diabetes patients (15, 17, 44, 48, 56, 57, 61, 62, 64, 66), patients with schizophrenia (45, 46), patients with major depressive disorder (42), patients with coronary artery disease (49), patients with refractory diabetic maculopathy (54), subjects with mild to moderate generalized anxiety disorder (50), individuals with metabolic syndrome (47, 51–53, 55), healthy subjects (40, 43), mildly overweight healthy women (41), patients under methadone maintenance treatment (58), overweight/obese prediabetic patients (60), patients with mild and moderate persistent allergic asthma (63), multiple sclerosis patients (59), patients with non-alcoholic fatty liver disease (67, 68), patients with active rheumatoid arthritis (65), and ulcerative colitis patients (69). All research was done in English. **Figure 2A** (TG), 2B (TC), 2C (LDL), 2D (HDL), 2E (FBG), 2F (insulin), 2G (HbA1c), 2H (HOMA-IR), 2I (SBP), 2J (DBP), 2K (CRP), 2L (IL-6), 2M (TNF-α), 2N (weight), 2O (BMI), 2P (WC), 2Q (FM%), 2R (MDA), 2S (TAC), 2T (ALT), 2U (AST), and 2V (ALP) depict the WMD and 95% CI for outcomes forest plots.

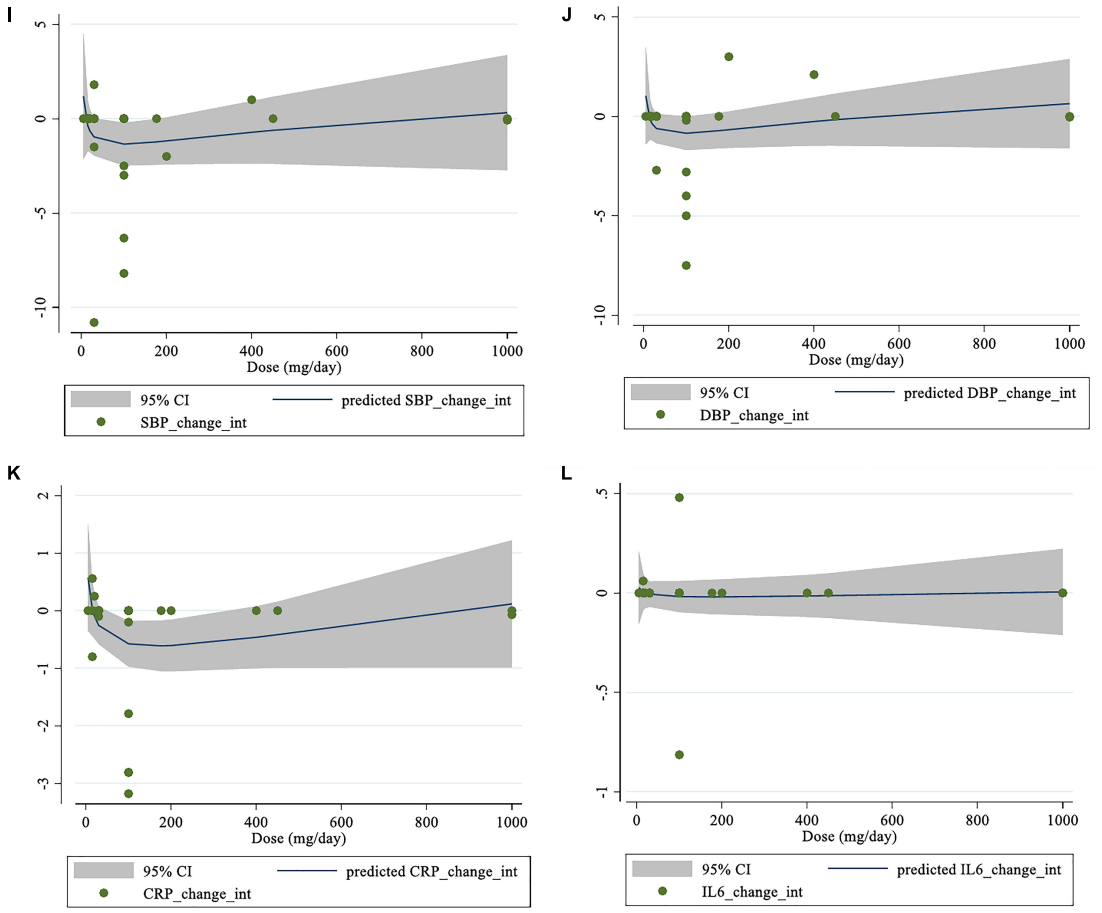

FIGURE 4
(Continued)

Adverse events

Most studies did not report specific side effects, but some side effects such as dry mouth, restlessness, anxiety, daily drowsiness, morning drowsiness (42), constipation, polydipsia, headache (17, 50), increased appetite, feet swelling, stomach ache, subconjunctival-hemorrhage, swelling, redness, and burning of the eyes (54), stomach pain (65), were reported in some studies.

Quality assessment

Out of the 32 studies examined for this review, 11 trials (15, 17, 50, 56, 57, 60–63, 68, 69) were rated as having good quality, 12 trials (40, 41, 45, 47, 49, 51, 54, 58, 64–67) as having medium quality, and 9 trials (42–44, 46, 48, 52, 53, 59, 63) as having low quality. The details of the risk of bias in studies according to the domains used by the Cochrane collaboration's tool are provided in **Table 2**.

Meta-analysis

Effect of saffron consumption on lipid profiles and subgroup analysis

In total, we pooled 24 effect sizes from 18 studies, with 1312 participants [intervention group (IG) = 655, control group (CG) = 657], to estimate the effect of saffron on plasma TG (15, 17, 40, 42, 44, 45, 47, 49, 51–55, 57, 58, 60, 61, 63, 67), and 23 effect sizes from 18 studies, for TC (15, 17, 40, 42, 44, 45, 47, 49, 51–55, 57, 58, 60, 61, 63) with 1208 participants (IG = 603, CG = 605), LDL (15, 17, 40, 44, 45, 47, 49, 51–55, 57, 58, 60, 61, 63, 67) with 1292 participants (IG = 645, CG = 647), and HDL (15, 17, 40, 44, 45, 47, 49, 51–55, 57, 58, 60, 61, 63, 67) levels with 1292 participants (IG = 645, CG = 647) (**Table 3**). According to the overall result of the meta-analysis, saffron significantly decreased serum TG (WMD = −8.81 mg/dl, 95%CI: −14.33, −3.28; P = 0.002; I^2 = 55.1%, P = 0.001; **Figure 2A**), TC (WMD = −6.87 mg/dl, 95%CI: −11.19, −2.56; P = 0.002; I^2 = 72.5%, P < 0.001; **Figure 2B**), and LDL (WMD = −6.71 mg/dl, 95%CI: −10.51, −2.91; P = 0.001;

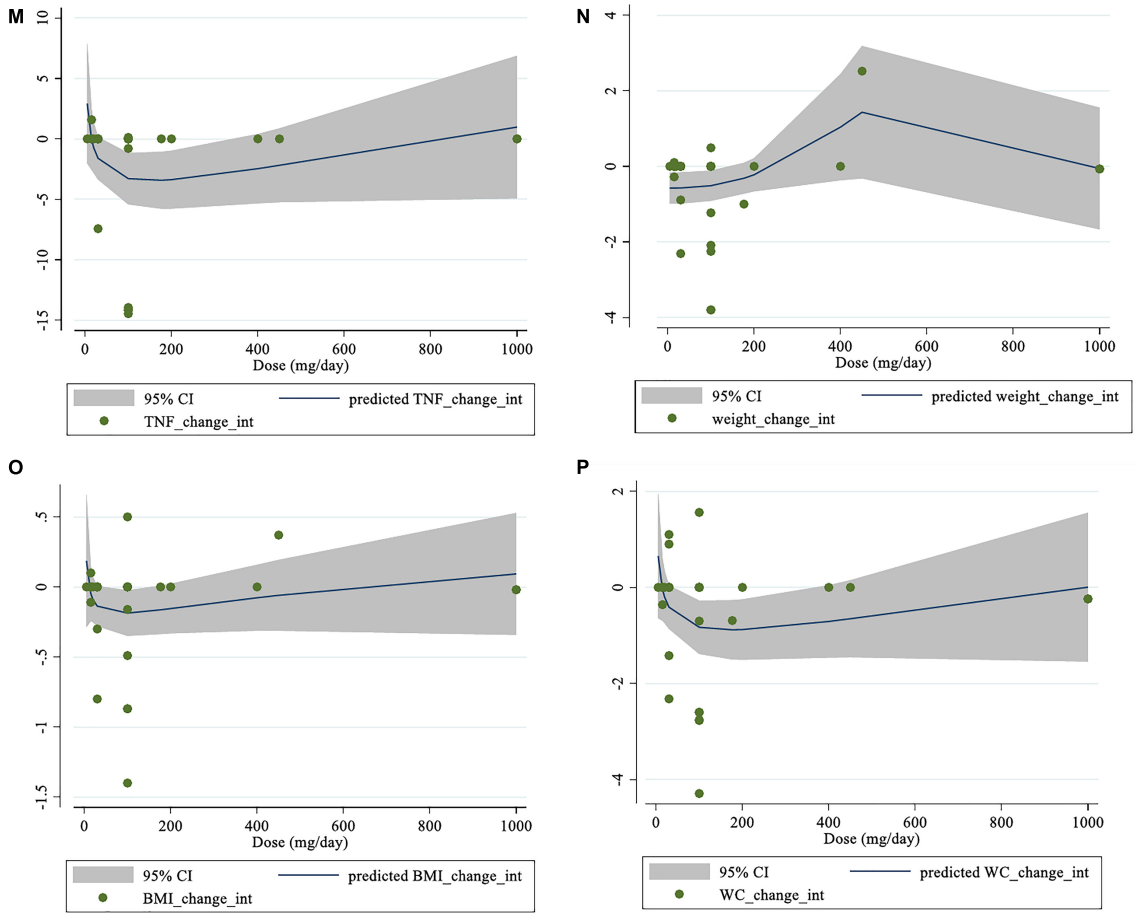

FIGURE 4
(Continued)

I^2 = 81.3%, P < 0.001; Figure 2C). However, saffron on HDL showed no significant effect (WMD = 0.21 mg/dl, 95%CI: −0.73, 1.16; P = 0.660; I^2 = 66.2%, P < 0.001; Figure 2D).

Different subgroup analyses were performed to determine the potential sources of heterogeneity among studies. The subgroup analysis revealed that saffron significantly decreased TG in studies with < 12 weeks of intervention (WMD = −11.18 mg/dl; 95%CI: −18.53, −3.84; P = 0.003), low (WMD = −7.80 mg/dl; 95%CI: −14.44, −1.16; P = 0.021) and high (WMD = −11.29 mg/dl; 95%CI: −20.69, −1.89; P = 0.019) doses of intervention, subjects with baseline TG < 150 (WMD = −4.65 mg/dl; 95%CI: −8.88, −0.43; P = 0.031), non-diabetic participants (WMD = −10.66 mg/dl; 95%CI: −17.70, −3.62; P = 0.003), and in studies that used saffron (WMD = −8.96 mg/dl; 95%CI: −16.01, −1.93; P = 0.013) as intervention, but when the baseline BMI was > 30 kg/m^2, saffron significantly increased TG levels (WMD = 17.93 mg/dl; 95%CI: −35.31, −0.55; P = 0.043). Also, the reduction in TC and LDL levels was significant in some subgroups. In studies with ≥ 12 weeks intervention duration (WMD = −11.21 mg/dl; 95%CI: −19.74, −2.69; P = 0.010), intervention dose ≥ 100 mg/day (WMD = −10.76 mg/dl; 95%CI: −18.12, −3.41; P = 0.004), studies that used crocin (WMD = −7.15 mg/dl; 95%CI: −9.98, −4.33; P < 0.001)], obese (WMD = −19.59 mg/dl; 95%CI: −33.56, −5.61; P = 0.006) and normal weight (WMD = −7.43 mg/dl; 95%CI: −10.52, −4.34; P < 0.001) participants, non-diabetic individuals (WMD = −7.77 mg/dl; 95%CI: −13.03, −2.50; P = 0.004), and subjects with baseline TC < 200 (WMD = −7.39 mg/dl; 95%CI: −13.16, −1.62; P = 0.012), TC levels were reduced. The following subgroups showed a reduction in LDL: baseline LDL ≥ 100 (WMD = −8.10 mg/dl; 95%CI: −14.38, −1.82; P = 0.011), intervention dose < 100 mg/day (WMD = −5.10 mg/dl; 95%CI: −5.10, −1.23; P = 0.010), using crocin (WMD = −6.58 mg/dl; 95%CI: −10.91, −2.25; P = 0.003) as an intervention, obese (WMD = −13.36 mg/dl; 95%CI: −23.68, −3.03; P = 0.011) and non-diabetic (WMD = −9.41 mg/dl; 95%CI: −14.17, −4.65; P < 0.001) participants (Table 3).

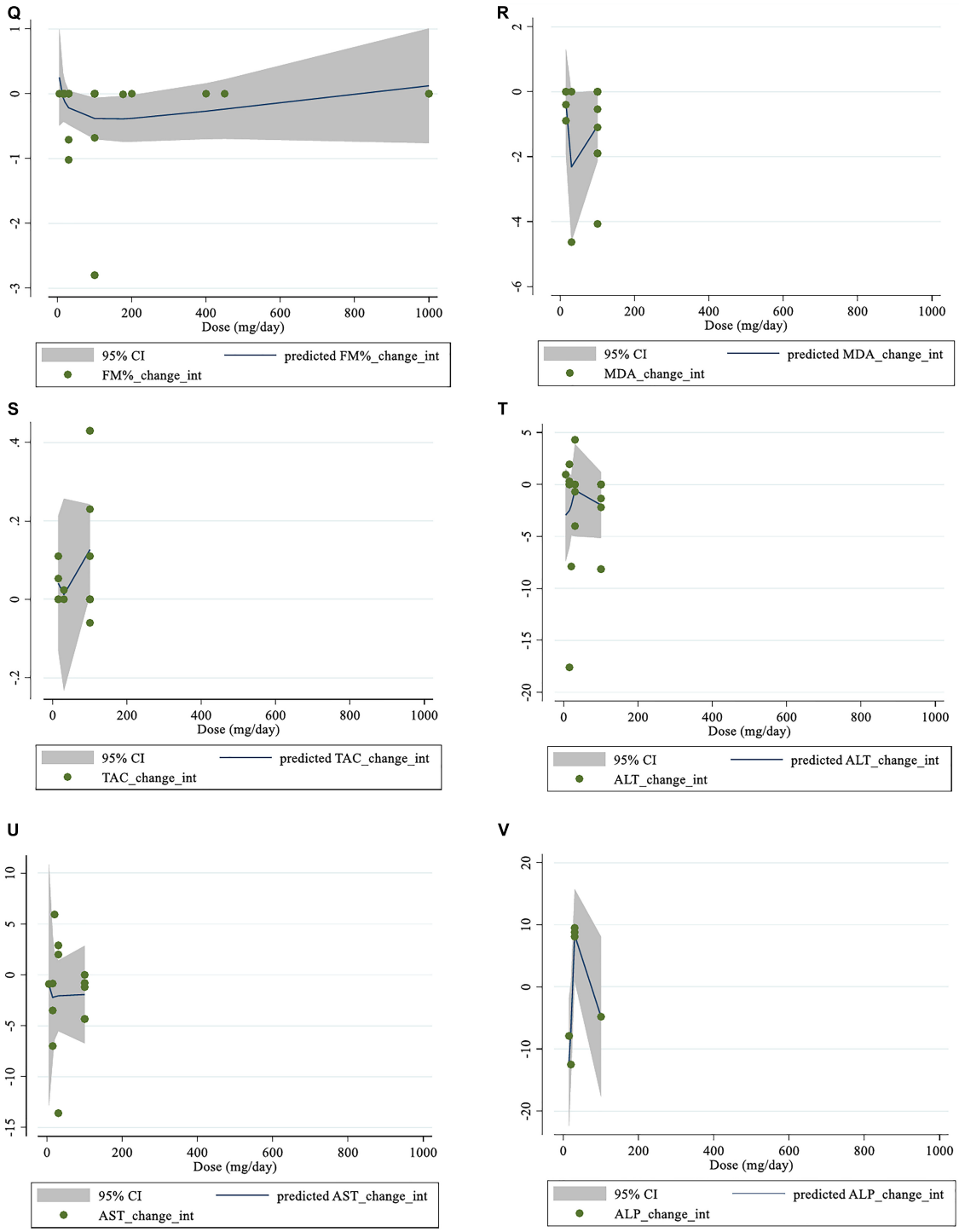

FIGURE 4
Non-linear dose-response relations between saffron consumption and absolute mean differences. Dose-response relations between dose (mg/day) and absolute mean differences **(A)** TG (mg/dl); **(B)** TC (mg/dl); **(C)** LDL (mg/dl); **(D)** HDL (mg/dl); **(E)** FBG (mg/dl); **(F)** Insulin (miu/ml); **(G)** HbA1c (%); **(H)** HOMA-IR; **(I)** SBP (mmHg); **(J)** DBP (mmHg); **(K)** CRP (mg/l); **(L)**; IL-6 (pg/ml); **(M)** TNF-α (pg/ml); **(N)** weight (kg); **(O)** BMI (kg/m^2); **(P)** WC (cm); **(Q)** FM (%); **(R)** MDA (uM/L); **(S)** TAC (mM/L); **(T)** ALT (U/L); **(U)** AST (U/L) and **(V)** ALP (U/L). TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1c, hemoglobin A1c; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor; TAC, total antioxidant capacity; BMI, body mass index; WC, waist circumference; FM, fat mass; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; MDA, malondialdehyde; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, confidence interval; WMD, weighted mean difference.

Effect of saffron consumption on glycemic profiles and subgroup analysis

Saffron's effects on FBG, insulin, HbA1c, and HOMA-IR were calculated in nineteen (22 effect sizes) (15, 17, 42, 44, 45, 47, 49, 51–54, 57, 58, 60–62, 64, 66, 67) with 1231 participants (IG = 616, CG = 615), six (7 effect sizes) (15, 44, 45, 57, 58, 64) with 422 participants (IG = 212, CG = 210), ten (12 effect sizes) with 761 participants (IG = 380, CG = 381) (15, 17, 44, 45, 54, 57, 60–62, 64) and six (7 effect sizes) (15, 45, 57, 58, 61, 64) trials with 405 participants (IG = 202, CG = 203), respectively. Pooled random-effects model analysis revealed significant decreasing effects of saffron on FBG level (WMD = −7.59 mg/dl, 95%CI: −11.88, −3.30; P = 0.001; I^2 = 93.3%, P < 0.001; **Figure 2E**), HbA1c (WMD = −0.18%, 95%CI: −0.21, −0.07; P < 0.001; I^2 = 56.9%, P = 0.008; **Figure 2G**), and HOMA-IR (WMD = −0.49, 95%CI: −0.89, −0.09; P = 0.016; I^2 = 70.8%, P = 0.002; **Figure 2H**). However, the effects of saffron on serum insulin level (WMD = −0.46 miu/ml, 95%CI: −1.00, 0.06; P = 0.088; I^2 = 75.6%, P < 0.001; **Figure 2F**) were not significant.

A subgroup analysis revealed that saffron at doses of less than 100 mg per day could considerably lower FBG levels (WMD = −10.05; 95%CI: −15.17, −4.92; P < 0.001), HbA1c (WMD = −0.21; 95%CI: −0.33, −0.09; P < 0.001) and HOMA-IR (WMD = −1.22; 95%CI: −2.42, −0.02; P = 0.045). The results also showed that saffron could significantly reduce FBG level and HbA1c, when the duration of intervention was ≥ 12 weeks (WMD $_{FBG}$ = −12.02 mg/dl; 95%CI: −18.28, −5.77; P < 0.001; WMD $_{HbA1c}$ = −0.27%; 95%CI: −0.45, −0.08; P = 0.004), and HOMA-IR, when the length of intervention was less than 12 weeks (WMD = −0.23; 95%CI: −0.41, −0.04; P = 0.013). Furthermore, both diabetic (WMD = −0.25%; 95%CI: −0.46, −0.03; P = 0.020) and non-diabetic (WMD = −0.17%; 95%CI: −0.22, −0.11; P < 0.001) participants who consumed saffron had significantly lower HbA1c levels. Saffron, however, only significantly affects FBG levels in diabetic patients (WMD = −14.08 mg/dl; 95%CI: −22.38, −5.78; P = 0.001). Additionally, the subgroup analysis showed that only the overweight patients' serum insulin concentrations (WMD = −0.00 miu/ml; 95%CI: −0.33, 0.33; P = 0.002) could be considerably lowered by saffron.

Both saffron (WMD = −7.49 mg/dl; 95%CI: −13.98, −1.01; P = 0.023) and crocin (WMD = −8.13 mg/dl; 95%CI: −15.41, −0.86; P = 0.028) consumption resulted in significantly lower FBG levels, however, only saffron consumption resulted in significantly lower HbA1c (WMD = −0.15%; 95%CI: −0.22, 0.08; P < 0.001) values (**Table 3**).

Effect of saffron consumption on blood pressure and subgroup analysis

In total, we pooled data from 9 (11 effect sizes) (40, 45, 48, 52, 53, 57, 63, 64, 66), six (7 effect sizes) with 564 participants (IG = 285, CG = 279), and 8 (9 effect sizes) with 476 participants (IG = 241, CG = 235), (40, 48, 52, 53, 57, 63, 64, 66) studies to evaluate the effect of saffron on SBP and DBP, respectively. The pooled effect demonstrated a significant reduction in SBP after consuming saffron (WMD = −3.42 mmHg, 95%CI: −5.80, −1.04; P = 0.005; I^2 = 82.5%, P < 0.001; **Figure 2I**). Saffron had not significant effect on DPB (WMD = −0.19 mmHg, 95%CI: −2.42, 2.03; P = 0.862; I^2 = 81.4%, P < 0.001; **Figure 2J**). A subgroup analysis revealed that saffron at doses of <100 mg/day (WMD = −4.97 mmHg; 95%CI: −8.06, −1.88; P = 0.002) for ≥ 12 weeks (WMD = −4.21 mmHg; 95%CI: −8.38, −0.05; P = 0.047) in patients with baseline SBP ≥ 120

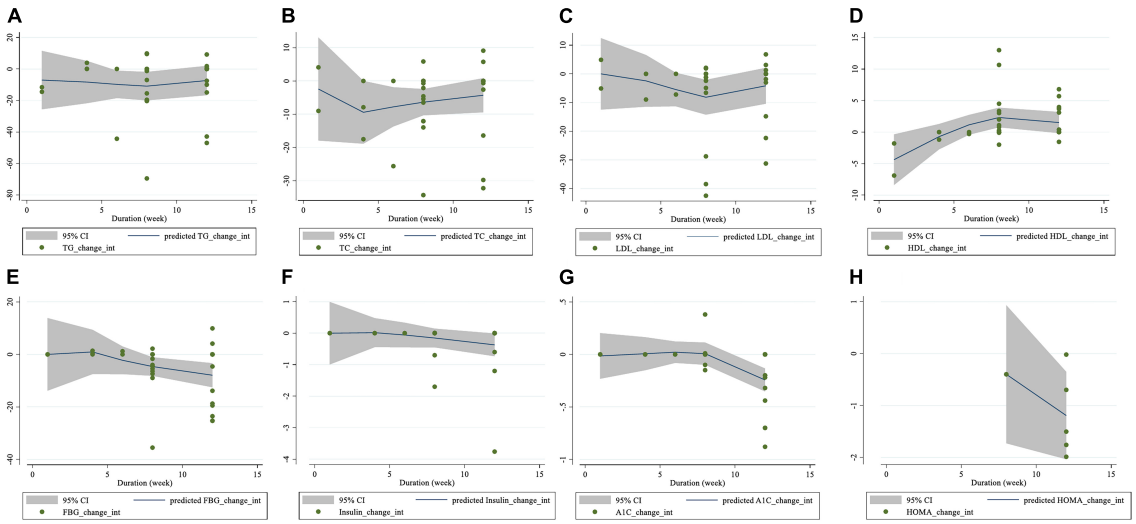

FIGURE 5
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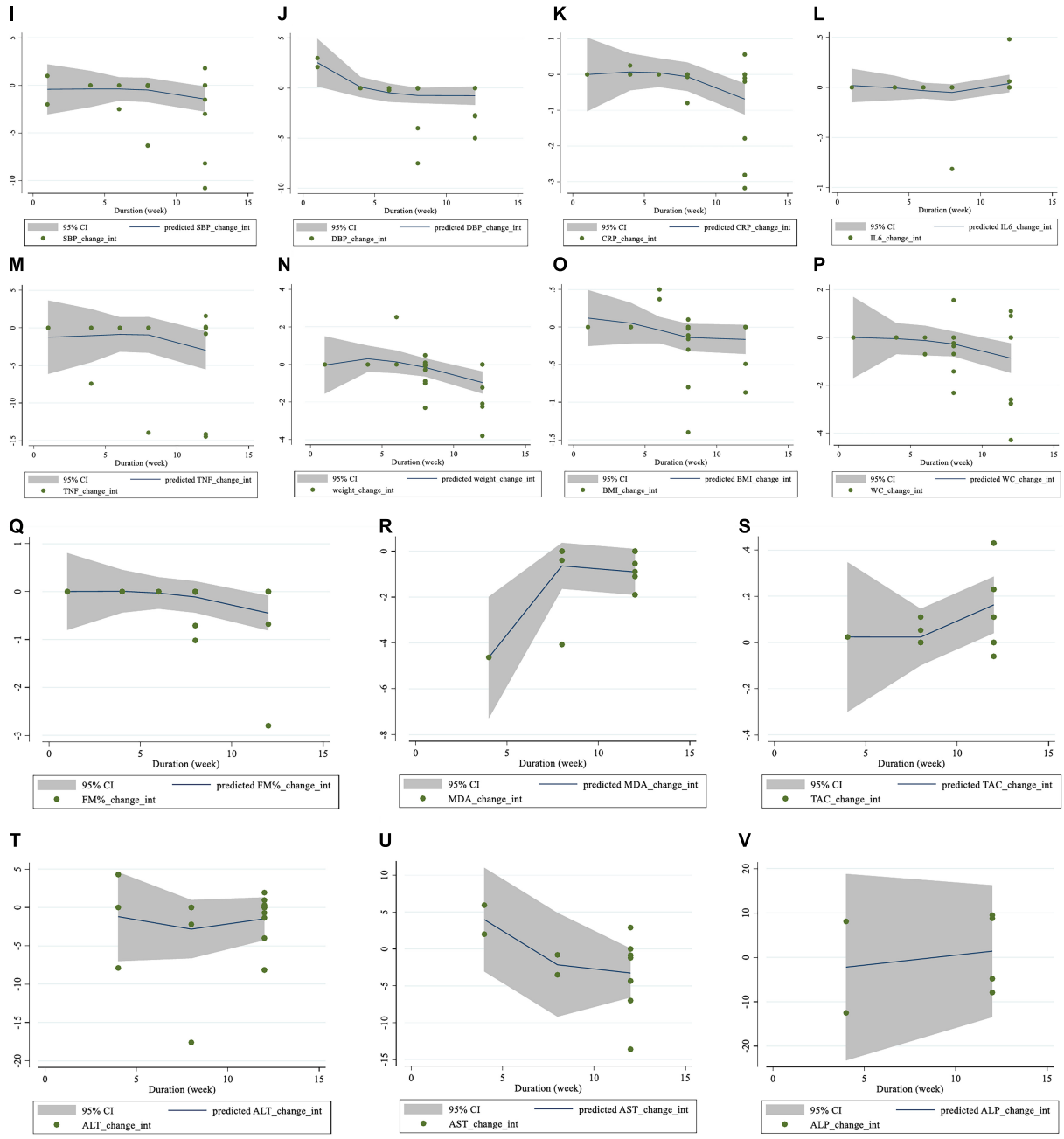

FIGURE 5
Non-linear dose-response relations between saffron consumption and absolute mean differences. Dose-response relations between duration of intervention (week) and absolute mean differences on **(A)** TG (mg/dl); **(B)** TC (mg/dl); **(C)** LDL (mg/dl); **(D)** HDL (mg/dl); **(E)** FBG (mg/dl); **(F)** Insulin (miu/ml); **(G)** HbA1c (%); **(H)** HOMA-IR; **(I)** SBP (mmHg); **(J)** DBP (mmHg); **(K)** CRP (mg/l); **(L)**; IL-6 (pg/ml); **(M)** TNF-α (pg/ml); **(N)** weight (kg); **(O)** BMI (kg/m^2); **(P)** WC (cm); **(Q)** FM (%); **(R)** MDA (uM/L); **(S)** TAC (mM/L); **(T)** ALT (U/L); **(U)** AST (U/L) and **(V)** ALP (U/L). TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1C, hemoglobin A1C; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor; TAC, total antioxidant capacity; BMI, body mass index; WC, waist circumference; FM, fat mass; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; MDA, malondialdehyde; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, confidence interval; WMD, weighted mean difference.

(WMD = −4.24 mmHg; 95%CI: −8.22, −0.25; $P = 0.037$), and when crocin (WMD = −6.41 mmHg; 95%CI: −9.12, −3.69; $P < 0.001$) was used as an intervention, could significantly lower SBP. The results also showed that saffron could significantly reduce DBP in diabetic patients (WMD = −1.23 mmHg; 95%CI: −1.41, −1.05; $P < 0.001$) (Table 3).

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TABLE 4 GRADE profile of saffron on CVD risk factors in adults.

Outcomes	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	WMD (95%CI)	Quality of evidence
TG	No serious limitation	serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−8.81 (−14.33, −3.28)	⨁◯◯◯ Very low
TC	No serious limitation	serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−6.87 (−11.19, −2.56)	⨁◯◯◯ Very low
LDL	No serious limitation	Very serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−6.71 (−10.51, −2.91)	⨁◯◯◯ Very low
HDL	No serious limitation	serious limitation [1]	No serious limitation	Serious limitation[2]	No serious limitation	0.21 (−0.73, 1.16)	⨁⨁◯◯ Low
FBG	No serious limitation	Very serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−7.59 (−11.88, −3.30)	⨁◯◯◯ Very low
Insulin	No serious limitation	Very serious limitation [1]	No serious limitation	Serious limitation[2]	serious limitation	−0.46 (−1.00, 0.06)	⨁⨁⨁◯ Moderate
HbA1c	No serious limitation	serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−0.18 (−0.21, −0.07)	⨁◯◯◯ Very low
HOMA−IR	No serious limitation	serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−0.49 (−0.89, −0.09)	⨁◯◯◯ Very low
SBP	No serious limitation	Very serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−3.42 (−5.80, −1.04)	⨁◯◯◯ Very low
DBP	No serious limitation	Very serious limitation [1]	No serious limitation	Serious limitation[2]	No serious limitation	−0.19 (−2.42, 2.03)	⨁⨁◯◯ Low
CRP	No serious limitation	Very serious limitation [1]	No serious limitation	Serious limitation[2]	No serious limitation	−0.20 (−0.46, 0.05)	⨁⨁◯◯ Low
IL-6	No serious limitation	Very serious limitation [1]	No serious limitation	Serious limitation[2]	No serious limitation	−0.12 (−0.83, 0.59)	⨁⨁◯◯ Low
TNF-α	No serious limitation	Very serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−2.54 (−4.43, −0.65)	⨁⨁◯◯ Low
Weight	No serious limitation	No serious limitation	No serious limitation	Serious limitation[2]	No serious limitation	−0.12 (−0.82, 0.58)	⨁◯◯◯ Very low
BMI	No serious limitation	No serious limitation	No serious limitation	Serious limitation[2]	No serious limitation	0.01 (−0.17, 0.21)	⨁⨁◯◯ Low
WC	No serious limitation	serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−1.50 (−2.83, −0.18)	⨁◯◯◯ Very low
FM	No serious limitation	No serious limitation	No serious limitation	Serious limitation[2]	serious limitation	−0.57 (−1.57, 0.42)	⨁⨁◯◯ Low
MDA	No serious limitation	serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−1.50 (−2.42, −0.57)	⨁◯◯◯ Very low
TAC	No serious limitation	Very serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	0.07 (0.01, 0.13)	⨁◯◯◯ Very low
ALT	No serious limitation	Very serious limitation [1]	No serious limitation	No serious limitation	No serious limitation	−2.16 (−4.10, −0.23)	⨁◯◯◯ Very low
AST	No serious limitation	Very serious limitation [1]	No serious limitation	Serious limitation[2]	No serious limitation	1.03 (−1.85, 3.92)	⨁⨁◯◯ Low
ALP	serious limitation	Very serious limitation [1]	No serious limitation	Serious limitation[2]	serious limitation	2.84 (−14.29, 19.97)	⨁⨁⨁⨁ High

[1]ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CI, confidence interval; CRP, c-reactive protein; FBG, fasting blood glucose; FM, fat mass; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; LDL, low-density lipoprotein; DBP, diastolic blood pressure; MDA, malondialdehyde; SBP, systolic blood pressure; TAC, total antioxidant capacity; TC, total cholesterol, TG, triglyceride; WC, waist circumference; IL-6, interleukin 6.
[2]There is significant heterogeneity for TG (I^2 = 55.1%), TC (I^2 = 72.5%), LDL (I^2 = 81.3%), HDL (I^2 = 66.2%), FBG (I^2 = 93.3%), Insulin (I^2 = 75.6%), HbA1C (I^2 = 56.9%), HOMA-IR (I^2 = 70.8%), SBP (I^2 = 82.5%), DBP (I^2 = 81.4%), CRP (75.4%), IL-6 (I^2 = 87.4%), TNF-α (I^2 = 92.5%), WC (I^2 = 70.3%), MDA (I^2 = 73.7%), TAC (I^2 = 77.3%), ALT (I^2 = 87.7%), and AST (I^2 = 95.9%) and ALP (I^2 = 82.6.9%).
There is no evidence of significant effects of saffron consumption on HDL, Insulin, DBP, CRP, IL-6, Weight, BMI, FM, AST, and ALP.

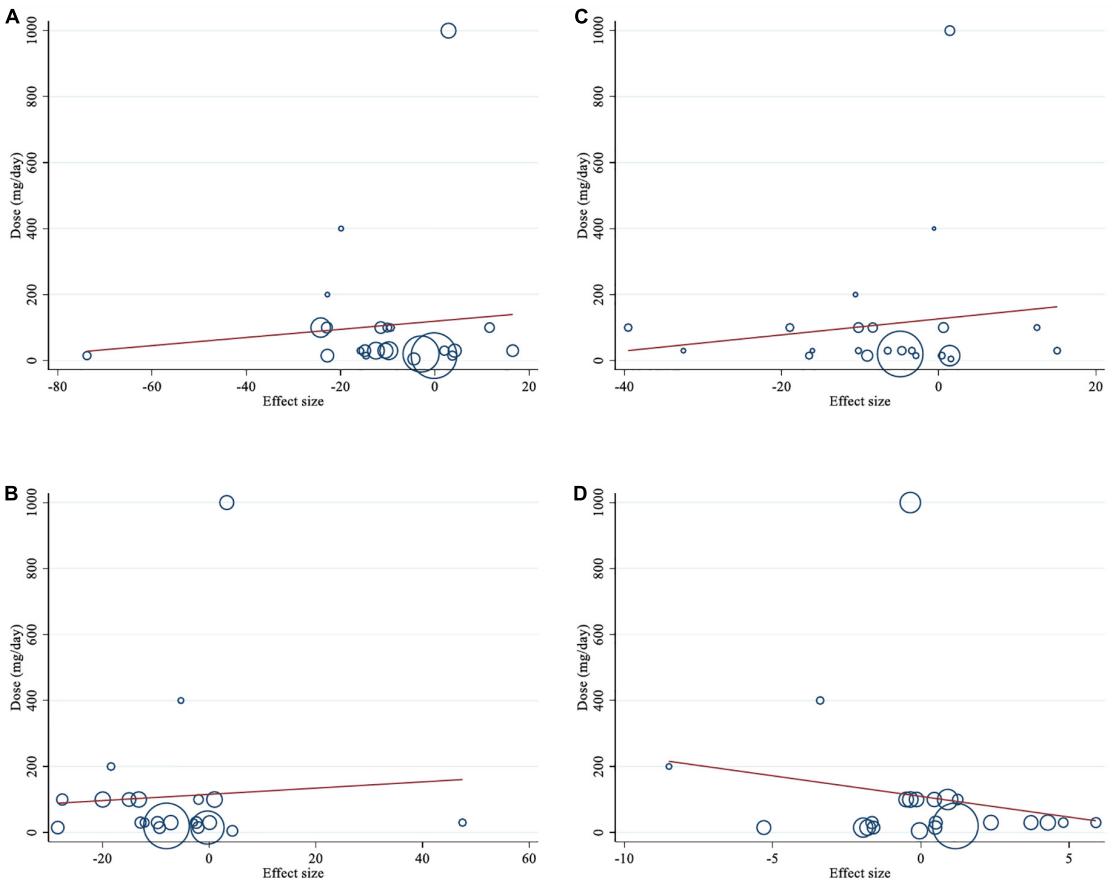

FIGURE 6
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Effect of saffron consumption on inflammatory markers and subgroup analysis

Saffron's effect on CRP, IL-6, and TNF-α was studied in 8 (10 effect sizes) (44, 46, 52, 57, 58, 62, 65, 68) with 596 participants (IG = 299, CG = 297), 3 (3 effect sizes) (53, 62, 66) with 165 participants (IG = 84, CG = 81), and 7 (7 effect sizes) studies with 427 participants (IG = 215, CG = 212), (53, 57, 59, 62, 65, 66, 68), respectively. A meta-analysis revealed that saffron significantly reduced TNF-α (WMD = −2.54 pg/ml, 95%CI: −4.43, −0.65; P = 0.008; I^2 = 93.6%, P < 0.001; **Figure 2M**), and a subgroup analysis revealed that saffron had a significant influence on TNF-α in studies with < 12 weeks of intervention (WMD = −6.22 pg/ml; 95%CI: −10.31, −2.14; P = 0.003), and high dose interventions (≥ 100 mg/day) (WMD = −4.02 pg/ml; 95%CI: −7.94, −0.10; P = 0.044).

The variations in CRP (WMD = −0.20 mg/l, 95%CI: −0.46, 0.05; P = 0.127; I^2 = 74.4%, P < 0.001; **Figure 2K**), and IL-6 (WMD = −0.12 pg/ml, 95%CI: −0.83, 0.59; P = 0.739; I^2 = 87.4%, P < 0.001; **Figure 2L**) when compared to controls were not significant. Saffron consumption, on the other hand, resulted in significant decreases in CRP in high dose interventions (≥100 mg/day) (WMD = −0.72 mg/l; 95%CI: −1.30, −0.14; P = 0.014), non-diabetic subjects (WMD = −0.52 mg/l; 95%CI: −0.94, −0.10; P = 0.015) and when saffron (WMD = −0.57 mg/l; 95%CI: −1.12, −0.02; P = 0.040) used as intervention (**Table 3**).

Effect of saffron consumption on anthropometric parameters and subgroup analysis

Changes in body weight, BMI, WC, and FM% were assessed in 12 (13 effect sizes) (41, 44, 48–50, 52, 55, 57, 58, 60, 68, 69) with 841 participants (IG = 425, CG = 416), 11 (12 effect sizes) with 785 participants (IG = 396, CG = 389) (44, 48–50, 52, 57, 58, 60, 63, 68, 69), 15 (7 effect sizes) with 884 participants (IG = 447, CG = 437) (41, 44, 45, 48, 49, 52, 53, 55, 57, 60, 68, 69), and 4 (5 effect sizes) (41, 49, 57, 68) trials with 100 participants (IG = 50, CG = 50), respectively. Overall, we observed no significantly different change in weight (WMD = −0.12 kg, 95%CI: −0.82, 0.58; P = 0.732; I^2 = 0.0%, P = 0.995; **Figure 2N**), BMI (WMD = 0.01 kg/m^2, 95%CI: −0.17,

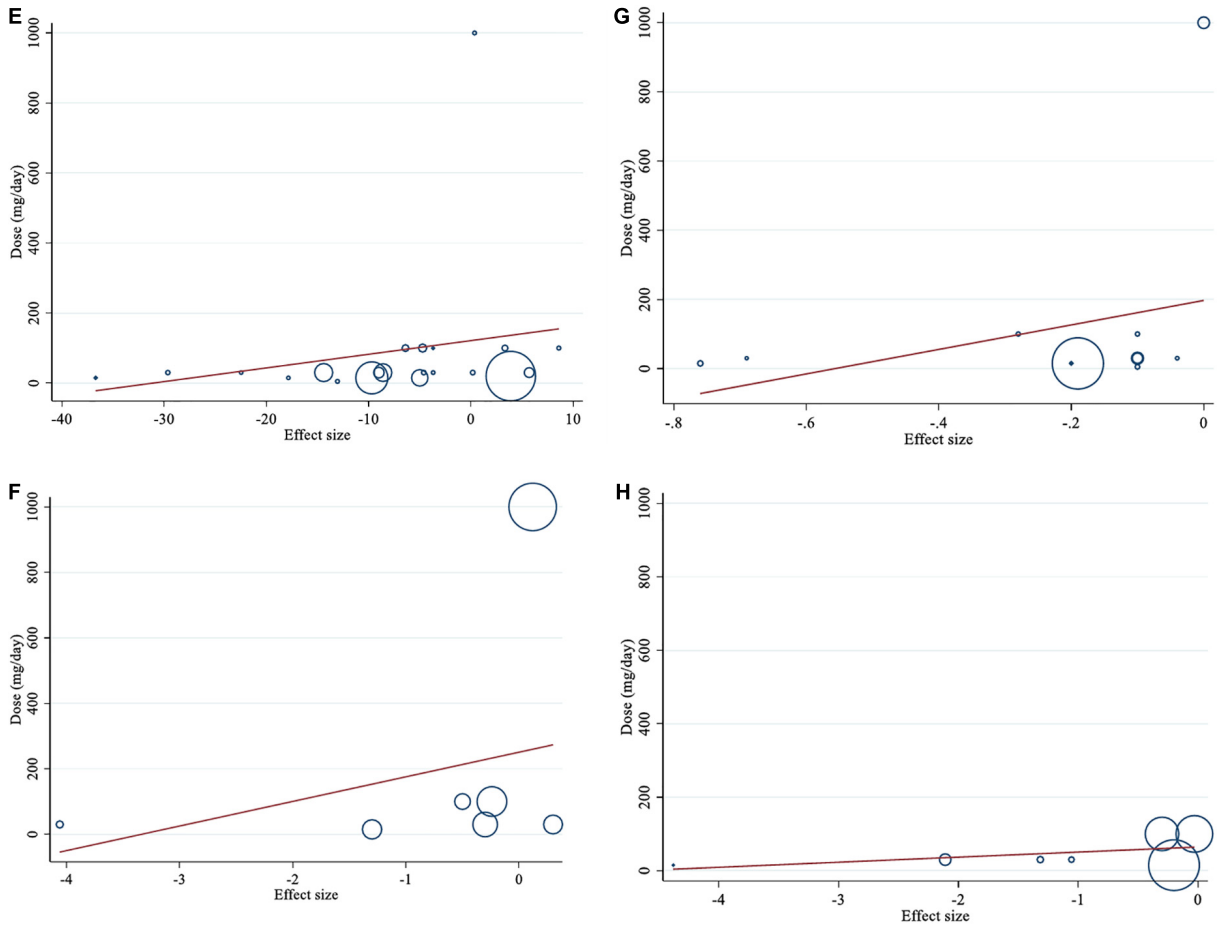

FIGURE 6
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0.21; P = 0.853; I^2 = 0.0%, P = 0.809; Figure 2O), and FM% (WMD = −0.57%, 95%CI: −1.57, 0.42; P = 0.262; I^2 = 0.0%, P = 0.599; Figure 2Q) between the intervention and control groups. However, pooled effect sizes showed a substantial decrease in WC after saffron consumption (WMD = −1.50 cm; 95%CI: −2.83, −0.18; P = 0.026; I^2 = 71.06%, P < 0.001; Figure 2P). A subgroup analysis revealed that saffron at doses of less than 100 mg per day (WMD = −2.68 cm; 95%CI: −4.88, −0.48; P = 0.017) could dramatically lower WC. Also, when crocin was used as an intervention, we saw a significant reduction in WC (WMD = −3.32 cm; 95%CI: −6.24, −0.40; P = 0.026) (Table 3).

Effect of saffron consumption on the immune system and subgroup analysis

For MDA and TAC, the study comprised and 455 subjects (IG: 230, CG: 225), and 454 subjects (IG:229, CG:225) from 7 trials (7 effect sizes) respectively (57–59, 62, 65, 68, 69), According to the meta-analysis, saffron had a decreasing effect on MDA (WMD = −1.50 uM/L, 95%CI: −2.42, −0.57; P = 0.001; I^2 = 77.4%, P < 0.001; Figure 2R) and an enhancing effect on TAC (WMD = 0.07 mM/L, 95%CI: 0.01, 0.13; P = 0.032; I^2 = 69.9%, P = 0.003; Figure 2S). The subgroup analysis revealed that MDA in both diabetic (WMD = −1.02 uM/L; 95%CI: −2.05, −0.01; P = 0.049) and non-diabetic (WMD = −1.52 uM/L; 95%CI: −2.48, −0.57; P = 0.002) patients decreased significantly after consuming saffron. Saffron also significantly raised TAC in non-diabetic subjects (WMD = 0.14 mM/L; 95%CI: 0.05, 0.23; P = 0.001), according to subgroup analysis. In studies which used saffron as an intervention (WMD $_{MDA}$ = −1.08 uM/L; 95%CI: −1.69, −0.46; P = 0.001; WMD $_{TAC}$ = 0.17 uM/L; 95%CI: 0.02, 0.31; P = 0.021), and studies with intervention doses of ≥100 (WMD $_{MDA}$ = −1.17 uM/L; 95%CI: −1.88, −0.47; P = 0.001; WMD $_{TAC}$ = 0.21 mM/L; 95%CI: 0.05, 0.37; P = 0.009) saffron significantly reduced MDA while increasing TAC. In studies with interventions lasting more than 12 weeks (WMD = −0.96 uM/L; 95%CI: −1.48, −0.43; P < 0.001), saffron dramatically decreased MDA, according to additional subgroup analyses (Table 3).

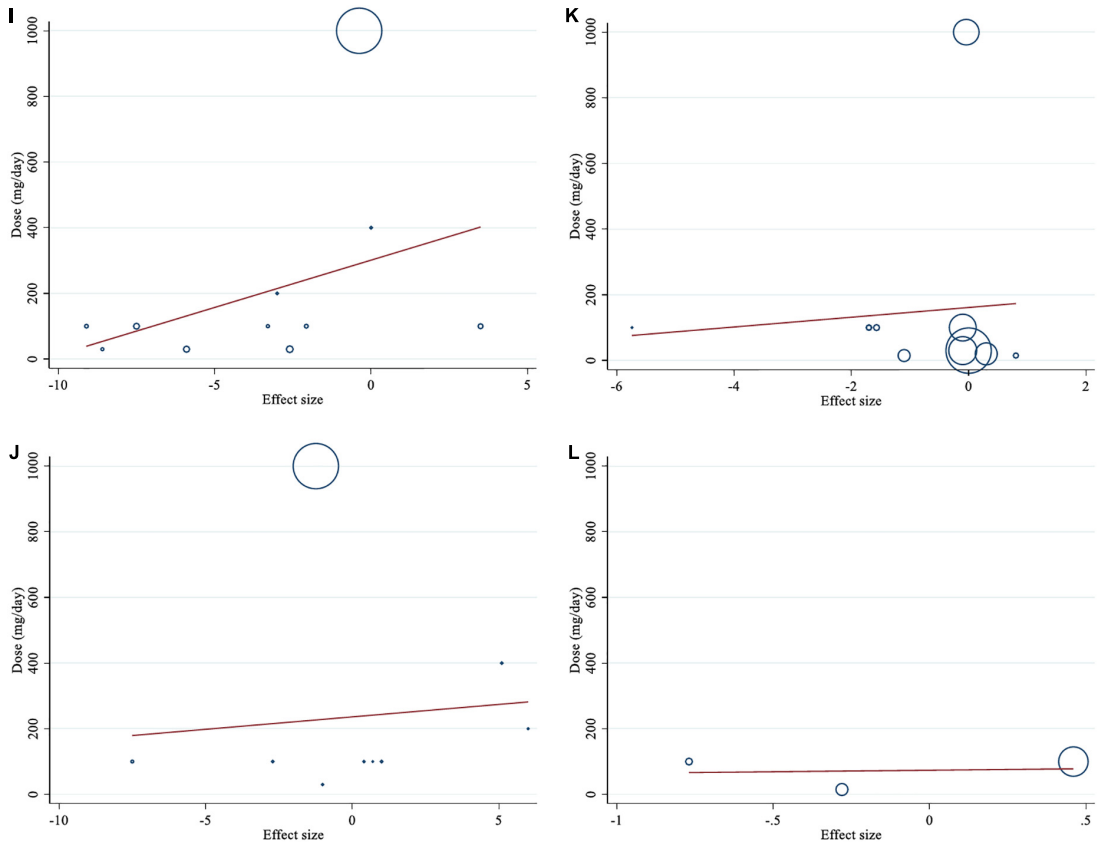

FIGURE 6
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Effect of saffron consumption on liver enzymes and subgroup analysis

Saffron significantly affected ALT (WMD = −2.16 U/L, 95%CI: −4.10, −0.23; P = 0.028; I^2 = 88.8%, P < 0.001; Figure 2T), according to the findings of a pooled analysis of 8 studies (11 effect sizes) (15, 42, 46, 54, 57, 61, 67, 68) with 637 participants (IG = 318, CG = 319). However, the results of a pooled analysis of 8 (12 effect sizes) (15, 42, 46, 54, 57, 61, 67, 68) with 637 participants (IG = 318, CG = 319) and 4 (5 effect sizes) (42, 46, 57, 61) trials with 296 participants (IG = 148, CG = 148), revealed no significant effect of saffron on AST (WMD = 1.03 U/L, 95%CI: −1.85, 3.92; P = 0.482; I^2 = 96.3%, P < 0.001; Figure 2U) and ALP (WMD = 2.84 U/L, 95%CI: −14.29, 19.97; P = 0.745; I^2 = 82.6%, P = 0.544; Figure 2V) respectively. The subgroup analysis revealed that saffron results in 5.58 (U/L) and 5.10 (U/L) reductions in ALT compared to controls in studies with a duration < 12 weeks (WMD = −5.58 U/L; 95%CI: −10.42, −0.75; P = 0.024) and non-diabetic patients (WMD = −5.10 U/L; 95%CI: −8.41, −1.78; P = 0.003), respectively. Crocin (WMD = −4.94 U/L; 95%CI: −9.38, −0.50; P = 0.029), when taken as an intervention, could dramatically lower AST. Additionally, after consuming saffron, the overweight individuals' AST levels (WMD = −1.26 U/L; 95%CI: −1.85, −0.66; P < 0.001) significantly decreased (Table 3).

Non-linear dose-response analysis

There was evidence of a non-linear relationship between saffron dosage and HDL (coefficients = 5.95, P = 0.049; Figure 4D), HOMA-IR (coefficients = 7.69, P = 0.002; Figure 4H), weight (coefficients = 0.06, P = 0.036; Figure 4N), and ALP (coefficients = 1.78, P = 0.016; Figure 4V). In addition, the non-linear dose-response analysis revealed a non-linear relationship between saffron dosage and FBG (coefficients = −0.67, P = 0.011; Figure 4E), HbA1c (coefficients = −0.02, P = 0.002; Figure 4G), and TNF-α (coefficients = −3.56, P = 0.042; Figure 4M).

Moreover, there was a non-linear relationship between the length of the intervention and HDL (coefficients = 3.20, P = 0.007; Figure 5D) and DBP (coefficients = −1.85, P = 0.033; Figure 5J). However, there was no evidence of a non-linear association between the duration of the intervention and other outcomes.

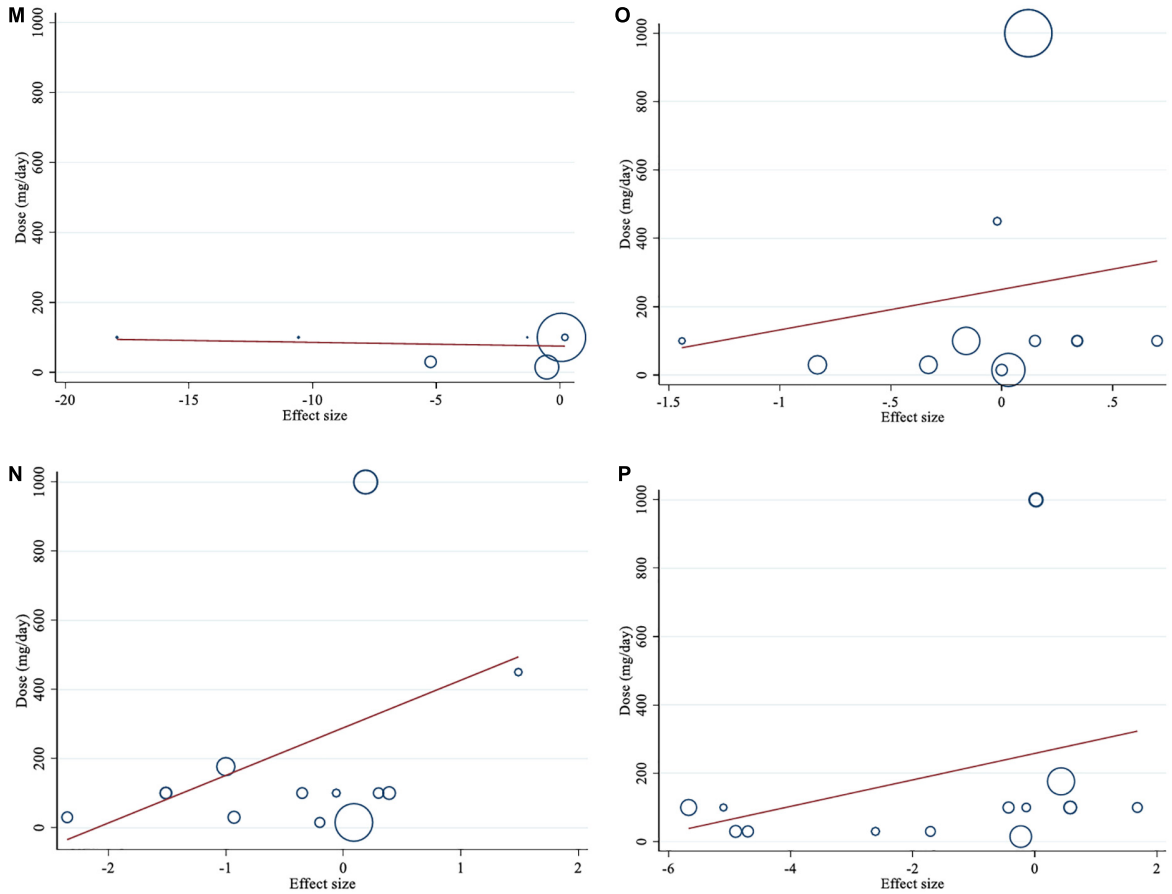

FIGURE 6
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Meta-regression analysis

Meta-regression analysis was used to assess how the dosage of saffron and the length of the intervention altered lipid profiles, glycemic profiles, blood pressure, inflammatory markers, anthropometric parameters, the immune system, and liver enzymes. Linear association was found between FBG and duration of intervention (coefficients = −0.29, P = 0.003; **Figure 7E**). There was no statistically significant linear association between the length and dosage of the intervention and changes in other outcomes (**Figures 6 A–V**, **7 A–D, F–V**).

Sensitivity analysis

Findings regarding saffron consumption and lipid profiles, blood pressure, FBG, HbA1c, HOMA-IR, IL-6, weight, BMI, FM%, MDA, TAC, AST, and ALP remained robust in the sensitivity analysis. However, the significant effect of saffron on TNF-α, WC, and ALT disappeared when excluding the studies done by Ghiasian et al. (59) (WMD = −0.86, 95%CI: −2.19, 0.46) and Hamidi et al. (65) (WMD = −1.72, 95%CI: −3.45, 0.01) for TNF-α; Fadai et al. (A) (45) (WMD = −1.11, 95%CI: −2.36, 0.13), Fadai et al. (B) (45) (WMD = −1.08, 95%CI: −2.31, 0.14), Abedimanesh et al. (B) (49) (WMD = −1.29, CI 95%: −2.58, 0.01), Kermani et al. (52) (WMD = −1.18, 95%CI: −2.44, 0.07), and Ebrahimi et al. (57) (WMD = −0.87, 95%CI: −1.90, 0.16) for WC; Mohamadpour et al. (43) (WMD = −1.51, 95%CI: −3.20, 0.16), Parsi et al. (67) (WMD = −1.24, 95%CI: −2.83, 0.35), and Tajaddini et al. (15) (WMD = −2.52, 95%CI: −5.11, 0.06) for ALP. Sensitivity analysis indicated that exclusion of the articles done by Mohamadpour et al. (43) (WMD = −0.36, 95%CI: −0.65, −0.06), Azimi et al. (44) (WMD = −0.33, 95%CI: −0.66, −0.01), Mousavi et al. (A) (46) (WMD = −0.38, 95%CI: −0.74, −0.02), Mousavi et al. (B) (46) (WMD = −0.33, 95%CI: −0.66, −0.00), Ebrahimi et al. (57) (WMD = −0.33, 95%CI: −0.66, −0.00), and Shahbazian et al. (62) (WMD = −0.29, 95%CI: −0.57, −0.02) altered the overall effect of saffron on CRP concentration to a significant value. Additionally, the total effect of saffron on insulin was significantly changed by excluding the study by Fadai et al. (A) (45) (WMD = −0.61, 95%CI: -1.21, -0.01).

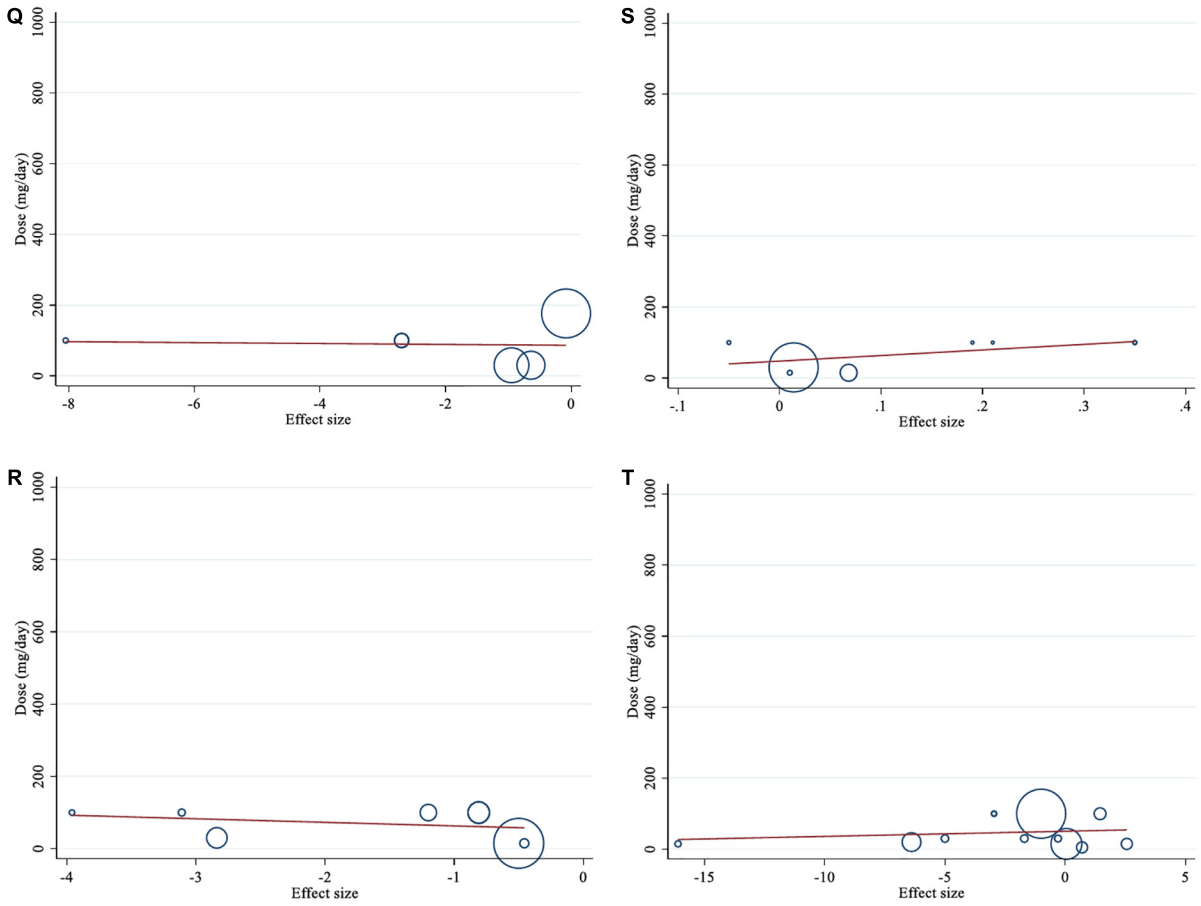

FIGURE 6
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GRADE assessment

The GRADE system is used to grade the quality of the evidence by the outcome in Table 4. For TG, TC, LDL, FBG, HbA1c, HOMA-IR, SBP, weight, WC, MDA, TAC, and ALT, the quality of the evidence was very low. Additionally, the HDL, DBP, CRP, IL-6, TNF-, BMI, FM%, and AST evidence quality was low. Only for insulin and ALP were the evidence quality levels moderate and high, respectively.

Publication bias

There was no evidence of publication bias among the included articles assessing the effect of saffron on TG (P_{Egger} = 0.077, P_{Begg} = 0.413; Figure 3A), TC (P_{Egger} = 0.950, P_{Begg} = 0.916; Figure 3B), LDL (P_{Egger} = 0.410, P_{Begg} = 0.958; Figure 3C), HDL (P_{Egger} = 0.352, P_{Begg} = 0.958; Figure 3D), FBG (P_{Egger} = 0.0.074, P_{Begg} = 1.00; Figure 3E), HbA1c (P_{Egger} = 0.866, P_{Begg} = 0.273; Figure 3G), HOMA-IR (P_{Egger} = 0.059, P_{Begg} = 0.133; Figure 3H), DBP (P_{Egger} = 0.529, P_{Begg} = 0.348; Figure 3J), IL-6 (P_{Egger} = 0.108, P_{Begg} = 1.00; Figure 3L), TNF-α (P_{Egger} = 0.130, P_{Begg} = 1.00; Figure 3M), weight (P_{Egger} = 0.183, P_{Begg} = 0.702; Figure 3N), BMI (P_{Egger} = 0.382, P_{Begg} = 0.542; Figure 3O), WC (P_{Egger} = 0.238, P_{Begg} = 0.216; Figure 3P), MDA (P_{Egger} = 0.105, P_{Begg} = 0.138; Figure 3R), TAC (P_{Egger} = 0.050, P_{Begg} = 0.621; Figure 3S), ALT (P_{Egger} = 0.403, P_{Begg} = 0.131; Figure 3T), and AST (P_{Egger} = 0.829, P_{Begg} = 784; Figure 3U) levels, using Begg's test and Egger's tests. But among articles evaluating the impact of saffron on insulin (P_{Egger} = 0.041, P_{Begg} = 0.072; Figure 3F), SBP (P_{Egger} = 0.042, P_{Begg} = 1.00; Figure 3I), CRP (P_{Egger} = 0.023, P_{Begg} = 0.697; Figure 3K) FM% (P_{Egger} = 0.001, P_{Begg} = 0.060; Figure 3Q), and ALP (P_{Egger} = 0.004, P_{Begg} = 0.462; Figure 3V), publication biases were found.

Discussion

The present study is a comprehensive systematic review and dose-response meta-analysis of the effects of saffron on all CVD risk factors. The results of 32 RCT with 1674

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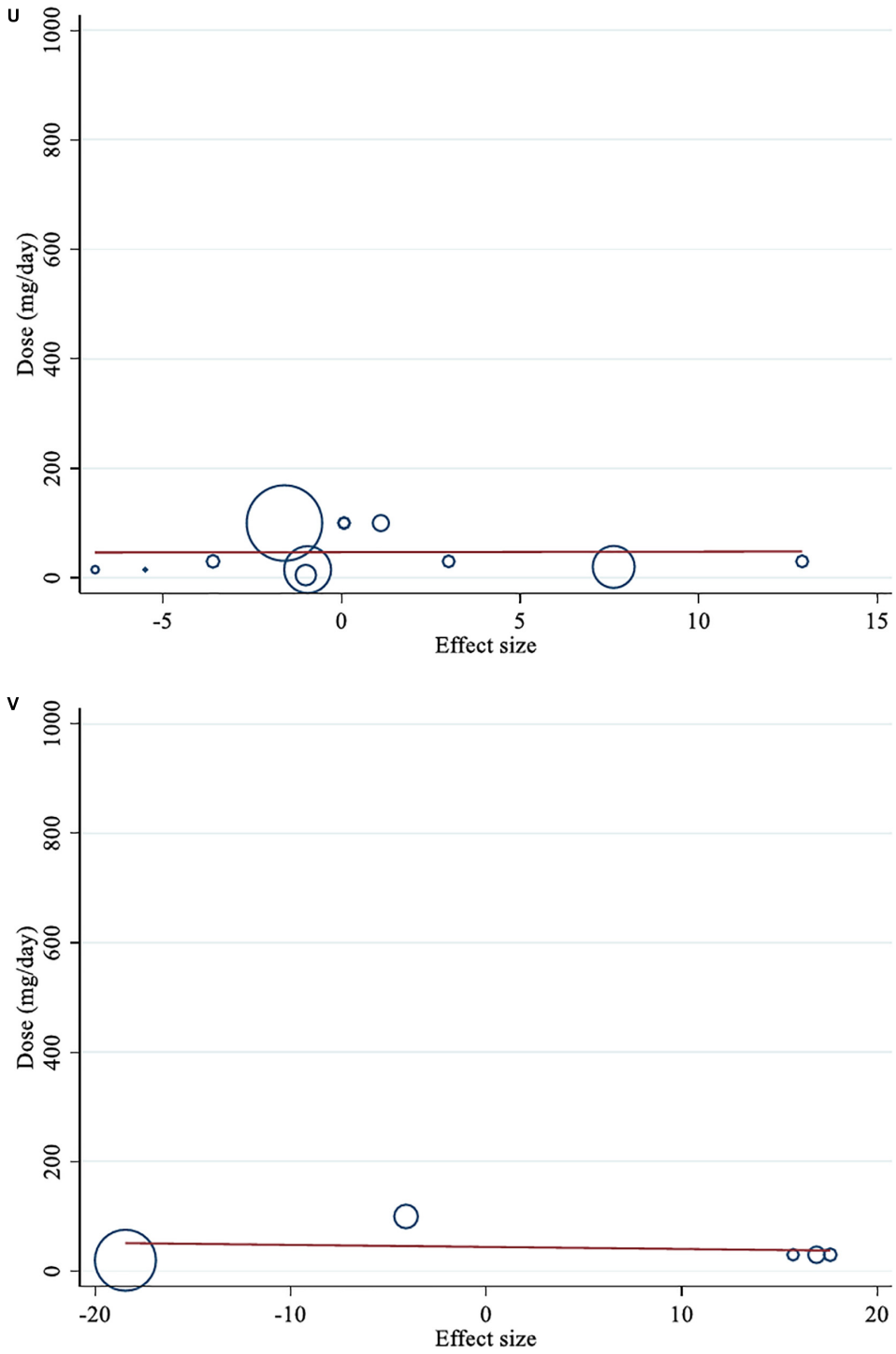

FIGURE 6
Linear dose-response relations between saffron consumption and absolute mean differences. Dose-response relations between dose (g/day) and absolute mean differences **(A)** TG (mg/dl); **(B)** TC (mg/dl); **(C)** LDL (mg/dl); **(D)** HDL (mg/dl); **(E)** FBG (mg/dl); **(F)** Insulin (miu/ml); **(G)** HbA1c (%); **(H)** HOMA-IR; **(I)** SBP (mmHg); **(J)** DBP (mmHg); **(K)** CRP (mg/l); **(L)**; IL-6 (pg/ml); **(M)** TNF-α (pg/ml); **(N)** weight (kg); **(O)** BMI (kg/m^2); **(P)** WC (cm); **(Q)** FM (%); **(R)** MDA (uM/L); **(S)** TAC (mM/L); **(T)** ALT (U/L); **(U)** AST (U/L) and **(V)** ALP (U/L). TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1C, hemoglobin A1C; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor; TAC, total antioxidant capacity; BMI, body mass index; WC, waist circumference; FM, fat mass; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; MDA, malondialdehyde; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, confidence interval; WMD, weighted mean difference.

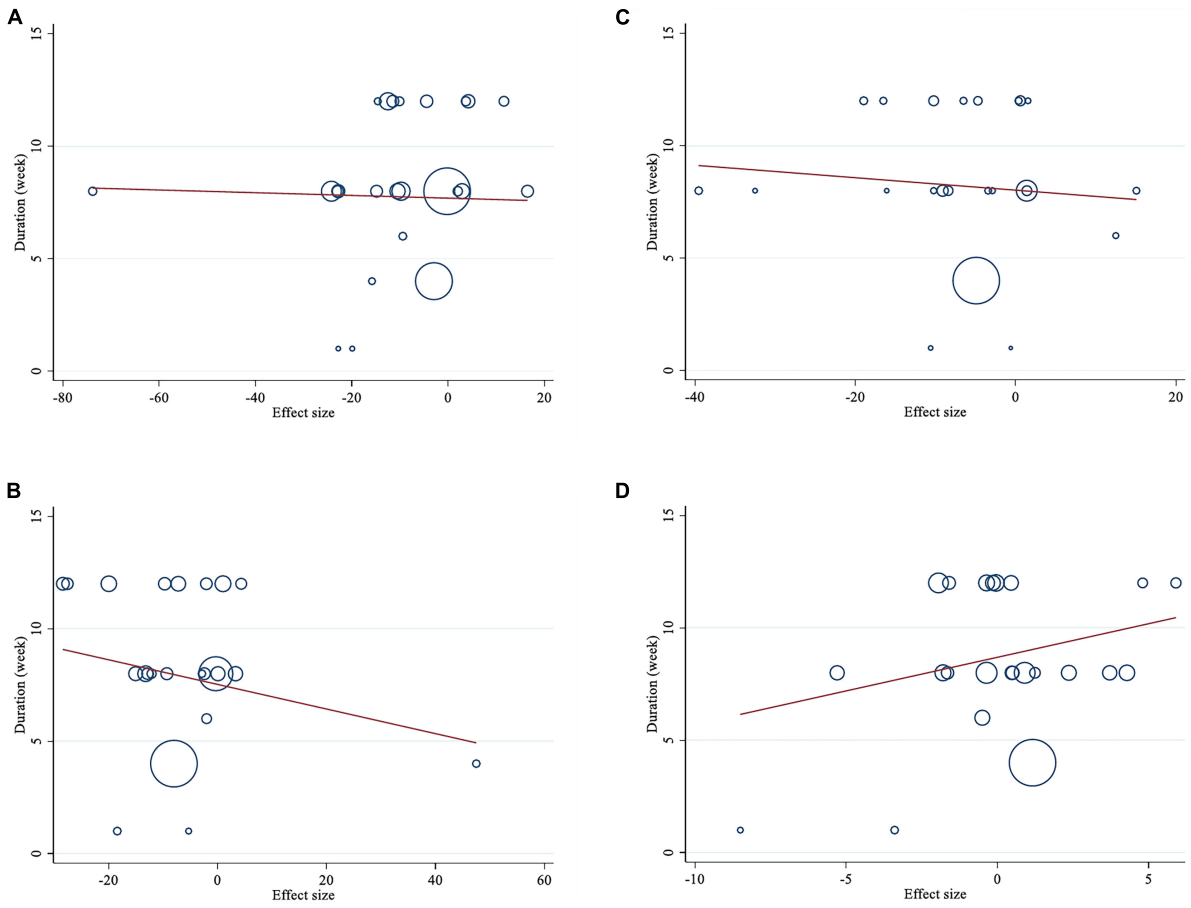

FIGURE 7
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individuals showed that saffron intake can reduce TG, TC, LDL, FBG, HbA1c, HOMA-IR, SBP, CRP, TNF-α, WC, MDA, and ALT, and can elevate TAC levels. According to the subgroup analysis TG, TC, and LDL were reduced significantly in individuals with obesity, and FBG was reduced in overweight individuals. Moreover, participants with diabetes showed a significant reduction in FBG, HBA1c, and MDA levels by saffron supplementation. Saffron supplementation reduced LDL and SBP in individuals with abnormal baseline levels (LDL $\geq$ 100 mg/dl and SBP $\geq$ 120 mmHg), and reduced TG and TC in the categories of lower levels (TG $<$ 150 mg/dl and TC $<$ 200 mg/dl). This supplementation also reduced FBG in both categories of baseline higher and lower than 100 mg/dl. In the non-linear dose-response analysis, between dose for saffron intake and HDL, HOMA-IR, ALP, HbA1c, TNF-α, FBG, and weight was a significant association, and a significant linear association was seen between FBG and duration of saffron supplementation.

Saffron (crocus sativus) is a nutraceutical containing three phytochemical compounds including carotenoids (crocin and crocetin) that are responsible for saffron color, volatile oil component (safranal) that produces odor, and glycoside (picrocrocin) that is the bitter precursor for safranal (70–72). These different subtypes have different tastes, odors, absorption ways, and bioavailability (21). When the hydrophobic crocetin is esterified with two water-soluble sugars (gentiobioses), crocin will be produced which is water soluble and has a high bioavailability. The included studies in this meta-analysis have used two types of substances (saffron or crocin) for supplementation. According to the subgroup analysis, TG, CRP, MDA, and TAC were reduced only in the saffron group while LDL SBP, WC, and ALT were reduced in the crocin group. Both of these compounds could effectively reduce TC, FBG, and HbA1c.

The effect of saffron on inflammatory markers

This study revealed reductions in CRP and TNF-α but no changes in IL-6 were seen following the saffron intervention.

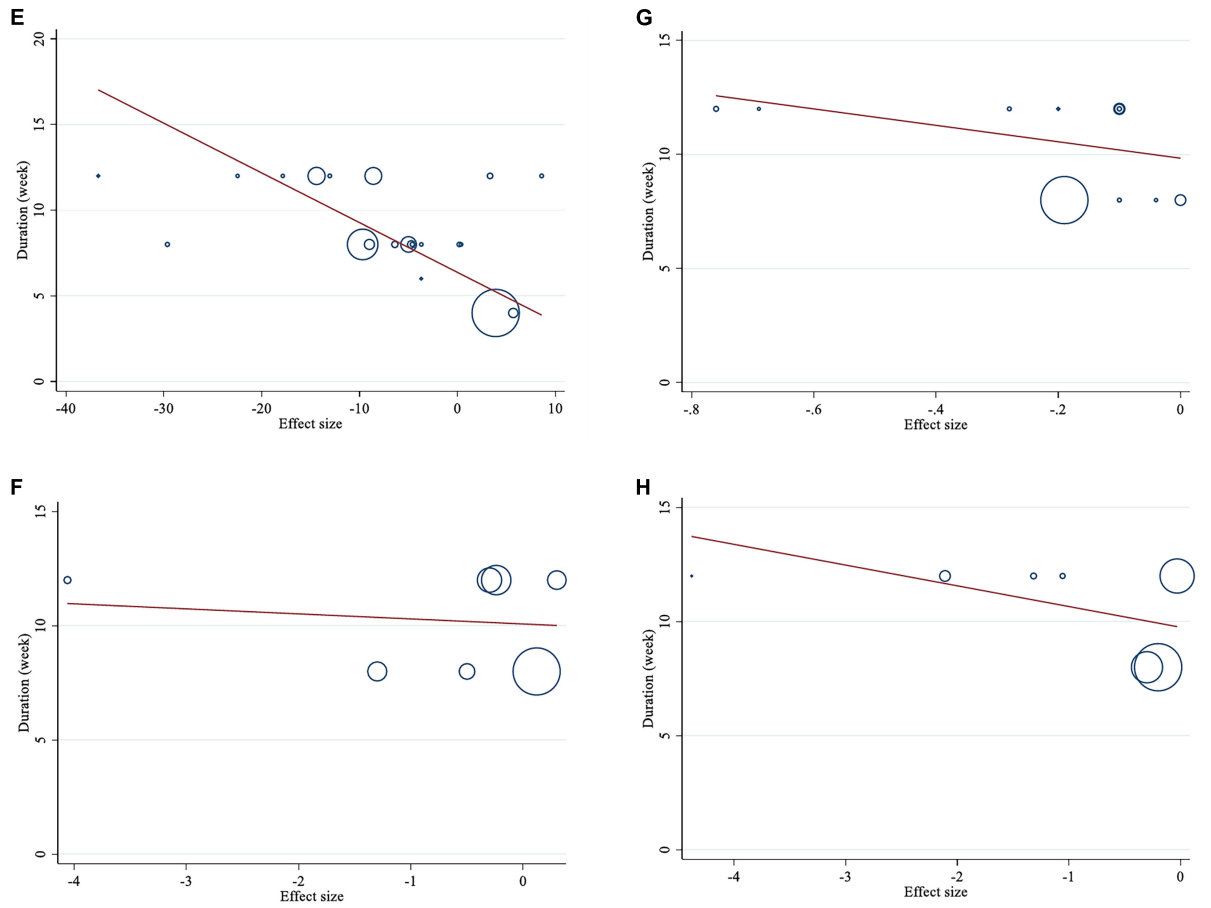

FIGURE 7
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A meta-analysis of 8 RCTs in 2021 by Asbaghi et al. did not reveal any significant impacts of saffron on CRP, TNF-α, and IL-6. However, significant reductions occurred in subgroups with higher baseline measures (CRP ≥ 3 mg/l and TNF-α ≥ 15 pg/ml), lower supplementation dosages (≤30 mg/day), and some other subgroups (26). The controversy can be due to the larger sample size of the present study. The limited number of included trials evaluating the effect of saffron on IL-6 (only three studies) hindered the implementation of subgroup analyses on IL-6. In the present study, the subgroups of non-diabetic individuals and intervention dosages of more than 100 mg/d showed significantly lower CRP and TNF-α levels. Moreover, there was a non-linear association between dose with TNF-α.

Saffron can inhibit serum levels of inflammatory markers such as nuclear factor kappa B (NF-kB), TNF-α, Interferon-gamma (IFN-γ), and some interleukins while acting as the agonist of peroxisome proliferator-activated receptor γ (PPARγ) (73). This medical spice can also downregulate key pro-inflammatory enzymes such as myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), phospholipase A2, and prostanoids (73).

The effect of saffron on antioxidant status

Saffron could reduce MDA levels, and enhance TAC according to our analysis. MDA and TAC reduced significantly in the subgroups of intervention dose of ≥100 mg/d and MDA reduced in the subgroup of trial duration ≥12 months. Oxidative stress, which means the loss of balance between oxidants and antioxidants in favor of oxidants, occurs when the environmental stressors become overwhelming or in case of not enough antioxidant capacity in the body (74). A meta-analysis by Morvaridzadeh et al in 2021 showed the beneficial effect of saffron on TAC and MDA in unhealthy patients (20).

The mechanism by which saffron can affect oxidative stress can be attributed to increasing the levels of glutathione

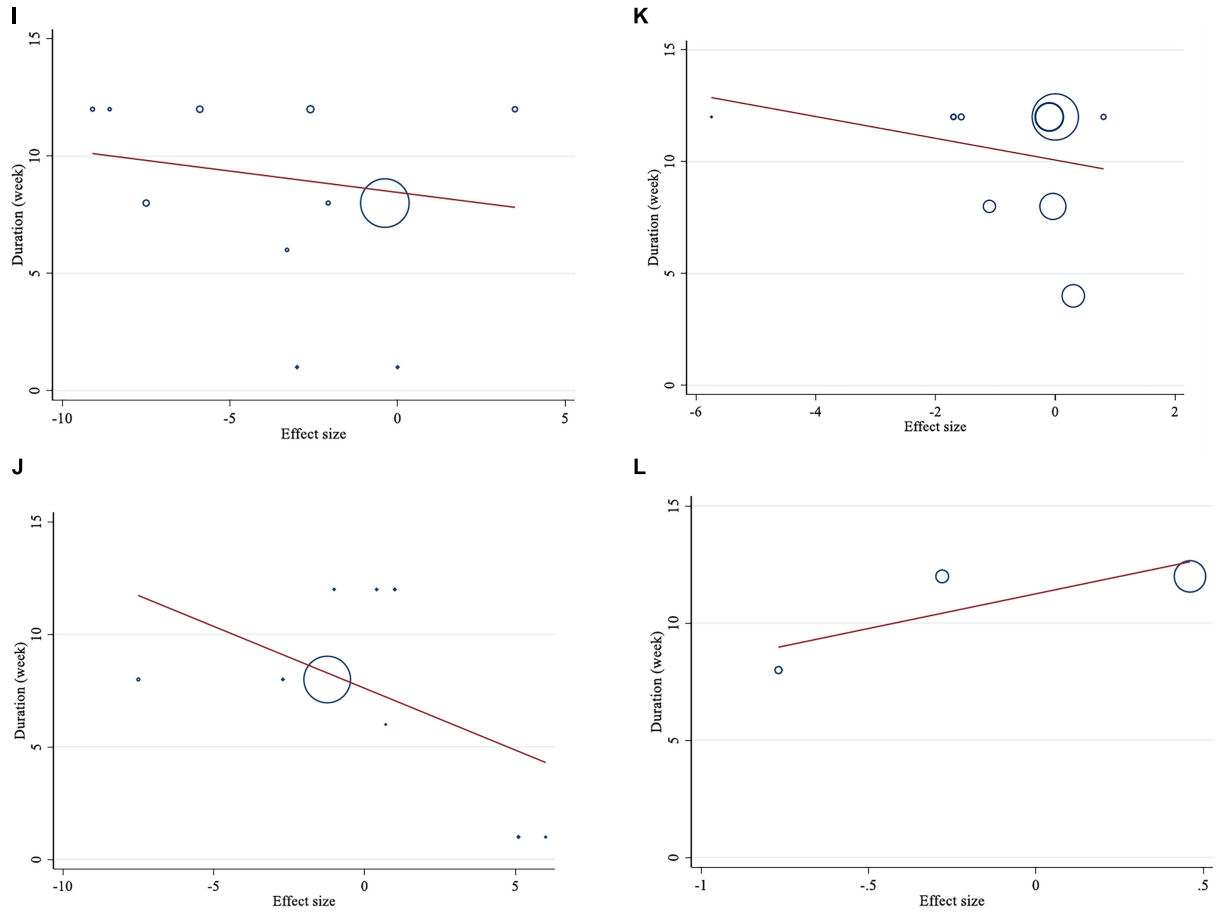

FIGURE 7
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reductase (75). Safranal is suggested to act against the aging process due to its antioxidant properties and can act as a remedy for hepatic ischemia-reperfusion (IR) injury via the prevention of high intracellular reactive oxygen species (ROS) concentration and restoring the content of antioxidant enzymes (76). Existing research shows that saffron can enhance some antioxidant enzymes such as catalase and superoxide dismutase (SOD) (77). Moreover, animal studies revealed the anti-toxicity effects of saffron in different tissues against natural or chemical toxins (78).

The effect of saffron on lipid profiles

The finding of this study shows significant reductions in TG, TC, and LDL after saffron supplementation. However, no significant changes in HDL levels were seen. A meta-analysis of six RCTs by Asbaghi et al. in 2019 demonstrated similar results in the reduction of TG and TC but showed a significant increase in HDL and no changes in LDL levels which are in contrast with this study (25). Another dose-response meta-analysis of 14 RCTs in 2019 by Rahmani et al. showed results similar to the Asbaghi et al. study in TC and TG reduction, no changes in LDL levels, and an increase in HDL levels after long-term consumption of saffron according to the meta-regression analysis (79). The optimum dose of saffron supplementation was 400 mg/d for TG reduction in this study, while dose-response analysis in the present study was not significant for TG (79). The controversy between these two studies in 2019 and the present study in 2022 can be related to the higher sample size of the present study owing to the recently published RCTs (15, 67). Moreover, another meta-analysis of ten studies also published in 2019 by Pourmasoumi et al. showed no effect of saffron on lipid profile (22). According to the subgroup analysis, the reduction in TC, TG, and LDL was significant in individuals with BMI $\geq$ 30 (obese). This can be explained by the anti-inflammatory properties of saffron (75) since inflammatory markers are higher in individuals with obesity compared to normal weight (80). A non-linear association has been seen

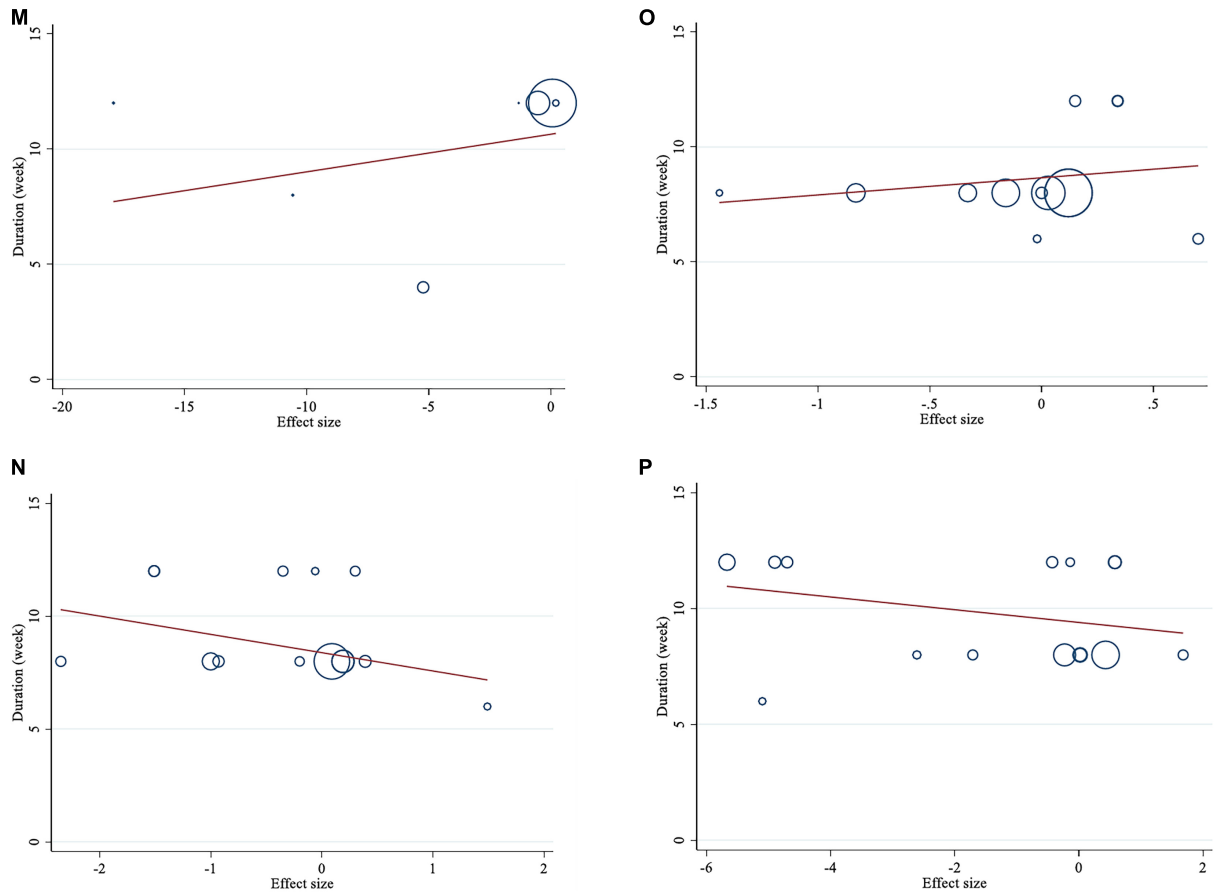

FIGURE 7
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between HDL levels with both the dose and duration of saffron intervention.

An animal study on the effect of saffron (crocetin) against alcoholic fatty liver showed that this substance can enhance mitochondrial-β-oxidation, decline fatty sediment, and prevent lipid peroxidation (19). Moreover, saffron and crocin could effectively reduce hyperlipidemia parameters in rats (81) and humans (79). Saffron also reduces cholesteryl ester transfer protein (CETP) which is involved in the regulation of serum lipid profile (51).

The effect of saffron on insulin resistance

In this study saffron significantly impacted FBG, HbA1c, and HOMA-IR while not affecting insulin levels. A meta-analysis of ten studies in 2019 by Pourmasoumi et al. showed a significant reduction in fasting plasma glucose (FPG) following saffron supplementation and no changes in fasting insulin level (22). Another meta-analysis by Rahmani et al. in 2020 also showed a reduction in FPG especially when the intervention duration exceeds 12 weeks, but could not show a reduction in HbA1c (23). In contrast, another meta-analysis of six RCTs by Asbaghi et al. in 2019 revealed no significant changes in FBG after the supplementation (25). This is in line with the meta-analysis by Roshanravan et al. in 2022 that could not reveal any impact of saffron on blood glucose (82). The existing controversy can be due to different sample sizes, different participant morbidities, or different types of supplementations. According to the subgroup analysis FBG and HbA1c reduced significantly in individuals with diabetes after the saffron intervention. This can be justified by the anti-inflammatory properties of saffron in individuals with diabetes (83). There was shown a non-linear association between FBG, HbA1c, and HOMA-IR with a dose of saffron supplementation.

Regarding the effect of saffron on blood glucose profile, this agent enhances glucose uptake and insulin sensitivity in muscle cells via the phosphorylation of AMPK (AMP-activated protein kinase), ACC (acetyl-CoA carboxylase), and MAPKs (mitogen-activated protein kinase) (18).

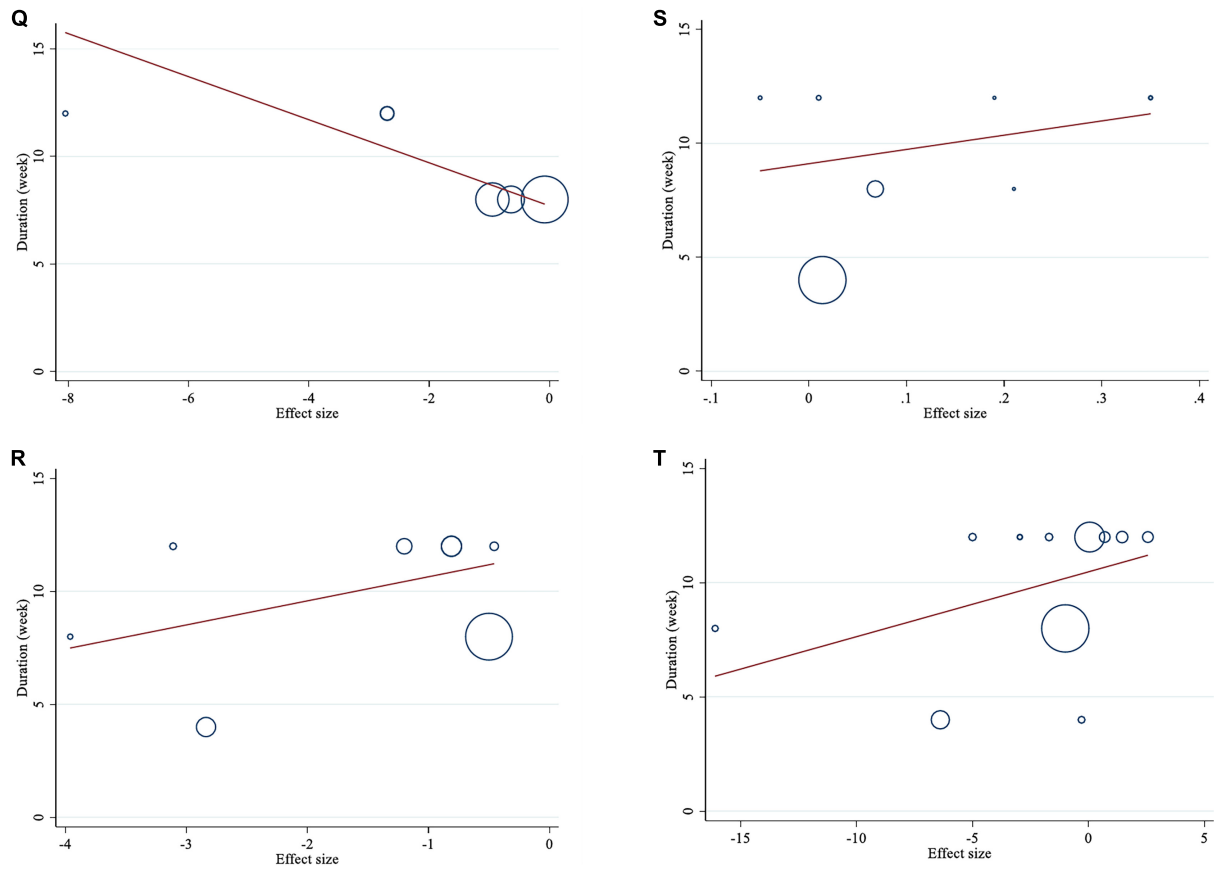

FIGURE 7
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The effect of saffron on blood pressure

Our results showed a significant reduction in SBP and a non-significant reduction in DBP. In contrast, a meta-analysis of ten publications by Pourmasoumi et al. in 2019 showed a significant reduction in DBP and no changes in SBP following saffron supplementation. Another dose-response meta-analysis by Setayesh et al. in 2021 showed the effective impact of saffron on both SBP and DBP and mentioned that the impact of the supplementation on DBP is dependent on the duration of the intervention and the effect would be more in case of longer durations (84). Subgroup analysis of the present study showed that SBP reduces in individuals with baseline SBP $\geq$ 120, intervention duration $\geq$ 12, and intervention dose of $<$100. DBP significantly decreased in the subgroup of diabetic patients. The dose-response analysis revealed a significant association in DBP in the optimum duration of 2 weeks.

The effect of saffron on endothelial nitric oxide (NO) synthases can lead to the elevation of NO production and the lowering of blood pressure (85). Moreover, Crocetin can down-regulate intracellular adhesion molecule-1 (ICAM-1) protein expression (86). This effect can affect the renin-angiotensin system and lead to hypertension suppression (87).

The effect of saffron on anthropometric measures

According to this meta-analysis, saffron intake can significantly reduce WC but has no significant effect on weight, BMI, and FM. However, the non-linear dose-response analysis showed that the optimum dose of 450 mg/d of the intervention can reduce weight. A dose-response meta-analysis of 14 studies by Rahmani et al. in 2019 could not show any significant effect of this intervention on weight (79). This result can be interpreted as the intervention dose being lower than optimum in this study. The mean dose of saffron administered in the meta-analysis by Rahmani et al. was 160 mg/d (79). An animal study by Mashmoul et al. in 2014 compared the anti-obesity effect of crocin and saffron. After inducing obesity in rats with a high-fat diet for 12 weeks, the supplementation showed a beneficial effect of saffron on prospective food consumption and LDL/HDL ratio while crocin had a beneficial effect on

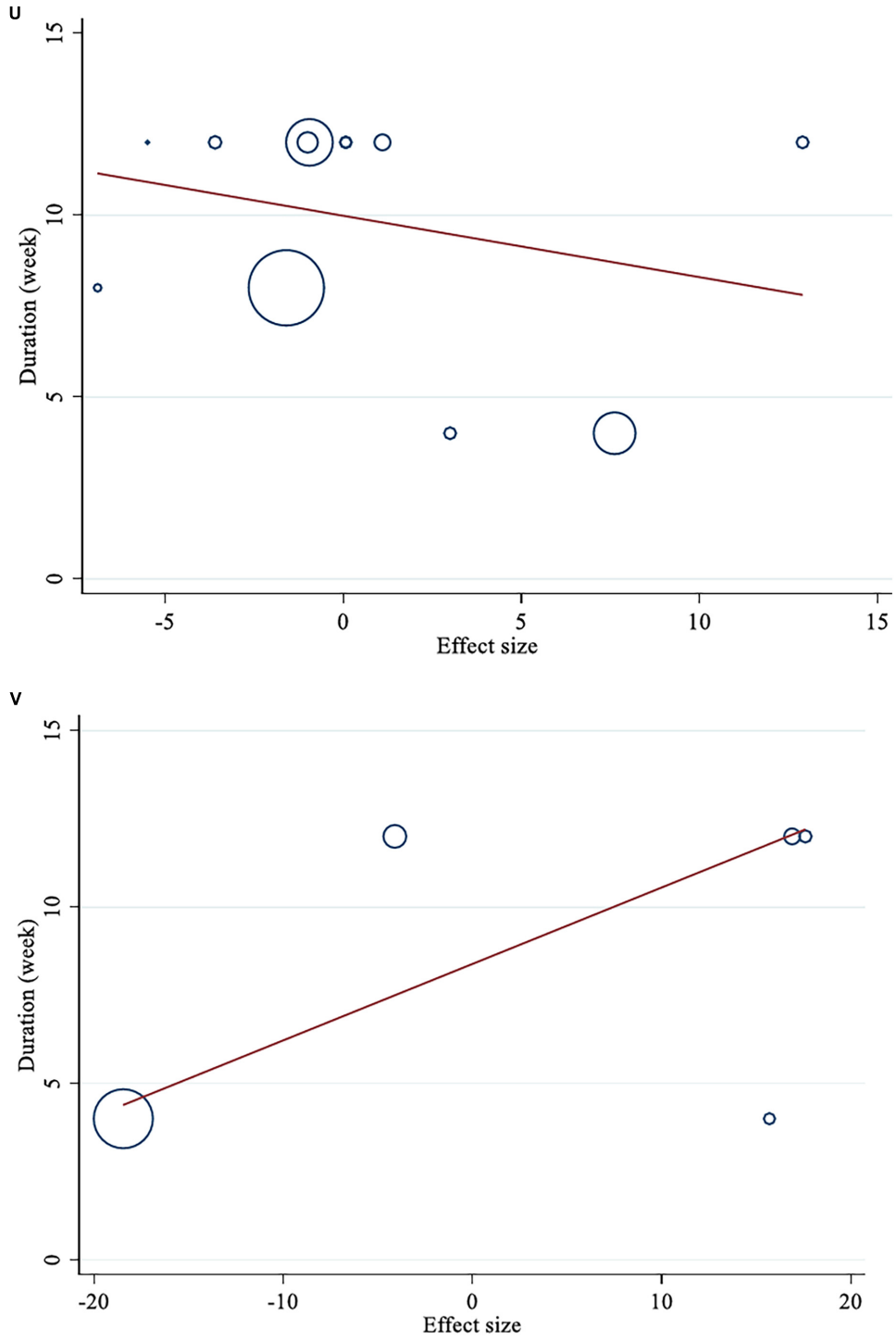

FIGURE 7
Linear dose-response relations between saffron consumption and absolute mean differences. Dose-response relations between duration of intervention (week) and absolute mean differences **(A)** TG (mg/dl); **(B)** TC (mg/dl); **(C)** LDL (mg/dl); **(D)** HDL (mg/dl); **(E)** FBG (mg/dl); **(F)** Insulin (miu/ml); **(G)** HbA1c (%); **(H)** HOMA-IR; **(I)** SBP (mmHg); **(J)** DBP (mmHg); **(K)** CRP (mg/l); **(L)**; IL-6 (pg/ml); **(M)** TNF-α (pg/ml); **(N)** weight (kg); **(O)** BMI (kg/m^2); **(P)** WC (cm); **(Q)** FM (%); **(R)** MDA (uM/L); **(S)** TAC (mM/L); **(T)** ALT (U/L); **(U)** AST (U/L) and **(V)** ALP (U/L). TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HbA1C, hemoglobin A1C; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor; TAC, total antioxidant capacity; BMI, body mass index; WC, waist circumference; FM, fat mass; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; MDA, malondialdehyde; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, confidence interval; WMD, weighted mean difference.

lipid profile (TG, and TC), and lowered the rate of body weight gain (88). This is in line with the present study in which WC was reduced only in the crocin subgroup but not in the saffron group. The justification can be related to the higher antioxidant properties of crocin compared to the same weight of saffron since crocin is the carotenoid component responsible for the color of saffron (72).

The medical herb of saffron can regulate the expression of leptin and adiponectin in adipose tissue (89) and inhibit the secretion of pancreatic and gastric lipase that regulates fat absorption (90). This effect can reduce central adipose tissue accumulation and decrease blood circulating leptin levels, leading to higher satiety perception (90).

The effect of saffron on liver enzymes

The liver enzymes ALT, AST, and ALP were assessed. Only ALT reduces after saffron supplementation according to this meta-analysis. However, the dose-response analysis showed that at the optimum dose of 20 mg/d ALP can be reduced. A meta-analysis of 12 RCTs by Karimi et al in 2021 showed no beneficial effect of saffron on the mentioned three liver function markers (91). Another meta-analysis of nine RCTs in 2022 by Mousavi et al. showed results similar to this analysis on AST, ALT, and ALP. However, the dose-response analysis did not show any relationships (92). The existing controversy can be due to different sample sizes or different supplementation types (crocin or saffron) in these studies.

Liver enzymes may rise above normal levels in healthy individuals or stay normal in liver diseases (93). Regarding this unstable nature of liver enzymes, the results of this study on the effect of saffron on ALT, ASP, and ALP should be interpreted carefully. Moreover, existing diseases can affect liver enzyme levels differently (93) and the participants of this meta-analysis had different morbidities.

This study is the first comprehensive systematic review and dose-response meta-analysis on the effect of saffron on all cardiovascular risk factors. The strengths of this study are the use of the risk of bias assessment, GRADE classification of quality of evidence, non-linear dose-response analysis, subgroup analysis, sensitivity analysis, and meta-regression analysis that enhance the accuracy of the results. Moreover, the adverse effects reported in the study were summarized. The studies were included based on inclusion criteria with a variety of participants which provided the possibility of subgroup analysis and also can make the results eligible to be generalized. The randomized placebo-controlled design of included studies and the double- or triple-blind design of most of them are other strengths. However, some limitations also exist. The contrasting findings may be due to different supplementation types of saffron (crocin, crocetin, safranal, and picrocrocin). Although all studies used randomization, information on allocation concealment, randomization efficiency, and withdrawal was not consistently reported. The included studies were significantly heterogeneous. Some of the current meta-analysis outcomes were secondary outcomes in RCTs. Moreover, regarding the considerable number of the included studies, the types of measurements for outcomes could be different. The intra-assay coefficient of variation and inter-assay variability for biochemical kits in different studies might lead to heterogeneous results. Most of the studies were conducted in Iran due to the use of this plant as a spice in cooked foods, and therefore it seems that it cannot be generalized to other countries. Similarly, the anthropometric indices were measured by different scales and differently trained persons in the included studies. In addition, the blood pressure had been taken in different positions (seated or standing posture, supine position) which is another limitation. It is suggested that combining saffron with starchy food can enhance its bioavailability (21). Therefore, different timing of supplementation in the included RCTs, whether it was consumed simultaneously with food or not, could lead to different results. Another point to be mentioned is the high risk of bias in some of the included trials, highlighting the need for more high-quality clinical trials in the future.

Conclusion

This systematic review and dose-response meta-analysis revealed the beneficial effects of saffron on cardiovascular risk factors including TG, TC, LDL, FBG, HbA1c, HOMA-IR, SBP, CRP, TNF-α, WC, MDA, TAC, and ALT. The non-linear dose-response analysis showed a significant association between dose for saffron intake with HDL, HOMA-IR, ALP, HbA1c, TNF-α, FBG, weight, and showed between the supplementation duration and HDL level, and DBP. Given the significant beneficial results, saffron seems to be an appropriate supplement and adjunct therapy along with other conventional medicine used for preventing or alleviating CVD risk factors.

Data availability statement

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

Author contributions

MoZ designed the study. MoZ and OA developed the search strategy. MaZ, MN, and MN-S extracted the data and conducted the analyses. MoZ, FG, and AH drafted the manuscript. MoZ, MN-S, and OA assessed the risk of bias of the meta-analyses. FS and OA

interpreted the results and revised the manuscript. All authors read and approved the final manuscript.

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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Fluorescent advanced glycation end products in type 2 diabetes and its association with diabetes duration, hemoglobin A1c, and diabetic complications

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Background: Fluorescent advanced glycation end products (fAGEs) are generated through the Maillard reaction between reducing sugars and amino compounds. fAGEs accumulation in human bodies have been confirmed to be related to many chronic diseases. To date, the correlations between serum fAGEs levels and clinical parameters or carotid intima media thickness (CIMT) in patients with T2DM remain unclear. Thus, this study aimed to investigate the relationship between serum AGEs levels and clinical parameters or CIMT in patients with T2DM.

Method: A total of 131 patients with diabetes and 30 healthy controls were enrolled. Patients were divided into three groups according to diabetes duration, including $\leq$5, 5–10, and $\geq$10 years. Serum fAGEs, protein oxidation products, clinical parameters, and CIMT were determined.

Results: The result showed that levels of fAGEs and protein oxidation products increased with the increasing duration of diabetics. Pearson correlation coefficients of fAGEs versus hemoglobin A1c (HbA1c) were $>$0.5 in patients with diabetes duration $\geq$10 years. A continued increase in fAGEs might cause the increase of HbA1c, urinary albumin/creatinine ratio (UACR) and CIMT in patients with T2DM.

Conclusion: Our study suggested that levels of fAGEs could be considered as an indicator for duration of diabetics and carotid atherosclerosis. Diabetes duration and smoking might have a synergistic effect on the increment of

fAGEs levels, as evidence by the results of correlation analysis in patients with long-duration diabetics (≥10 years) and smoking. The determination of fAGEs might be helpful to advance our knowledge on the overall risk of complications in patients with T2DM.

KEYWORDS

fluorescent advanced glycation end products (fAGEs), type 2 diabetes mellitus, glycosylation, carotid intima media thickness (CIMT), diabetic complications

1 Introduction

Advanced glycation end products (AGEs) are generated through the Maillard reaction between reducing sugars (such as glucose and fructose) and amino compounds (1, 2). This reaction occurs both in heat processed foods (3) or *in vivo* (4). It has been reported that dietary AGEs might be released into the blood stream or directly gain entry into the systemic circulation (5). Accumulation of dietary AGEs in blood stream have been confirmed to be related to many chronic diseases, such as kidney disease (6), diabetes (7), atherosclerosis (8), Alzheimer's disease (AD) (9) or tumor (10). Therefore, AGEs have received much attention not only in food science but also in clinical research.

Usually, AGEs in the body are mainly obtained primarily through dietary intake (exogenous AGEs) or self-metabolism (endogenous AGEs). Exogenous AGEs were generated in foods high in fat and protein content (11, 12), and endogenous AGEs are formed in body due to altered glucose metabolism (13, 14). These compounds would eventually enter the blood circulatory system through digestion and absorption (**Figure 1**). Therefore, a high-AGEs diet or higher levels of endogenous AGEs would induce the accumulation of AGEs in human tissues, resulting in the organ injury and dysfunction (such as pancreas, carotid, liver, and kidney). At present, relationships between AGEs and human disease have been previously discussed. Koska et al. (15) investigated the relationship between AGEs and incident cardiovascular disease (CVD) in patients with type 2 diabetes mellitus (T2DM), which showed that higher levels of AGEs are associated with increased incident CVD. Akram et al. (16) investigated that AGEs levels in gingival crevicular fluid of chronic periodontitis, which indicated that AGEs contents are higher in patients with T2DM. Cai et al. (17) also suggested that binding of AGEs and AGEs receptors could induce oxidative stress, leading to islet cell dysfunction and insulin resistance (18). Uribarri et al. (19) investigated that the relationship between dietary intake of AGEs and insulin resistance, which indicated that exogenous AGEs might contribute to insulin resistance in patients with T2DM. Diet-derived AGEs might be released into the systemic circulation, which might participate in the progress of diabetes and uremia (20). In addition to endogenous AGEs and dietary AGEs, cigarette smoke is also one source of AGEs and the main induction factors for AGEs formation (21, 22). These studies mainly focused on the pathophysiological effect of AGEs in diseases and influencing factors of AGEs generation *in vivo*. However, the correlations between serum AGEs levels and clinical parameters, carotid intima media thickness (CIMT), or smoking in patients with T2DM remain unclear.

According to different spectral fluorescence properties, AGEs could be divided into non-fluorescent AGEs (N^{ε} -carboxymethyl-lysine, N^{ε} -carboxyethyl-lysine, and pyrraline) and fluorescent AGEs (fAGEs) (**Figure 2**; 23, 24). Many AGEs are capable of forming cross-links between proteins and most of them have fluorescent properties (such as pentosidine, lys-hydroxy-triosidine, and argpyrimidine). The fluorescence intensity was then used to measure the fAGEs concentrations in serum due to the autofluorescence characteristics of fAGEs (25). Serum fAGEs levels could be used as a reference for long-term blood glucose control in diabetes (26). Therefore, the objective of this work was to evaluate the correlation between serum fAGEs levels and CIMT, smoking or clinical parameters, such as hemoglobin A1c (HbA1c), serum uric acid (UA), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), cholesterol (CHO), urinary albumin/creatinine ratio (UACR), in patients with T2DM, thereby providing some valuable references and guidelines for understanding the development and progression of diabetic complications.

2 Materials and methods

2.1 Subjects

According to the diagnostic criteria of WHO1999, all the subjects were 131 patients with type 2 diabetes hospitalized in Shanxi People's Hospital from June 2021 to December 2021. A total of 30 healthy subjects who for health check-up were enrolled as the control group. These patients were divided into four groups based on the different duration of diabetics: (1) control (n = 30), (2) ≤5 years (n = 49), (3) 5–10 years (n = 33), and (4) ≥10 years (n = 49). Besides, these patients were also

classified into two groups according to carotid intima media thickness (CIMT $\geq$ 1 mm or CIMT $<$ 1 mm) and smoking (smoking or no smoking), respectively.

2.2 Ethical guidelines

The present study was approved by the Institutional Ethics Committee of Shanxi Provincial People's Hospital, Taiyuan, Shanxi Province, China (approval no. 2022023), and was conducted in accordance with the Helsinki Declaration. The main purpose and method of the study were explained to all participants. All participants signed the informed consent form, and they could withdraw from the study, at any time.

2.3 Inclusion and exclusion criteria

The inclusion criteria in this study were as follows: (1) signing the written informed consent form, (2) T2DM, (3) BMI: 19–35 kg/m^2. Exclusion criteria: (1) other types of diabetes; (2) ketosis or ketoacidosis, hyperosmolar coma, and severe stress within half a year, (3) family history of mental illness or alcohol users, (4) individuals that had used antibiotics, probiotics, non-steroidal anti-inflammatory drugs, and/or steroids within the past 3 months, (5) lactating or pregnant females, (6) patients with other serious illnesses.

2.4 Patients and blood sample collections

This study was designed to assess the correlation between serum fAGEs and AGEs-related complications in patients with T2DM, thereby predicting the severity of diabetic complications by fAGEs levels. Briefly, 131 T2DM patients and 30 healthy subjects (75 males and 86 females) in the department of endocrinology from Shanxi Provincial People's Hospital were selected. All blood tests were determined after an overnight fast of $>$8 h.

2.5 Measurements

Hemoglobin A1c levels of diabetics patients were determined by the method reported by Thevarajah et al. (27) with slight modification. Briefly, the centrifuged blood samples were analyzed by a trained and calibrated investigator using ion-exchange high-performance liquid chromatography (ARKRAY Inc. Kyoto, Japan). Individuals with HbA1c levels of $<$ 6.0 and $\geq$6.0% were considered normoglycemic and hyperglycemic, respectively. Blood and urine tests were performed at the clinical laboratory. That is to say, blood and urine sample were tested at a certified central laboratory for levels of UA, TG, CHO, HDL, LDL, and UACR according to standard procedures.

2.6 CIMT

Carotid intima media thickness were scanned by the method reported by Jun et al. (28). The subjects were supine, and the neck was fully exposed. Meanwhile, the head of subjects turned to the side away from the ultrasound physician. CIMT measurement was taken using LOGIQ 7 machine equipped with a 10 MHz linear transducer (GE, Healthcare, Milwaukee, WI, USA). CIMT value was scanned at three points: the far wall of the mid and the distal common carotid artery, and 1.0 cm proximal to the carotid bulb. The mean value of the three measurements on each side was used as the CIMT value (29). Usually, focal wall thickening $>$50% of the surrounding CIMT, or its CIMT of 1.5 mm was identified as carotid plaque (30).

2.7 Fluorescence intensity of AGEs

The fluorescence intensity of AGEs in serum samples was determined using the method described by Ferrer et al. (31) with some modifications. Briefly, 5 ml fasting blood was collected, and centrifuged at 1000 g for 10 min to separate the serum. FAGEs levels were evaluated on a fluorescence spectrometer (PerkinElmer LS-55). The excitation and emission wavelength was 370 and 440 nm, respectively. The slit width was 5.0 nm. The fluorescence intensities of AGEs were measured against reagent blank prepared with the same reagent concentrations.

2.8 Determination of protein oxidation products

In general, POPs include dityrosine, N'-formylkynurenine, and kynurenine (25). These compounds were quantified by the method reported by Ou et al. (25) with modifications. Based on different fluorescence intensities of POPs, the excitation wavelengths were chosen at 330, 325, and 365 nm, respectively; the emission wavelengths were recorded at 415, 434, and 480 nm, respectively, for the quantification of dityrosine, N'-formylkynurenine, and kynurenine, respectively. Then, 5 ml fasting blood was collected, and centrifuged at 1000 g for 10 min to separate the serum.

The fluorescence intensities of POPs were measured against reagent blank prepared with the same reagent concentrations. Besides, a fluorescence spectrometer (PerkinElmer LS-55) was used to quantify the POPs contents.

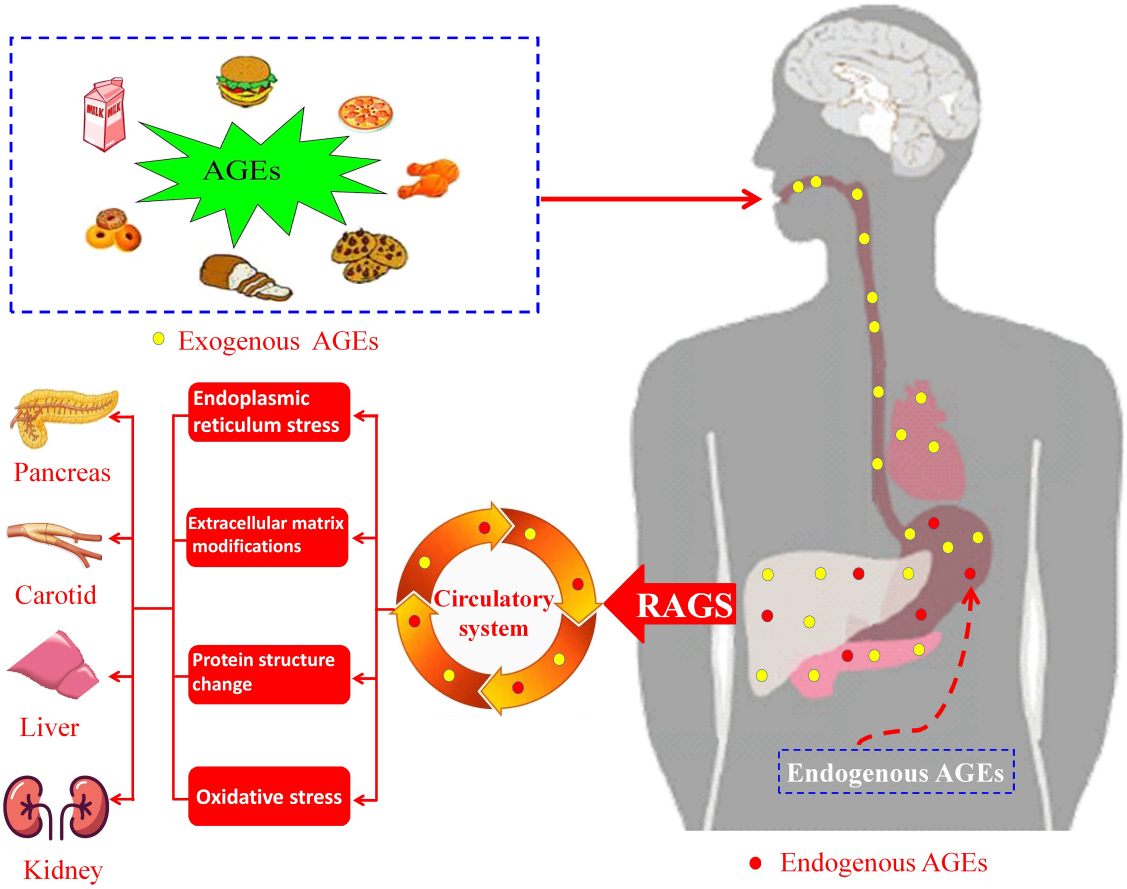

FIGURE 1
Schematic pathway of advanced glycation end products (AGEs) intake and formation in human body and AGEs-induced diseases.

2.9 Statistics

All statistical analyses were performed using the statistix version 9.0 software (Analytical Software, Tallahassee, FL, USA) and GraphPad Prism 8.0 software (GraphPad, San Jose, CA, USA). Continuous data with a normal distribution were expressed as mean value ± SE, whereas non-normal distributed data are expressed as medians (quartile). The statistical significance ($P < 0.05$) was evaluated using unpaired Student's *t*-test and Pearson's correlation coefficient r.

3 Results

3.1 Baseline characteristics

Baseline characteristics were showed in Table 1. A total of 161 individuals were enrolled in this study: 131 patients with type 2 diabetes and 30 healthy subjects, including 75 males and 86 females. According to the duration of diabetics, 131 patients with T2DM were divided into three groups: ≤5 years ($n = 49$), 5–10 years ($n = 33$), and ≥10 years ($n = 49$). The baseline characteristics showed that there was no significant difference in age, sex and BMI among the groups. There were significant differences in HbA1c and UACR ($P < 0.05$), but no significant differences were found in UA, TG, LDL, HDL, and CHO (Table 1).

3.2 POPs and fAGEs

As shown in Table 2, levels of dityrosine, N'-formylkynurenine, and kynurenine in patients with long-duration diabetics (≥10 years) were significantly higher than that in patients with short-duration diabetics (≤5 and 5–10 years), which meant that duration of diabetics could significantly affect protein oxidation. Furthermore, a similar trend was observed in the amounts of fAGEs. In patients with long-duration diabetics (≥10 years), serum fluorescence intensity of AGEs (34.7 ± 1.2) were significantly ($P < 0.05$) higher than that in patients with short-duration diabetics (5–10 and ≤5 years) (24.7 ± 1.5 and 19.7 ± 1.2, respectively).

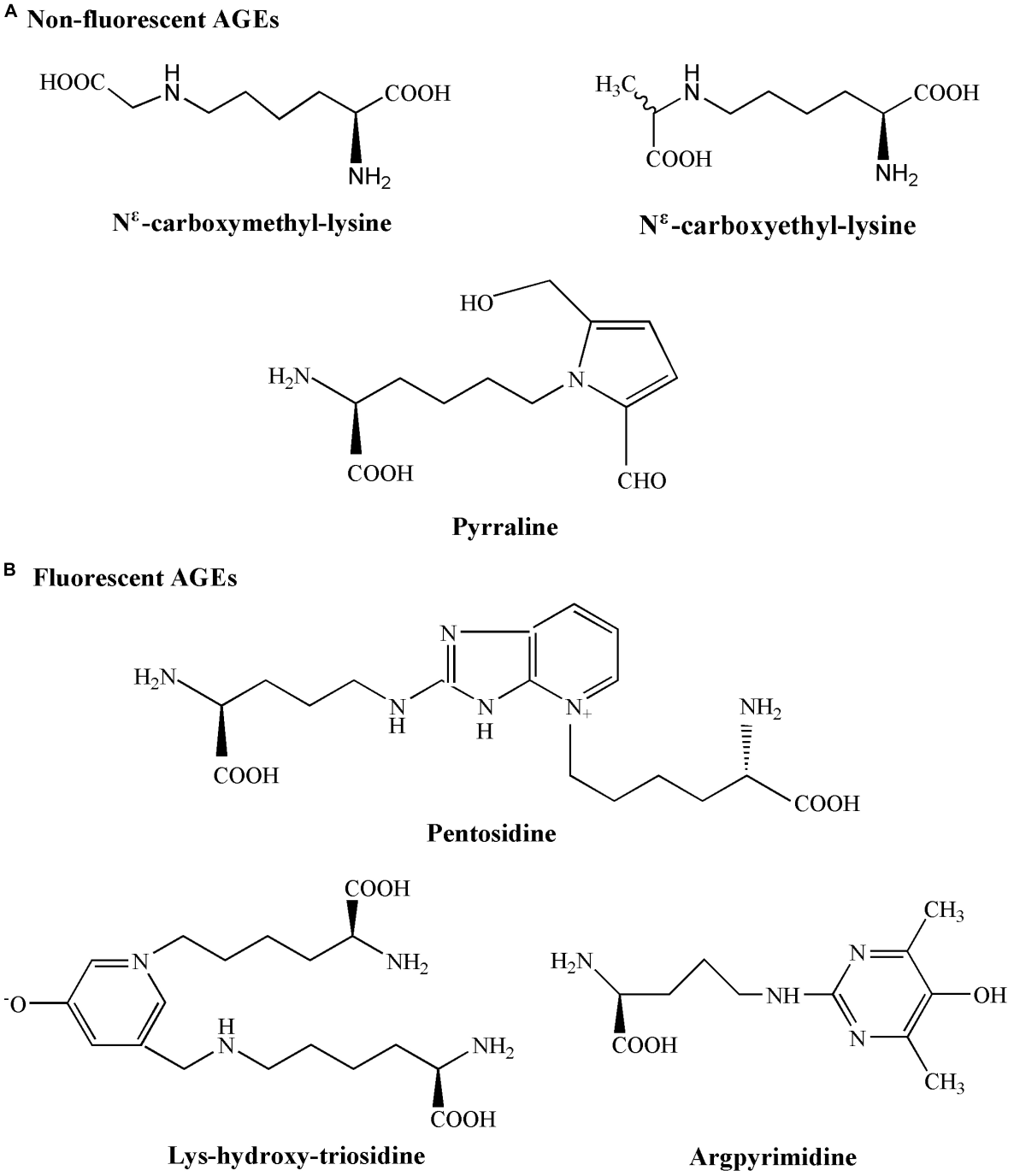

FIGURE 2
Chemical structures of non-fluorescent advanced glycation end products (AGEs) and fluorescent AGEs.

3.3 Correlation between fAGEs and clinical parameters in patients with T2DM

In order to investigate whether there was a relationship between clinical parameters (HbA1c, UACR, UA, TG, LDL, HDL, and CHO) and serum fAGEs, a correlation analysis was performed in patients with different durations of diabetics. Compared to the patients with diabetes duration <10 years (**Figures 3A, B**), it was worth noting that a significant correlation was observed between HbA1c and fAGEs or POPs in patients with diabetes duration ≥10 years (**Figure 3C**).

TABLE 1 Clinical and biochemical characteristics of participants.

Characteristic	Control subjects	Diabetes duration			*P*-value
		≤5	5–10	≥10	
Subjects (*n*)	30	49	33	49	NA**
Age (years)	41 ± 13	51 ± 17	59 ± 13	64 ± 15	0.3063
Sex (male/female)	12/18	28/21	13/20	22/27	0.1332
BMI (kg/m^2)	24.3 ± 6.3	32.4 ± 2.4	30.8 ± 3.7	27.1 ± 4.4	0.3454
HBA1c	5.6 ± 0.6	5.9 ± 2.1	6.7 ± 1.6	9.2 ± 1.7	0.0484*
UACR	4.0 ± 1.3	9.9 ± 2.7	11.1 ± 2.4	18.4 ± 3.7	0.0013*
UA	306 ± 44	329 ± 59	336 ± 48	439 ± 53	0.0526
TG	2.8 ± 1.1	2.4 ± 0.5	2.0 ± 1.2	1.6 ± 0.8	0.4806
LDL	2.7 ± 0.6	2.8 ± 0.7	3.0 ± 1.2	2.7 ± 0.8	0.9594
HDL	1.2 ± 0.3	1.0 ± 0.1	1.1 ± 0.4	1.1 ± 0.3	0.8740
CHO	4.3 ± 1.4	4.4 ± 1.0	4.6 ± 1.1	4.3 ± 0.6	0.9828

*P-values reflect differences between means of control subjects and diabetes at baseline ($P < 0.05$).
**NA, not applicable.

TABLE 2 Fluorescence intensities of advanced glycation end products (AGEs) and protein oxidation products (POPs) in healthy subjects and patients with type 2 diabetes mellitus (T2DM)*.

Fluorescence intensity	Healthy subjects (*n* = 30)	Diabetes duration		
		≤5 (*n* = 49)	5–10 (*n* = 33)	≥10 (*n* = 49)
fAGEs*	17.7 ± 1.6^c	19.7 ± 1.2^c	24.7 ± 1.5^b	34.7 ± 1.2^a
Dityrosine*	19.7 ± 2.0^b	19.1 ± 1.6^b	22.6 ± 1.9^b	33.2 ± 1.6^a
N'-formylkynurenine*	24.6 ± 2.2^b	20.9 ± 1.7^b	26.0 ± 2.1^b	36.8 ± 1.7^a
Kynurenine*	23.6 ± 1.8^d	29.4 ± 1.4^c	34.8 ± 1.8^b	43.7 ± 1.4^a

*Different letters (a–d) in the same row indicate significant differences ($P < 0.05$).

Furthermore, a similar result was also observed between UACR and fAGEs or POPs (Figure 3C). These findings indicated that the increase of fAGEs and protein oxidation products might lead to higher concentrations of HbA1c and UACR in patients with diabetes duration ≥10 years.

3.4 Effect of smoking on the formation of fAGEs in patients with T2DM

Effect of smoking on the generation of fAGEs was investigated in patients with T2DM. As presented in Table 3, there was no significant difference in the amounts of serum fAGEs and POPs in patients with diabetes duration ≤5 years. In addition, in patients with diabetes duration >5 years, intensities of fAGEs and POPs in smokers with T2DM were significant higher compared to non-smokers with T2DM (Table 3). To further elucidate the reason for increasing fluorescence intensity of AGEs in smokers with T2DM, the relationship between smoking and fAGEs is also evaluated. Compared to the no smoking patients (Figure 4A), a significant correlation was found between fluorescence intensity of AGEs, POPs, and HbA1c or UACR in the smoking patients (Figure 4B). Additionally, the fluorescence intensity of AGEs in smoking patients with diabetes duration ≥10 years was higher compared to the smoking patients with diabetes duration <10 years (Table 3).

3.5 Correlation between fAGEs and CIMT in patients with T2DM

In order to research the effect of fAGEs on the increment of CIMT, the intensities of fAGEs and protein oxidation products (dityrosine, N'-formylkynurenine, and kynurenine) were investigated in patients with CIMT ≥1 mm and CIMT <1 mm. As presented in Table 4, compared with patients (CIMT < 1 mm), a significant increase of fAGEs was observed in patients (CIMT ≥ 1 mm) with diabetes duration ≥10 years. There was no significant difference in the amounts of fAGEs in patients (diabetes duration <10 years). Besides, in order to investigate whether there was a relationship between serum fAGEs formation and HbA1c, a correlation analysis was performed in patients with T2DM. As shown in Figure 4, compared to the patients with CIMT <1 mm (Figure 4C), a significant correlation was observed between HbA1c and

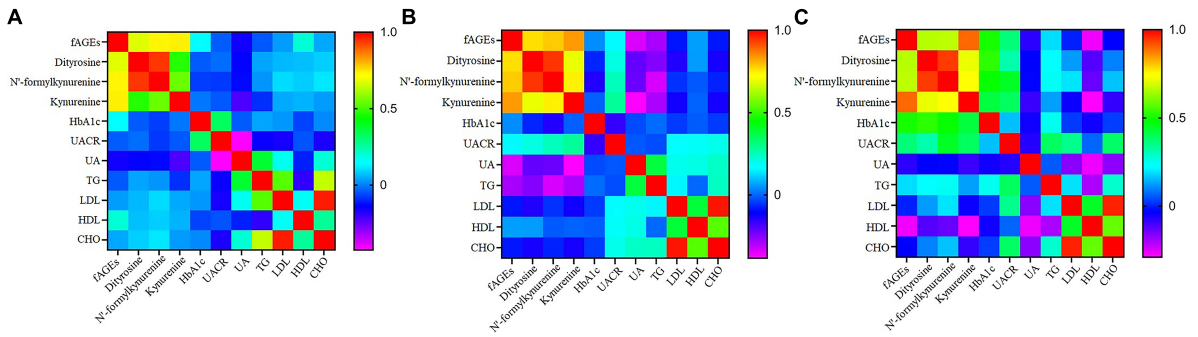

FIGURE 3
The heatmap of correlation coefficient of fluorescent advanced glycation end products (fAGEs), dityrosine, N'-formylkynurenine and kynurenine, HbA1c, UACR, UA, TG, LDL, HDL, and CHO in patients with diabetes duration ≤5 years **(A)**, 5–10 years **(B)**, and ≥10 years **(C)**.

TABLE 3 Fluorescence intensities of advanced glycation end products (AGEs) and protein oxidation products (POPs) in smokers and non-smokers with type 2 diabetes mellitus (T2DM).

	fAGEs**	Dityrosine**	N'-formylkynurenine**	Kynurenine**
Healthy subjects (n = 30)*				
Smokers (n = 6)	$^{BC}21.7 \pm 4.4^{a}$	$^{B}24.9 \pm 5.3^{a}$	$^{B}30.1 \pm 5.5^{a}$	$^{C}24.4 \pm 4.8^{a}$
Non-smokers (n = 24)	$^{C}16.7 \pm 1.4^{a}$	$^{B}18.4 \pm 1.9^{a}$	$^{B}23.2 \pm 2.1^{a}$	$^{C}23.4 \pm 1.7^{a}$
Diabetes duration ≤5 years (n = 49)*				
Smokers (n = 19)	$^{C}19.6 \pm 2.5^{a}$	$^{B}20.7 \pm 3.0^{a}$	$^{B}22.3 \pm 3.1^{a}$	$^{C}28.7 \pm 2.7^{a}$
Non-smokers (n = 30)	$^{BC}19.8 \pm 1.2^{a}$	$^{B}18.0 \pm 1.7^{a}$	$^{B}20.0 \pm 2.0^{a}$	$^{B}29.8 \pm 1.5^{a}$
Diabetes duration 5–10 years (n = 33)*				
Smokers (n = 10)	$^{B}29.1 \pm 3.4^{a}$	$^{B}27.9 \pm 4.1^{a}$	$^{B}32.8 \pm 4.2^{a}$	$^{B}42.0 \pm 3.7^{a}$
Non-smokers (n = 23)	$^{B}22.8 \pm 1.4^{b}$	$^{B}20.3 \pm 2.0^{b}$	$^{B}23.0 \pm 2.2^{b}$	$^{B}31.7 \pm 1.8^{b}$
Diabetes duration ≥10 years (n = 49)*				
Smokers (n = 14)	$^{A}42.7 \pm 2.9^{a}$	$^{A}39.5 \pm 3.5^{a}$	$^{A}44.1 \pm 3.6^{a}$	$^{A}52.3 \pm 3.1^{a}$
Non-smokers (n = 35)	$^{A}31.6 \pm 1.1^{b}$	$^{A}30.7 \pm 1.6^{a}$	$^{A}33.9 \pm 1.8^{a}$	$^{A}40.2 \pm 1.4^{b}$

*Different letters (a, b) in patients with the same diabetes duration (column) indicate significant differences ($P < 0.05$).
**Different letters (A–C) in smokers or non-smokers with T2DM indicate significant differences ($P < 0.05$).

fluorescence intensities of AGEs or POPs in patients with CIMT ≥1 mm (Figure 4D). Moreover, a significant increase of fAGEs was found in patients with CIMT >1 compared to the patients with CIMT <1 (diabetes duration ≥10 years) (Table 4).

4 Discussion

As indicated above, the present study suggested that duration of diabetics could significantly affect protein oxidation. Generally, carbonylation was recognized as one of the most important oxidative modifications of protein (32), and the oxidative degree of lipid was evaluated by levels of malondialdehyde (MDA) (33). Pan et al. (34) investigated the relationship between the oxidative stress status and diabetes complications in patients with T2DM, which found that diabetes duration significant positively correlated with MDA, advanced oxidation protein products and protein carbonyl ($P < 0.05$). These findings discussed here indicated oxidative stress was correlated to diabetes duration. Increased oxidative stress in patients with T2DM could induce the oxidation of protein (35, 36), resulting in higher levels of protein oxidation products in patients with long-duration diabetics. Furthermore, duration of diabetics could significantly promote the formation of fAGEs. This result seems to be reasonable because protein oxidation products was immediate precursor of fAGEs, higher levels of protein oxidation products would promote the formation of fAGEs (37). Usually, fAGEs can be formed via the Maillard reaction or lipid oxidation pathway (38). In this study, increased oxidative stress in patients with T2DM could induce the formation of free radicals (such as hydroxyl radical, superoxide radical and cross-linked radical cation) and lipid oxidation, which promoted the generation of fAGEs (39). The fluorescence intensities of AGEs and protein oxidation products increased with the increasing duration of diabetics ($P < 0.05$), probably due to the increased oxidative stress and lipid oxidation. Therefore, it was reasonable that fAGEs in patients with diabetes

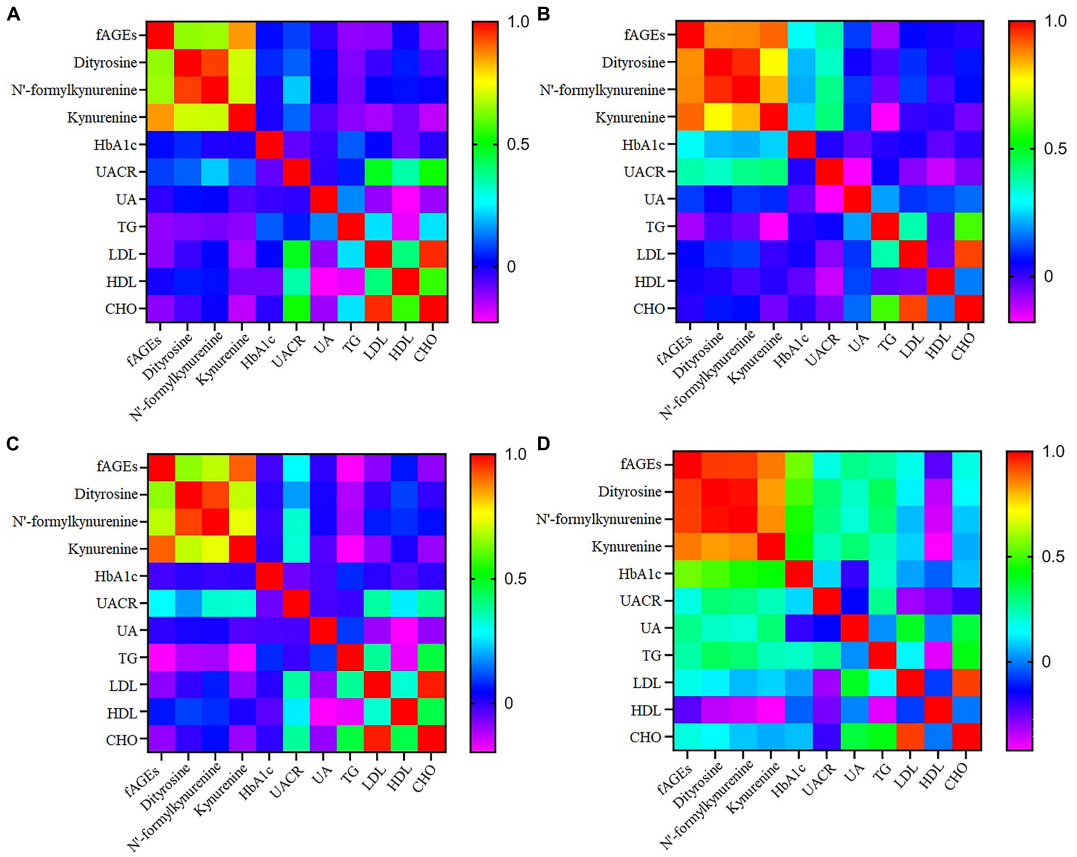

FIGURE 4
The heatmap of correlation coefficient of fluorescent advanced glycation end products (fAGEs), dityrosine, N'-formylkynurenine and kynurenine, HbA1c, UACR, UA, TG, LDL, HDL, and CHO in T2DM with non-smokers **(A)**, smokers **(B)**, CIMT < 1 **(C)**, and CIMT ≥ 1 **(D)**.

duration $\geq$10 years was higher compared to the patients with diabetes duration $<$10 years. Diabetes duration could significantly promote the formation of serum fAGEs and protein oxidation products.

Changes in the physiology or pathology of patients could be reflected in clinical parameters. For example, HbA1c is an important indicator of long-term glycemic control, which could reflect the cumulative glycemic history of the preceding 2–3 months (40). Glycation is also a major cause of spontaneous damage to extracellular and cellular proteins of living organisms (41). The present study indicated that higher levels of fAGEs and protein oxidation products might promote the increase of HbA1c level. The result was consistent with previous findings that correlation coefficient between AGEs and HbA1c in patients with T2DM was 0.661 (42). In general, the formation of AGEs contain three main steps: (1) carbonyl group of lipid oxidation products or reducing sugar react with protein to form Schiff's base, which becomes to Amadori products after rearrangement; (2) Amadori products dehydration and rearrangement generates highly reactive dicarbonyl compounds, such as 3-deoxyglucosone (3-DG), glyoxal (GO), and methylglyoxal (MGO); and (3) these carbonyl compounds react with arginine and lysine residues of proteins to form a stable AGEs (8, 43, 44). Similarly, HbA1c is also the product of Amadori rearrangement that formed during the process of glycation (45). Therefore, higher levels of HbA1c probably due to increased overall protein glycation reactions (46). Besides, Chao et al. (42) also reported that AGEs might enhance glycation reactions of hemoglobin, which subsequently enhance the formation of HbA1c in patients with T2DM. The fluorescence intensity of AGEs could be considered as a marker of long-term glycemic control in patients with T2DM.

Not only is smoking a risk factor for developing diabetes (47), smoking also affects the formation of AGEs (48). Therefore, the impact of diabetes and some general habits, such as smoking, on the formation of fAGEs also needs to be further investigated. The present study indicated fAGE levels in smokers were significantly higher than those in non-smokers (diabetes duration $\geq$10 years). This result meant that smoking might contribute to levels of fAGEs in patients with T2DM. Cerami et al. (48) reported that reactive glycation products were present in aqueous extracts of tobacco and in tobacco

TABLE 4 Fluorescence intensities of advanced glycation end products (AGEs) and protein oxidation products (POPs) in patients with carotid intima media thickness (CIMT) ≥1 and <1 mm.

	fAGEs**	Dityrosine**	N'-formylkynurenine**	Kynurenine**
Healthy subjects (n = 30)*				
CIMT ≥ 1 mm (n = 0)	–	–	–	–
CIMT < 1 mm (n = 30)	$^{C}17.7 \pm 1.3$	$^{B}19.7 \pm 1.8$	$^{B}24.6 \pm 2.1$	$^{BC}32.4 \pm 2.7$
Diabetes duration ≤ 5 years (n = 49)*				
CIMT ≥ 1 mm (n = 6)	$^{B}20.2 \pm 2.4^{a}$	$^{B}18.8 \pm 3.2^{a}$	$^{B}21.9 \pm 3.5^{a}$	$^{B}36.6 \pm 3.1^{a}$
CIMT < 1 mm (n = 43)	$^{C}19.6 \pm 1.1^{a}$	$^{B}19.1 \pm 1.5^{a}$	$^{B}20.8 \pm 1.7^{a}$	$^{C}28.3 \pm 2.3^{b}$
Diabetes duration 5-10 years (n == 33)*				
CIMT ≥ 1 mm (n = 6)	$^{B}26.4 \pm 2.5^{a}$	$^{B}23.1 \pm 3.3^{a}$	$^{B}26.8 \pm 4.1^{a}$	$^{AB}38.2 \pm 3.9^{a}$
CIMT < 1 mm (n = 27)	$^{B}24.4 \pm 1.4^{a}$	$^{B}22.5 \pm 1.9^{a}$	$^{B}25.8 \pm 2.2^{a}$	$^{B}36.0 \pm 2.9^{a}$
Diabetes duration ≥ 10 years (n = 49)*				
CIMT ≥ 1 mm (n = 11)	$^{A}46.4 \pm 3.6^{a}$	$^{A}41.8 \pm 4.6^{a}$	$^{A}45.1 \pm 5.0^{a}$	$^{A}51.1 \pm 4.0^{a}$
CIMT < 1 mm (n = 38)	$^{A}31.4 \pm 1.2^{b}$	$^{A}30.8 \pm 1.6^{b}$	$^{A}34.4 \pm 1.8^{b}$	$^{A}48.3 \pm 2.4^{a}$

*Different letters (a, b) in patients with the same diabetes duration (column) indicate significant differences ($P < 0.05$).
**Different letters (A–C) in patients with CIMT ≥ 1 mm or CIMT < 1 mm indicate significant differences ($P < 0.05$).

smoke in a form that could rapidly react with proteins to form AGEs, resulting in a significant increase in serum AGEs contents in smokers. Besides, AGEs in tobacco and tobacco smoke were diet-derived AGEs, and dietary AGEs might be released into the blood stream or directly gain entry into the systemic circulation (5). Therefore, consumption of tobacco diet resulted in increase of plasma levels of AGEs. Furthermore, smoking and diabetes duration might have a synergistic effect on the formation of fAGEs in patients with T2DM, as evidence by the results of correlation analysis in patients with long-duration diabetics (≥10 years). Facchini et al. (49) investigated that relationship between insulin resistance and cigarette smoking, which suggested that chronic cigarette smokers were insulin resistant and hyperinsulinaemic compared with a matched group of non-smokers. This result might help to explain why smoking could increase levels of HbA1c and fAGEs in patients with T2DM. The results are consistent with a previous report which demonstrated that smoking rates had a good correlation with HbA1c levels in patients with T2DM (50).

Diabetes complications have been paid attention because of harmful effects on human health (51). Carotid atherosclerosis, as one of the complications of diabetes, has been widely studied (26). CIMT is the most often used for carotid atherosclerosis evaluation in clinical trials (52). The present results demonstrated that fAGEs could significantly affect the increase of CIMT in patients with diabetes duration ≥10 years. It should be noted that HbA1c in patients with diabetes duration ≥10 years was higher than those in patients with diabetes duration <10 years (**Table 1**) ($P < 0.05$). Indyk et al. (45) reported that fAGEs was generated through various chemical pathways, such as Schiff's base and Amadori rearrangement, which might promote the increment of HbA1c level (53). Chao et al. (42) has been documented that fAGEs could enhance glycation reactions of hemoglobin, which promote the formation of HbA1c in patients with T2DM. James et al. (54) also reported that glycosylation of hemoglobin could alter nitric oxide binding to hemoglobin thiols and impair vasodilatation, which lead to an increase in CIMT. Higher levels of fAGEs might cause the increase of HbA1c, which further lead to an increase in CIMT (55). Therefore, the fluorescence intensities of AGEs could be considered as a marker for carotid atherosclerosis.

5 Conclusion

In summary, the results of this study demonstrate the relationships between serum fAGEs levels and CIMT or clinical parameters in patients with T2DM. The fluorescence intensities of AGEs and POPs increased with the increasing duration of diabetics. Diabetes duration and smoking markedly promoted the accumulation of fAGEs and POPs. Higher concentrations of fAGEs might cause the increase of HbA1c and UACR levels. A continued increase in fluorescence intensity of AGEs might cause the increase of CIMT in patients with T2DM. These findings reflected that increasing fAGEs might enrich circulating AGEs levels and contribute to impair vasodilatation progression in patients with T2DM, which subsequently lead to a significant increment in CIMT. Therefore, the fluorescence intensity of AGEs could be considered as a marker for the duration of diabetics and carotid atherosclerosis. This work might be helpful to advance our knowledge on the overall risk of complications in 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 Ethics Committee of Shanxi Provincial People's Hospital (approval no. 2022023). The patients/participants provided their written informed consent to participate in this study.

Author contributions

RL: conceptualization, funding acquisition, and writing—original draft. MZ and LX: investigation. JL and PY: data curation. ML: project administration. JQ: writing—review and editing. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1083872/full#supplementary-material

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Genetic heritability as a tool to evaluate the precision of 24-hour recall dietary questionnaire variables in UK Biobank

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A variety of statistical approaches in nutritional epidemiology have been developed to enhance the precision of dietary variables derived from longitudinal questionnaires. Correlation with biomarkers is often used to assess the relative validity of these different approaches, however, validated biomarkers do not always exist and are costly and laborious to collect. We present a novel high-throughput approach which utilizes the modest but importantly non-zero influence of genetic variation on variation in dietary intake to compare different statistical transformations of dietary variables. Specifically, we compare the heritability of crude averages with Empirical Bayes weighted averages for 302 correlated dietary variables from multiple 24-hour recall questionnaires in 177 K individuals in UK Biobank. Overall, the crude averages for frequency of consumption are more heritable than their Empirical Bayes counterparts only when the reliability of that item across questionnaires is high (measured by intra-class correlation), otherwise, the Empirical Bayes approach (for both unreliably measured frequencies and for average quantities independent of reliability) leads to higher heritability estimates. We also find that the more heritable versions of each dietary variable lead to stronger underlying statistical associations with specific genetic loci, many of which have well-known mechanisms, further supporting heritability as an alternative metric for relative validity in nutritional epidemiology and beyond.

KEYWORDS

heritability, nutrigenomics, nutritional epidemiology, 24-hour diet recall questionnaires, relative validity, phenotyping, empirical bayes, longitudinal data

Introduction

Dietary data are commonly collected longitudinally to enhance precision of dietary intake estimates. A variety of statistical approaches have been developed to best use this type of data in nutritional epidemiology. The simplest univariate approach is to collapse the data points per individual into a single aggregate mean or median, most appropriate when the variable is expected to be stable over time (Schober and Vetter, 2018). However, usual dietary intake is often estimated from unstable dietary questionnaire data with high day-to-day variation, such as from the 24-hour recall (24HR) questionnaire which records all foods and beverages consumed in a single day. For foods and beverages not consumed on a daily basis, the simple average approach over a small number of days is typically not adequate for capturing true habitual intake (Dodd et al., 2006). Nutritional epidemiologists and statisticians have developed methods to best handle this specific problem, most often applying sophisticated methods that estimate the true distribution of usual intake after accounting for within-person variability or for the correlation between observations in a mixed effects model (Dodd et al., 2006; Tooze et al., 2006). Extensions of these approaches apply the regression calibration approach for measurement error correction to estimate individual usual intake as the estimated conditional expectation given the empirically observed 24HR data [i.e., the Empirical Bayes (EB) method], which then allows for downstream association with an outcome of interest (Kipnis et al., 2009).

A key outstanding challenge addresses how best to evaluate and compare the performance of these various methods in increasing phenotype precision. The most common approach in epidemiology to assess relative validity is to demonstrate an improvement in the correlation of the processed phenotype with a "gold standard" measurement. The correlation of total energy intake or protein intake with doubly-labeled water (International Atomic Energy Agency, 1990) or urine protein levels (Greenwood et al., 2019), respectively, are classic examples of evaluating the validity of dietary intake derived from diet questionnaires. The EB method for estimating individual usual intake along with the incorporation of key covariates has also demonstrated improved phenotype precision when specifically testing the association between fish intake and blood mercury levels (Kipnis et al., 2009). In principle, the strength of association becomes stronger when noise and measurement error is reduced (Paeratakul et al., 1998; Willett, 2012). However, these approaches are only viable when a known gold standard measurement of the outcome of interest exists; these gold-standard methods are often laborious and time-intensive, and thus an alternative high-throughput approach is needed.

Genetics, and in particular genetic heritability, can be used as an unbiased and high-throughput metric to quantitatively benchmark and compare different phenotyping approaches, because nearly all human traits, including dietary intake, are influenced by genetic variation, either directly or indirectly (Ge et al., 2017a; Cole et al., 2020). This ubiquity of an underlying biological association allows our approach to use a common multi-variable human reference (i.e., the human genome) to estimate a summary aggregate variable of association (i.e., heritability) rather than rely on phenotype-specific gold-standard correlates. Furthermore, unlike other biological -omics datasets, genotypes also benefit from being captured in an unbiased and accurate manner nearly evenly throughout the genome, their easy accessibility, their increasing affordability, and their stability through an individual's lifetime with their consequent robustness to environmental confounders.

In this brief report we outline a preliminary investigation of genetic heritability as an anchor to compare relative validity of the same phenotypes derived using different statistical transformations, and we test its use at scale on hundreds of longitudinal 24HR questionnaire dietary variables from approximately 176 K individuals in UK Biobank (UKB). A flow chart of the study design is included in Supplementary Figure S1. Specifically, we derive a set of EB food intake variables over multiple 24HR questionnaires per person and compare these EB weighted values to crude unweighted estimates of either how often the food or beverage was consumed (proportions: Number of times consumed/number of questionnaires taken) or how much was consumed (averages: average amount over multiple questionnaires) using heritability analysis. Ultimately, we use heritability as a proxy for phenotype quality to determine if and when the EB method outperforms its crude counterpart across multiple variables simultaneously without the need for known gold standard correlates.

Methods

UK Biobank sample

UK Biobank is a prospective cohort of 500 K adults ages 40–69 at baseline collected from 2006 to 2010 across the UK. This large biomedical and research resource contains biological samples used to derive genetics, metabolomics, proteomics, and biomarkers as well as detailed phenotyping information spanning physical measures, imaging, lifestyle questionnaires, and health outcomes from multiple sources (self-reported, nurse interviews, and linked medical records). Extensive details on the genotyping, imputation, and quality control of this data, in addition to methodological details on deriving a subset of individuals of European ancestry (N = 455,146) used herein have been described elsewhere (Bycroft et al., 2018; Cole et al., 2020). All individual-level analyses were conducted under UKB

application 11898 in compliance with UKB regulations and all participants provided informed consent.

Dietary phenotype derivation

UKB contains data from two distinct dietary intake questionnaires. The first is a brief modified food frequency questionnaire (FFQ) of roughly 30 questions pertaining to habitual intake and frequency of foods and beverages over the previous year, asked of all participants in-person using a touchscreen at their baseline assessment center visit. The second, which is the sole dietary data source for this study, is a detailed 24HR questionnaire in which a subset of participants answered over 200 questions on specific foods and beverages consumed (with quantities) in the preceding 24-hour day. The 24HR was implemented as a questionnaire for the final 70 K in-person baseline assessment center participants from 2009–2010 and emailed four times to 320 K participants who consented to re-contact *via* email between February 2011 and April 2012. Approximately 200 K individuals have at least one and up to five recorded 24HR questionnaires.

Each questionnaire was filtered for credible estimates of total energy intake [≥1,000 kJ (UKB field 100002) and ≤20 MJ for males and ≤18 MJ for females (UKB field 100026)], typical dietary intake (UKB fields 100020 and 20085), completion duration greater than or equal to 5 min (UKB field 20082), and overall completion (UKB field 20081). Additionally, the participant could not be pregnant within 1 year of taking the 24HR nor have a cancer diagnosis within the previous year (UKB fields 3,140 and 40005). All 24HR questions were converted into 1/0 for yes/no to consumption; each categorical response was coded similarly [e.g., UKB field 20086 for special diet was converted into six binary variables for each response (gluten-free, lactose-free, low calorie, vegetarian, vegan, and a combined vegetarian or vegan field)]. 24HR questions pertaining to quantity consumed were also included as continuous variables.

After individual questionnaire pre-processing, all available data from all questionnaires were combined into two types of phenotypes: "proportions" for all food items representing the number of times consumed over the total number of questionnaires taken, and "averages" of continuous items only (i.e., quantities) which are simply averages over multiple questionnaires taken. Each phenotype type (proportions and averages) was derived using two approaches for comparison: "crude," representing the simple un-weighted derivations as indicated above, and "Empirical Bayes (EB)," which applies the EB method to weight individual responses based on the number of questionnaires taken.

EB proportions were calculated by first estimating empirical distribution parameters (alpha and beta) from a zero-one inflated distribution fit using the gamlssInf0to1 function in the gamlss.inf and gamlss R packages (Stasinopoulos et al., 2017), then calculating an EB proportion as follows: (number of successes + alpha)/(total number of questionnaires + alpha + beta). Of note, two nearly homogenous variables did not converge (UKB field 100920 milk type: "any" and a combined total drinks variable); for these we used parameters estimated from a similar variable distribution (UKB field 100920 any dairy milk type: "semiskimmed," "skimmed," and/or "whole"). EB averages were calculated by first fitting a Dirichlet-multinomial mixture model to all continuous variables as a matrix of possible responses and counts for each response as implemented in the DirichletMultinomial R package (Morgan, 2022) This fit model empirically estimates an alpha parameter to update each individual response based on the raw values and counts. Once a weighted value is obtained for each possible response, each individual's EB average is obtained by summing their weighted values over the total number of questionnaires plus the sum of the alpha estimates. A detailed explanation with R code has been described previously (Robinson, 2017).

Using genetics to benchmark phenotype precision

To estimate heritability, we first conducted genome-wide association study (GWAS) analysis on each phenotype using REGENIE whole genome regression software (version1.0.6.7), which allows for the inclusion of related individuals using a model similar to a linear mixed model (Mbatchou et al., 2021). Briefly, we first prepared a set of quality-controlled markers by filtering to genotyped markers with minor allele frequency >0.5%, minor allele count >10, and missingness <10% in samples of genetically determined European ancestry (see above) with less than 10% genotype missingness (M = 784,256). We next conducted REGENIE as directed in two steps, first fitting a whole genome regression model capturing the phenotypic variance attributable to genetic effects, followed by testing the association between each 24HR diet phenotype and 58,299,817 imputed markers conditional upon the model in step one. The resulting genetic variants were filtered for imputation INFO score ≥0.6 and common variants with minor allele frequency ≥0.5%, resulting in genome-wide summary statistics for 11,006,968 variants across 1,288 total phenotypes.

Our specific analysis presented here was computationally intense and required a high-performance computing environment. REGENIE linear mixed model GWAS for 1,288 phenotypes in ~200 K individuals required splitting the data into six sets, each requiring approximately 30 GB of memory and 10 days of compute time. For more information on performance, please see the REGENIE documentation (https://rgcgithub.github.io/regenie/). Several factors would improve the computational cost of this approach including more heritable

phenotypes in smaller sample sizes and the use of traditional (and not mixed) linear or logistic GWAS models.

The following covariates were included in the genetic model: sex, average age in months, average age in months squared, assessment center (UKB field 54 as a factor), birthplace (UKB field 1,647 as a factor), self-reported ethnicity (UKB field 21000 as a factor), proportion of questionnaires taken on a weekend (Friday, Saturday, or Sunday; UKB field 20080), duration of questionnaire in minutes winsorized at 25 min (UKB field 20082), the proportion of questionnaires taken with a duration ≥25 min (UKB field 20082), average hour of the day completed (UKB field 20081), total number of questionnaires taken, ten genetic principal components derived previously (Cole et al., 2020), and genotyping array. We used LD score regression software (version1.0.0) and LD scores computed using 1,000 Genomes European data to extract heritability estimates of each 24HR dietary phenotype. A Bonferroni heritability significance threshold was obtained by dividing 0.05 by the number of effectively independent phenotypes (N = 46.1) as estimated by the "eigenvalue formula" (Bretherton et al., 1999) on eigenvalues obtained from a principal components analysis on all covariate-adjusted dietary variables.

Intra-class correlation, a measurement of consistency across measures (e.g., across multiple 24HR questionnaires), was calculated as the between-subject variance/(between-subject variance + within-subject variance) from individuals of European ancestry that took the questionnaire all 5 times (N = 2,066) in R with the "irr" package. The clump command within the PLINK2 software (Chang et al., 2015) and the 1000 Genomes Project phase 3 European reference (Auton et al., 2015) was used to determine the number of independent genome-wide significant loci ($P < 5 \times 10^{-8}$) in 500 kb windows in each GWAS, followed by collapsing signals across all GWAS together to leave only one lead SNP-phenotype association per window.

Results

The 24HR questionnaire contains over 200 questions on foods and beverages consumed in the previous 24-hour day. After individual 24HR questionnaire quality control and filtering, there were 176,858 individuals remaining for all downstream analysis. Among these individuals, over half took the questionnaire at least twice (N = 95,777; 54%) with 46,893 completing two, 31,818 completing three, 15,000 completing four, and 2,066 completing all five 24HR questionnaires (Supplementary Figure S2).

From 264 UKB 24HR questionnaire fields, many with multiple categorical responses, we derived 158 binary variables (yes/no to consumption) and 243 continuous variables (quantities). All variables were converted to proportions (how often a food/beverage was consumed over questionnaires taken) and all continuous quantities were also averaged over questionnaires taken. Finally, all variables underwent an EB transformation as described in the *Methods* section, resulting in both a crude and EB version for each phenotype, for a total of 1,288 phenotypes tested for downstream analysis (Supplementary Table S1; Figure 1; Supplementary Figure S3). Note, averages were calculated from all questionnaires taken for each individual, even when that food or beverage was not consumed (i.e., a quantity of 0). Therefore, the accuracy of average quantities of foods and beverages that are episodically consumed on an irregular basis will likely be lower than the accuracy of average quantities of foods and beverages that are more regularly consumed. An alternative averaging approach, which was not taken in this study, would be to average quantities of foods only from questionnaires in which the food was consumed or apply more sophisticated approaches for episodic foods as previously developed (Kipnis et al., 2009).

After limiting to phenotypes in which at least one approach (crude or EB) had a significant heritability estimate based on a multiple testing threshold corrected for effectively independent phenotypes ($p < .05/46.1 = 0.00108$; see *Methods*), 200 proportion and 102 average quantity phenotypes remained. The EB approach led to higher heritability for well over half the phenotypes (209/302 = 69%), and the improvement in heritability was much more prominent in the average quantity (91/102 = 89%) compared with the proportion phenotypes (118/200 = 59%; Figure 2).

Upon closer examination of the dietary proportion phenotypes, we noticed that the EB approach led to higher heritability estimates at the lower end of the heritability spectrum, while the crude proportions led to higher heritability estimates at the higher end of the spectrum. We hypothesized that foods and beverages that are consumed on a more regular basis and have less questionnaire-to-questionnaire variability would have the highest heritability estimates and benefit the least from our version of the EB approach. To test this, we calculated intra-class correlation, a measure of reliability across multiple measures, on all raw dietary variables from a subset of individuals that took all five 24HR questionnaires (N = 2,066; Supplementary Table S1). Not surprisingly, there is a strong correlation between the ICC (i.e., the reliability from questionnaire to questionnaire) and the estimated crude SNP heritability (overall correlation = 0.61, proportions = 0.54, averages = 0.74; Figure 3), with the highest heritability among the most reliable phenotypes.

Furthermore, as seen in Figure 3A and Supplementary Table S1, the crude approach consistently leads to higher heritability estimates than the EB approach among the most reliable phenotypes such as coffee intake, and *vice versa* among the least reliable phenotypes, such as chocolate intake. Specifically, for the proportions with ICC in the top quartile (ICC ≥ 0.513), the crude proportion leads to higher heritability 86% of the time

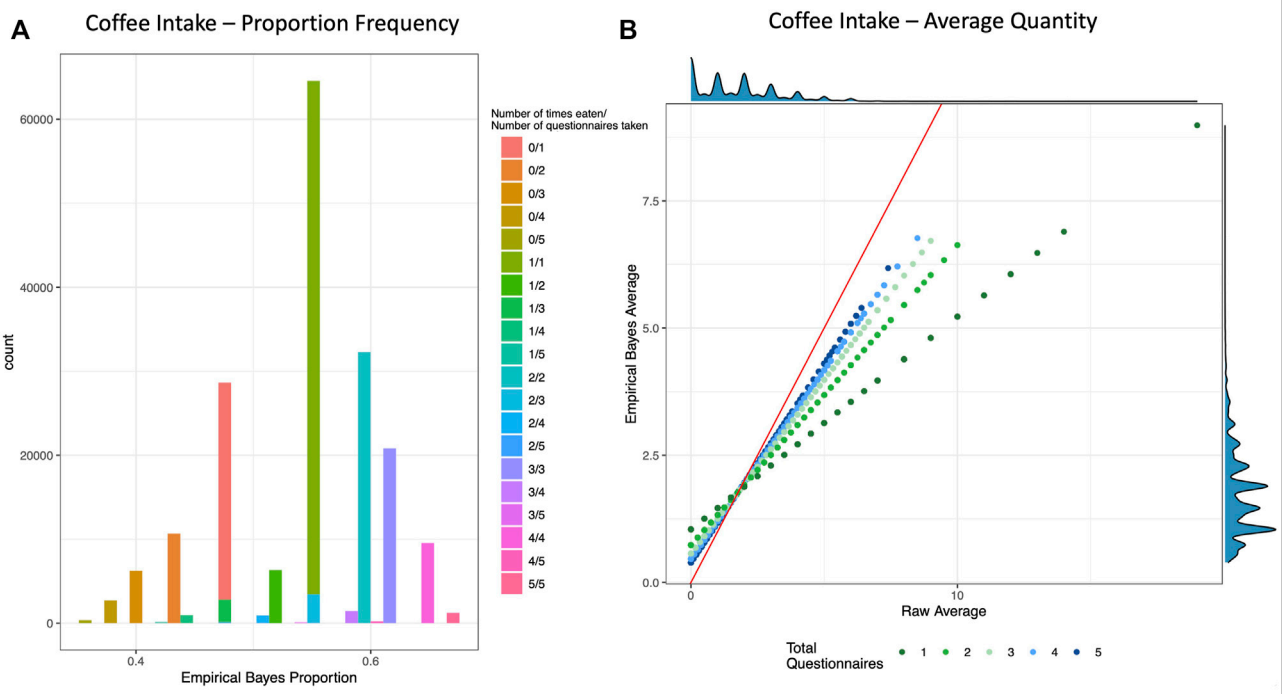

FIGURE 1
Empirical Bayes Proportion and Average Transformations: Coffee Intake. These example visualizations of coffee intake depict the shrinking of raw values for individuals with fewer total questionnaires. **(A)** Histogram of Empirical Bayes proportion colored and stacked by the crude proportion (number of times an individual reported drinking coffee out of how many total questionnaires that individual took). **(B)** Scatter plot of the crude average (*x*-axis) vs. the Empirical Bayes Average (*y*-axis) of cups of coffee per day, colored by total number of questionnaires taken. The red line is the line of identity, and the density plots are depicted on the top and right borders. See Supplementary Figure S3 for more examples.

(43/50 phenotypes), whereas for those derived from the least reliably reported foods and beverages in the bottom quartile (ICC ≤ 0.178), the EB proportion leads to higher heritability 84% of the time (42/50 phenotype comparisons). On the other hand, average quantities of foods and beverages, whether reliably reported from questionnaire to questionnaire or not, have consistently higher heritability estimates using the EB approach: 91% (21/26) in the top quartile (ICC ≥ 0.458) and 92% (24/26) in the bottom quartile (ICC ≤ 0.188) (Figure 3B). Habitually consumed beverages (e.g., coffee, water, tea, and alcohol) are among the most reliable (i.e., high ICC) and heritable phenotypes, and demonstrate this phenomenon well (Supplementary Figure S4). Although crude proportions of habitually consumed beverages have higher heritabilities, the EB version leads to higher heritability among the average quantity phenotypes, and even more so when the ICC is low.

Although gold standards are typically not available for most dietary phenotypes, some dietary phenotypes have strong associations at genetic loci with well-established mechanisms, which can serve as "genetic gold standards" for this subset of phenotypes. More broadly, if heritability were an appropriate metric to confidently assign and rank phenotype quality among different processing approaches, we would expect the more heritable version to have a stronger statistical association at genetic loci, particularly those with established biological mechanisms. To evaluate this question, we investigated the top associations from our GWAS data. Overall, we find that 208/379 (55%) of our independent loci associated with dietary intake (See *Methods*) are more strongly associated with the more heritable phenotype version (164 crude and 214 EB). Notably, these loci include well-known genetic gold standard associations such as SNP rs2472297 near the *CYP1A2* caffeine metabolism gene associated with coffee intake (Faber et al., 2005) and SNP rs2708381 in the *TAS2R46* bitter taste receptor gene (Andres-Barquin and Conte, 2004) associated with adding sugar or artificial sweetener to different beverages and foods. When filtering to dietary traits with the largest percent difference in heritability between the two versions (top 25% and top 10%), this concordance increases to 67% and 77%, respectively. This suggests that heritability may need to be substantially different to increase GWAS association strength.

Discussion

The overall goal of our study was to apply an EB approach to account for variability in number of repeated measures in dietary data and use an unbiased metric for assessing its utility in a high-throughput manner. While gold standard measurements are

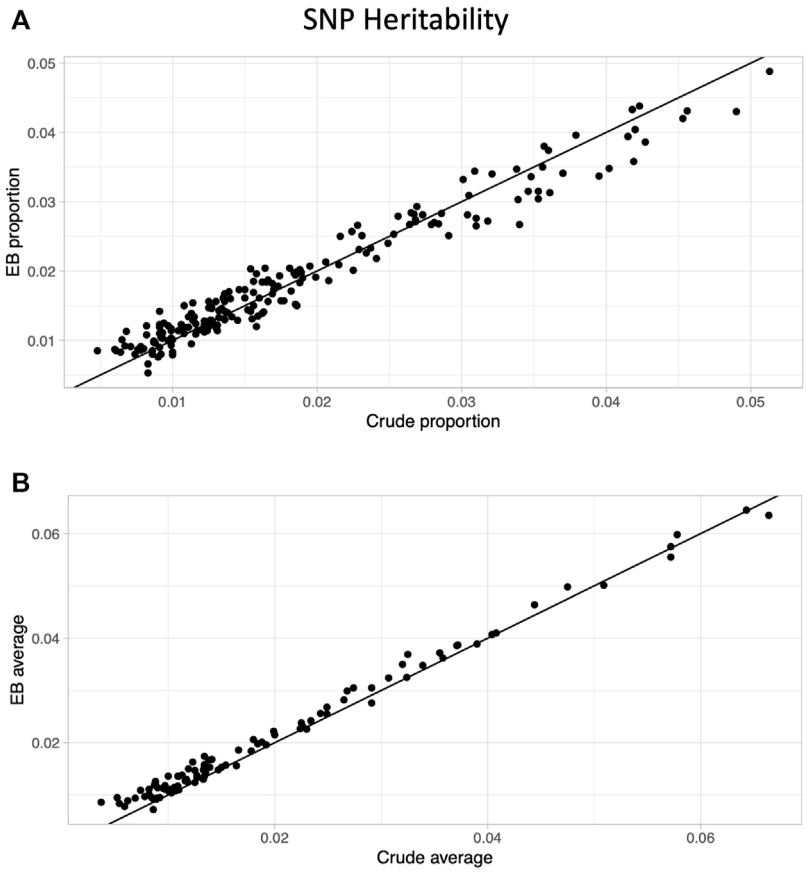

FIGURE 2
Heritability comparison between crude and Empirical Bayes approaches. Scatter plots of SNP heritability estimates comparing crude (*x*-axis) and Empirical Bayes (*y*-axis) for proportion phenotypes **(A)** and average phenotypes **(B)**. The black line is the line of identity.

often used in epidemiology to assess validity, they are often limited, unknown, or unmeasured in practice. Heritability provides a simple and broadly applicable extension of this approach that capitalizes on the measurable, non-zero heritability of the great majority of phenotypes (Ge et al., 2017a), meaning that a portion of their phenotypic variance is explained by genetic variance. Even if this heritability derives from a different, heritable mediator phenotype (as is often the case with largely environmentally-driven traits like dietary intake), increased precision in phenotypic measurement will result in reduced observed phenotypic variance and hence increased estimated heritability. Here, we use heritability estimates as an unbiased metric to compare the relative validity of phenotype processing approaches, and apply this standard simultaneously across hundreds of dietary variables.

Unlike the dietary data in UKB, typical nutritional epidemiology-focused cohorts capture dietary intake more often, at regularly spaced intervals, and validate with multiple different questionnaires (WILLETT et al., 1985; Ocké et al., 1997). Still, previous work has found that dietary variables derived from the 24HR questionnaire in UKB have ICC and correlations with biomarkers comparable to those derived from the more burdensome conventional studies (Carter et al., 2019; Greenwood et al., 2019). Furthermore, although dietary intake is a behavioral trait that is influenced by many external health and socio-cultural factors, we find that 302 of our overlapping derived dietary phenotypes have significant, albeit modest, heritabilities. Together, these findings support the utility of the UKB 24HR questionnaire data for capturing meaningful information for future studies, potentially in combination with the UKB FFQ, which alone does not contain enough information to estimate energy and nutrient intake.

We apply a Bayesian approach using the empirical data at hand to estimate distribution parameters and update individual estimates of proportion and average quantity phenotypes, representing how often and how much a food or beverage is consumed, respectively. The EB approach leads to higher heritability estimates more often than its crude counterpart, most often when considering average quantities consumed, and least often when examining yes/no questionnaire variables for foods and beverages that are

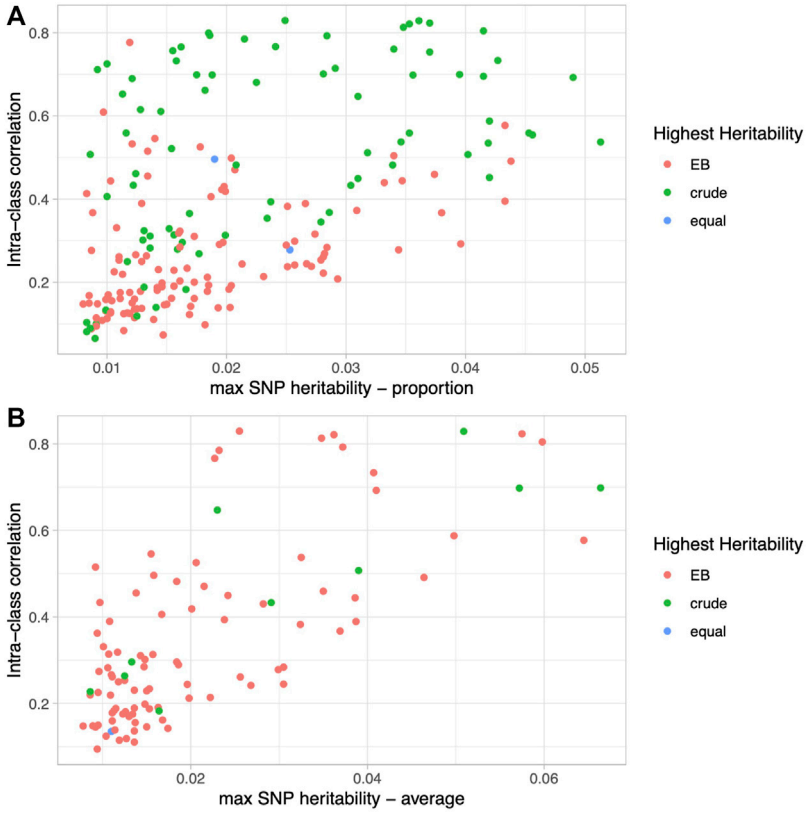

FIGURE 3
Relationship between phenotype reliability and heritability. Scatter plots of intra-class correlation (*y*-axis) versus SNP heritability estimates (*x*-axis) colored by the method (EB, crude, or equal) that led to the higher heritability for proportion phenotypes **(A)** and average phenotypes **(B)**.

consumed habitually with high reproducibility. There is a wide array of research and literature on accounting for measurement error in 24HR questionnaires, and future work could expand upon this brief report to compare these additional approaches to each other under different circumstances, such as among different dietary intake classes (e.g., foods, food groups, nutrients, and dietary patterns) or underlying frequency (e.g., habitual and episodic) (Kipnis et al., 2009; Bennett et al., 2017). We speculate, based on the findings within, that the more stable and reliable the dietary trait, such as with macronutrient levels, the less of a noise reduction and power gain would be seen using the Empirical Bayes and other measurement error correction methods.

In summary, we provide support for using heritability estimates as a novel tool for assessing phenotype quality in a high throughput manner, leveraging relationships with genetic variation on thousands of individuals as a common reference for hundreds of traits. A key feature that makes this type of analysis a viable and scalable approach is the stable and consistent genetic backbone that all individuals share, which genome-wide genotyping data are making more readily available in many large cohorts and biobanks throughout the world. Together with a thoughtful understanding of the biological question at hand, heritability can be used to optimize dietary variable processing and phenotype derivation. This approach can be extended to many traits and phenotype processing approaches beyond the field of nutritional epidemiology, as the principle of this work only hinges on a non-zero heritability. However, a key limitation to this approach is that heritability must be detectable. We demonstrate that the large sample size of the UKB allowed us to detect even modest heritability for many but not all noisy and environmentally mediated dietary traits derived from UKB's 24HR questionnaire. Furthermore, unlike correlations with known biomarkers, our use of heritability only quantifies the relative precision of dietary phenotypes, and does not discern their accuracy, particularly if mediated (and to different extents) through another heritable trait, as is often the case with dietary intake (e.g., health conditions and socioeconomic status) (Pirastu et al., 2022). Complete mediation of the relationship between genetic variants and dietary intake by heritable health conditions (e.g., medical advice that changes eating habits) would limit the use of this approach in a population free of the condition at hand. In the end, heritability is a metric of an underlying biological relationship, direct or indirect,

with the phenotypes at hand; therefore, a key assumption when comparing the same phenotype processed in two different ways is that the same genetic variants are at play, and the heritability estimate is capturing phenotype precision alone. As discussed, the use of heritability as a precision metric is best suited for comparing different transformations of the same phenotype, but an important next question is then how to compare heritability between two different phenotypes with both different levels of phenotype precision *and* different underlying genetic determinants. Furthermore, applying a recently developed approach that estimates heritability after correcting for measurement error (Ge et al., 2017b) to the nutritional data in UKB is a compelling and complementary next step to truly determine which dietary traits are more heritable.

Data availability statement

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

Author contributions

JC conceptualized and designed the project, conducted analyses, and wrote and edited the manuscript. KW designed the project and edited the manuscript. AM, JF, and JH oversaw the design of the project and edited the manuscript.

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Conflict of interest

JNH has equity in Camp4 Therapeutics.

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.

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Supplementary material

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Birth outcomes, puberty onset, and obesity as long-term predictors of biological aging in young adulthood

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Background: Biological aging and particularly the deviations between biological and chronological age are better predictors of health than chronological age alone. However, the predictors of accelerated biological aging are not very well understood. The aim was to determine the role of birth outcomes, time of puberty onset, body mass index (BMI), and body fat in accelerated biological aging in the third decade of life.

Methods: We have conducted a second follow-up of the Czech part of the European Longitudinal Study of Pregnancy and Childhood (ELSPAC-CZ) prenatal birth cohort in young adulthood (52% male; age 28–30; n = 262) to determine the role of birth outcomes, pubertal timing, BMI, and body fat on biological aging. Birth outcomes included birth weight, length, and gestational age at birth. Pubertal timing was determined by the presence of secondary sexual characteristics at the age of 11 and the age of first menarche in women. Biological age was estimated using the Klemera-Doubal Method (KDM), which applies 9-biomarker algorithm including forced expiratory volume in one second (FEV1), systolic blood pressure, glycated hemoglobin, total cholesterol, C-reactive protein, creatinine, urea nitrogen, albumin, and alkaline phosphatase. Accelerated/decelerated aging was determined as the difference between biological and chronological age (BioAGE).

Results: The deviations between biological and chronological age in young adulthood ranged from −2.84 to 4.39 years. Accelerated biological aging was predicted by higher BMI [in both early (R^2_{adj} = 0.05) and late 20s (R^2_{adj} = 0.22)], subcutaneous (R^2_{adj} = 0.21) and visceral fat (R^2_{adj} = 0.25), puberty onset (η_p^2 = 0.07), birth length (R^2_{adj} = 0.03), and the increase of BMI over the 5-year period between the two follow-ups in young adulthood (R^2_{adj} = 0.09). Single hierarchical model revealed that shorter birth length, early puberty onset, and greater levels of visceral fat were the main predictors, together explaining 21% of variance in accelerated biological aging.

Conclusion: Our findings provide comprehensive support of the Life History Theory, suggesting that early life adversity might trigger accelerated aging, which leads to earlier onset of puberty but decreasing fitness in adulthood, reflected by more visceral fat and higher BMI. Our findings also suggest that reduction of BMI in young adulthood slows down biological aging.

KEYWORDS

biological aging, BMI, obesity, puberty, birth outcomes, life history theory

1. Introduction

We live in an era of unprecedented aging (1). The percentage of people aged 65 and higher worldwide was 9% in 2019 and is expected to rise to 12% by 2030 and to 16% by 2050 (2). This increase in lifespan brings a proportional increase in age-related disease (3, 4). Previous research suggested that age-related changes in the organism accumulate well before the onset of disease and that even early life factors contribute to the speed of aging (5–7). In order to intervene early, a better understanding of the age-related changes and early detection of the altered aging trajectory is crucial (8).

To measure the aging process, US National Health and Nutrition Survey (NHANES) studied participants aged 30–75 years and developed a 10-biomarker-based measure of "Biological Age", which was more successful in predicting mortality in the 20-year follow-up than chronological age (9). Using the NHANES algorithm, Belsky et al. (8) calculated the Biological Age of Dunedin Study members and found variations in biological aging in young individuals of the same chronological age. While all participants were 38 years old, their biological age varied from 28 to 61 years (8). Higher biological vs. chronological age was associated with poorer physical fitness, appearance, and cognitive decline (8). The current study aims to find predictors of such accelerated biological aging.

Growing evidence in the last decade suggests that higher Body mass index (BMI) can have detrimental effect on life expectancy (10–12). Obesity has been linked to multiple chronic diseases, reduced functional capacity and lower quality of life (11–14). It is thus of no surprise that anti-aging strategies proposed to extend lifespan focus on caloric restriction (15). Promising results have been reported in primates, but their effectiveness is yet to be verified. However, shared epigenetic signatures (e.g., histone modification, DNA methylation, non-coding RNAs, and chromatin remodeling) have been reported in obesity and aging (16), suggesting BMI might be a possible predictor of biological aging.

Higher BMI in adulthood was associated with earlier onset of puberty (17, 18), another important predictor of all-cause and cardiovascular mortality (19, 20). According to Belsky (21, 22) early pubertal maturation and accelerated biological aging are part of the same evolutionary-developmental process, i.e., Life History Theory. Recent research supported this theory by demonstrating accelerated epigenetic aging in women with earlier onset of puberty (5, 23).

According to Belsky and Shalev (22, 24), earlier pubertal maturation is the result of faster biological aging that stems from adverse/stressful events early in life. Further research on aging and timing of puberty reported that child maltreatment (sexual, physical, or emotional abuse) predicts earlier onset of puberty in women (25) and is associated with accelerated epigenetic aging (5). Earlier pubertal maturation was also found in women who reported more risky and uncertain environments early in life (26) and in the offspring of mothers who reported depression symptoms, marital conflict, and financial stress during pregnancy (27). Consistently, an independent line of research associated higher mortality with preterm birth (28) and small body size indicated by small ponderal index (29), suggesting that birth outcomes might be among the key predictors of biological aging.

This emerging evidence suggests that higher mortality in adulthood is associated with accelerated biological aging, which might have its roots in early life. The current study aims to use data from the European Longitudinal Study of Pregnancy and Childhood (ELSPAC-CZ) prenatal birth cohort (30) and its two follow-ups in young adulthood [VULDE, Health Brain Age (7)] to determine the role of birth outcomes, time of puberty onset, BMI, and body fat in accelerated biological aging in the third decade of life. Since earlier pubertal development has been reported in women compared to men (31) and previous studies showed different trajectory of fat distribution between men and women during pubertal development (32) that continue with aging (33), we also considered potential sex differences in the relationships.

2. Materials and methods

2.1. Participants

A total of 262 young adults (52% men, 28–30 years of age; all of European ancestry) participated in the Health Brain

Age project at the Central European Institute of Technology, Masaryk University (CEITEC MU), a follow-up of the Czech part of the European Longitudinal Study of Pregnancy and Childhood (ELSPAC-CZ) (30), a prenatal birth cohort born in the South Moravian Region of the Czechia between 1991 and 1992. A subset of these participants (n = 110, 51% men) also took part in the first follow-up of this prenatal birth cohort at the age of 23–24 years, entitled Biomarkers and Underlying Mechanisms of Vulnerability to Depression (VULDE; n = 131) (7), and thus have a within-subject design data regarding anthropometrics in young adulthood. A diagram illustrating the sample size of the different studies as well as the final sample of the current study is provided in **Supplementary Figure 1**. Men and women did not differ in any of the demographic variables; detailed characteristics of the Health Brain Age sample can be found in **Table 1** and descriptive statistics and sample size included in the different analyses can be found in **Table 2**. All participants gave written informed consent for participation in Health Brain Age and VULDE (when applicable) and agreed to merge their historic data from ELSPAC-CZ and the subsequent studies. Informed consent was approved by the ELSPAC Ethics Committee.

2.2. Procedures

2.2.1. Anthropometric measures

Weight and height were measured once at birth and twice in young adulthood (age 23–24, age 28–30). BMI in young adulthood was calculated as the ratio of the participant's weight (kg) and height (m^2). Total body fat and amount of visceral fat in young adulthood were estimated by bio-impedance using the scale Tanita BC-545 N. The bio-impedance scale was used in a standardized manner for all participants; the procedure followed the collection of fasting blood sample, and all participants were provided water during the consent procedure. All participants were also instructed not to drink alcohol the day before. Subcutaneous fat in young adulthood was measured with skinfold calipers at four locations (biceps, triceps, suprailia, and under scapula) using a standard procedure and the mean of these four measures (in millimeters) was used in the subsequent analyses.

2.2.2. Gestational age

Gestational age was calculated as the difference between the date of birth and the ultrasound-based date of conception.

2.2.3. Puberty development and onset of puberty

At the age of 11, pediatricians assessed the development of secondary sexual characteristics (breasts in women, penis in men, and pubic hair in both sexes) on a scale from 1 (least developed) to 4 (most developed). Participants with less developed secondary sexual characteristics at the age of 11 were classified as the early puberty onset group. In women, the age of menarche served as an additional predictor of puberty onset.

2.2.4. Biomarkers in young adulthood

In the late 20s, forced expiratory volume in one second (FEV1) was calculated using MIR Smart One Spirometer. Systolic and diastolic blood pressure were assessed according to standard protocols. Blood samples were taken in the morning before the first meal. Cholesterol, C-reactive protein (CRP), glucose, albumin, creatinine, urea nitrogen serum levels (mg/dL) as well as alkaline phosphatase activity in serum (U/L) were measured on ROCHE analyzer (Cobas Integra 400, Roche diagnostics). The percentage of glycated hemoglobin was calculated based on glucose levels according to published equations and recommendations of the international consensus statement (34–37).

2.2.5. Calculation of biological age and BioAGE in young adulthood

Biological age was calculated using Klemera-Doubal Method (KDM), available through the R package "BioAge" (9) that applies a 9-biomarker algorithm including forced expiratory volume in one second (FEV1), blood pressure (systolic), glycated hemoglobin, total cholesterol, C-reactive protein, creatinine, urea nitrogen, albumin, and alkaline phosphatase (see **Supplementary Table 1** for descriptive statistics of biomarkers). The difference between biological age and chronological age (BioAGE) thus reflects accelerated/decelerated aging.

2.3. Statistical analysis

All statistical analyses were performed in SPSS version 28.0.0 (IBM SPSS Statistics). First, we assessed the distribution of data, and variables that did not follow a normal distribution were transformed using logarithmic transformation. Outliers that were greater than three standard deviations were removed from the analysis.

Measures of secondary sexual characteristics were fed into Two-Step Cluster Analysis (separate for both sexes) using Schwarz's Bayesian Criterion to automatically detect clusters. Linear regression was used to assess the predictors of BioAGE. In each model where men and women were treated as one group, sex and the interaction between sex and the predictor were treated as covariates. The significant predictors were then used in a hierarchical multiple linear regression to assess the multiple predictors of BioAGE within a single model. Predictors entered the model following the order of the lifetime: 1. Birth length, 2. Puberty onset, 3. Fat measures in adulthood (visceral and subcutaneous simultaneously). Two analogous models were estimated: one for the whole group with sex as a covariate,

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TABLE 1 Demographic information.

Demographics	Men	Women	Between group differences
	(*N* = 136)	(*N* = 126)	
Ethnicity			
White Caucasian	100%	100%	n/a
Age			
in years	M = 28.96 (±0.67)	M = 28.99 (±0.69)	t(260) = 0.34, $p = 0.732$
Education			
Not completed high school	2%	2%	$\chi^2(3) = 5.94$, $p = 0.110$
Completed high school	29%	18%	
Completed university	66%	79%	
Completed postgradual education	2%	1%	
Missing	0%	0%	
Maternal education			
Not completed high school	12%	18%	$\chi^2(4) = 7.59$, $p = 0.108$
Completed high school	34%	33%	
Completed university	26%	23%	
Completed postgradual education	4%	1%	
Missing	25%	25%	
Materinal smoking			
Not smoking during pregnancy	66%	67%	$\chi^2(1) = 0.10$, $p = 0.748$
Smoking during pregnancy	9%	10%	
Missing	25%	22%	

and another one for women only, where the year of the first menarche was used as a more precise measure of puberty onset. Simple group differences were analyzed using an independent samples *t*-test. Group by puberty onset interaction was assessed using two-way ANOVA. Multiple comparisons were corrected using the False Discovery Rate (FDR) method and thus FDR-corrected *p*-values larger than 0.05 were considered significant.

3. Results

3.1. Biological aging in late 20s

While all participants were 28–30 years old, their current biological age ranged from 26.07 to 34.20 years, and thus their BioAGE ranged from −2.84 to 4.39 years (**Figure 1**).

3.2. Does BMI and body fat in the late 20s predict biological aging in the late 20s?

BioAGE in the late 20s (**Figure 2A**) was predicted by higher BMI [$R^2_{adj} = 0.22$, $F(3,256) = 25.03$, $\beta = 0.10$, $p < 0.001$], overall body fat [$R^2_{adj} = 0.17$, $F(3,256) = 19.22$, $\beta = 0.07$, $p < 0.001$], subcutaneous fat [$R^2_{adj} = 0.21$, $F(3,256) = 23.35$, $\beta = 0.08$, $p < 0.001$] and visceral fat [$R^2_{adj} = 0.25$, $F(3,256) = 30.44$, $\beta = 0.16$, $p < 0.001$]. In addition, we found an interaction effect between sex and BMI (for every unit of BMI increase, BioAGE in women increased 0.08 years more than in men, $\beta = 0.79$, $p = 0.022$) and between sex and visceral fat (for every percent increase in visceral fat, BioAGE in women increased 0.2 years more than in men, $\beta = 0.35$, $p < 0.001$).

Post-hoc regressions in each sex revealed that BioAGE in late 20s was predicted by BMI in both women [$R^2 = 0.33$, $F(1,123) = 61.20$, $\beta = 0.58$, $p < 0.001$] and men [$R^2 = 0.10$, $F(1,133) = 15.13$, $\beta = 0.32$, $p < 0.001$], and by visceral fat in both women [$R^2 = 0.35$, $F(1,123) = 68.09$, $\beta = 0.60$, $p < 0.001$] and men [$R^2 = 0.15$, $F(1,133) = 23.92$, $\beta = 0.39$, $p < 0.001$].

3.3. Does BMI and body fat in the early 20s predict biological aging in the late 20s?

BioAGE in the early 20s (**Figure 2B**) was significantly associated with higher BMI [$R^2_{adj} = 0.05$, $F(3,105) = 3.09$, $\beta = 0.04$, $p = 0.037$], overall body fat [$R^2_{adj} = 0.09$,

TABLE 2 Biological aging and its predictors–descriptive statistics.

	Descriptive Statistics					Women					Men				
Measure	*N*	Min	Max	Mean	SD	*N*	Min	Max	Mean	SD	*N*	Min	Max	Mean	SD
Biological aging															
BioAGE	260	−2.84	4.39	0.00	1.19	125	−2.84	4.20	−0.03	1.27	135	−2.36	4.39	0.03	1.12
Current biological age	260	26.07	34.20	28.98	1.37	125	26.07	34.20	28.95	1.42	135	26.17	33.39	29.00	1.33
BMI and body fat in late 20s															
BMI	262	16.3	40.3	24.26	4.03	126	16.3	40.3	23.54	4.15	136	16.8	38.8	24.93	3.80
Overall body fat	262	5.0	47.4	23.04	9.07	126	7.9	47.4	29.35	7.34	136	5.0	35.2	17.20	6.14
Subcutaneous fat	262	3.9	33.8	13.14	5.95	126	6.1	33.8	14.72	6.07	136	3.9	33.1	11.68	5.46
Visceral fat	262	1.0	15.5	3.86	2.62	126	1.0	11.5	3.13	2.11	136	1.0	15.5	4.53	2.87
BMI and body fat in early 20s															
BMI	110	15.1	37.2	23.12	3.39	54	15.1	28.0	22.01	2.81	56	18.5	37.2	24.20	3.56
Overall body fat	109	14.7	45.0	28.30	6.49	54	14.7	45.0	29.79	6.62	55	17.4	40.0	26.84	6.06
Subcutaneous fat	110	5.5	28.5	13.57	5.33	47	6.3	28.5	14.25	4.91	63	5.5	27.8	13.07	5.61
Change in BMI and body fat (early–late 20s)															
BMI	110	−10.8	8.2	0.84	2.41	54	−4.8	8.2	0.77	2.24	56	−10.8	7.7	0.90	2.58
Overall body fat	110	−55.70	22.70	−5.93	8.59	54	−18.70	22.70	−1.66	6.30	56	−55.70	1.40	−10.06	8.53
Subcutaneous fat	110	−18.13	17.13	−1.38	5.62	47	−15.63	13.50	−1.04	5.28	63	−18.13	17.13	−1.63	5.90
Puberty development															
First period (years)	117	10	15	12.79	1.12	117	10	15	12.79	1.12					
Women: breast	62	1	4	2.10	0.84	62	1	4	2.10	0.84					
Women: pubic hair	62	1	4	1.94	1.02	62	1	4	1.94	1.02					
Men: genital	71	1	3	1.77	0.68						71	1	3	1.77	0.68
Men: pubic hair	69	1	3	1.39	0.57						69	1	3	1.39	0.57
Birth outcomes															
Birth weight (g)	256	1780	4600	3316	502	123	1780	4600	3159	476	133	1850	4600	3461	483
Bright length (cm)	256	40.0	56.0	50.25	2.36	123	40.0	54.0	49.46	2.45	133	43.0	56.0	50.98	2.01
Gestation (weeks)	133	37.43	42.43	39.97	1.12	66	37.43	42.00	39.88	1.08	67	37.43	42.43	40.05	1.17

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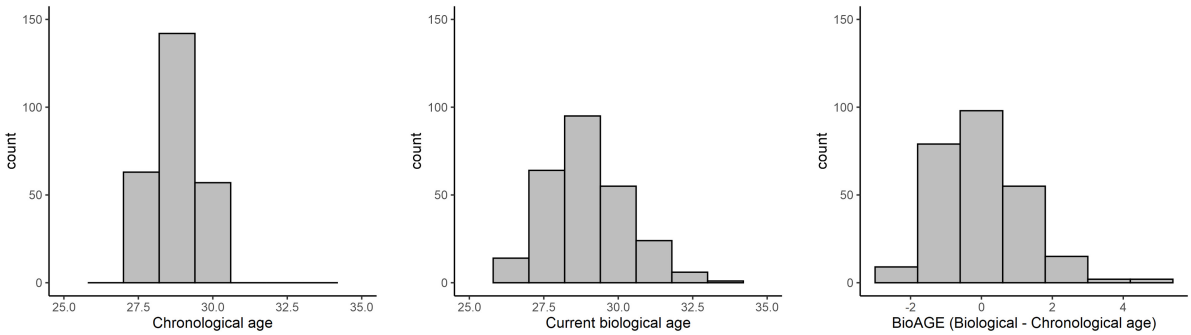

FIGURE 1
Distribution of chronological age, current biological age, and BioAGE.

F(3,104) = 4.54, β = 0.04, p = 0.009] and subcutaneous fat [R^2_{adj} = 0.05, F(3,105) = 2.78, β = 0.02, p = 0.049]. There were no interactions with sex (p = 0.022).

3.4. Does the change in BMI and body fat from the early to late 20s predict biological aging in the late 20s?

Decrease of BMI [R^2_{adj} = 0.09, F(3,105) = 4.39, β = 0.08, p = 0.009] and subcutaneous fat [R^2_{adj} = 0.07, F(3,105) = 3.77, β = 0.04, p = 0.018], but not of overall body fat (p = 0.431) over the 5-year period in young adulthood were associated with lower BioAGE in late 20s (Figure 2C). There were no interactions with sex (p = 0.189).

3.5. Does the timing of puberty onset predict biological aging in the late 20s?

The puberty data were classified into two categories based on the development of secondary sexual characteristics at the age of 11 in both women (late onset of puberty: n = 17, early onset of puberty, n = 45) and men (late onset of puberty: n = 26, early onset of puberty: n = 43).

Two-way ANOVA revealed a significant effect of puberty timing on BioAGE, BMI as well as body fat. Early puberty onset group had more accelerated BioAGE [η_p^2 = 0.07, F(1,125) = 10.01, p = 0.004], higher BMI [η_p^2 = 0.12, F(1,127) = 17.90, p < 0.001], overall body fat [η_p^2 = 0.07, F(1,127) = 10.05, p = 0.004], subcutaneous fat [η_p^2 = 0.12, F(1,127) = 17.99, p < 0.001] as well as visceral fat [η_p^2 = 0.09, F(1,127) = 12.11, p = 0.003] in late 20s than the late puberty onset group (Figure 3A). There was no significant interaction between puberty timing and sex on any of the dependent variables (p = 0.053).

Sex-specific *post-hoc* analyses showed that the effects of puberty onset were driven by women (Supplementary Figure 2). In women, early puberty onset group had more accelerated BioAGE [η_p^2 = 0.11, F(1,125) = 16.06, p = 0.001], higher BMI [η_p^2 = 0.13, F(1,127) = 18.62, p = 0.001], higher overall body fat [η_p^2 = 0.07, F(1,127) = 9.85, p = 0.009], subcutaneous fat [η_p^2 = 0.11, F(1,127) = 16.33, p = 0.001] and visceral fat [η_p^2 = 0.08, F(1,127) = 10.83, p = 0.007] than late puberty onset group (Supplementary Figure 2). No similar effects of puberty onset were found in men (p = 0.261). Complete statistics is reported in Supplementary Table 3.

Women with earlier onset of puberty experienced earlier first menarche [Cohen's d = 1.03, t(56) = 3.93, p < 0.001] (Figure 3B) and earlier first menarche predicted higher BioAGE [R^2 = 0.04, F(1,114) = 6.13, β = 0.26, p = 0.015] (Figure 3C).

3.6. Does birth weight, length, or gestational age predict accelerated biological aging in the late 20s?

Shorter birth length was associated with higher BioAGE [R^2_{adj}=0.03, F(3,250)=3.61, β=−0.08, p = 0.042], but no significant relationship emerged between birth weight (p = 0.127) or the duration of gestation (p = 0.843) and BioAGE (see Figure 4). There were no interactions with sex (p = 0.382).

3.7. Single model of accelerated biological aging combining predictors from birth to adulthood

Multiple regression evaluating the effects of birth length, puberty onset, and visceral and subcutaneous fat in the late 20s on BioAGE in the whole sample showed that all the predictors together explained 21% of the variance in biological aging [R^2_{adj} = 0.21, F(5,119) = 7.59, p > 0.001]. While

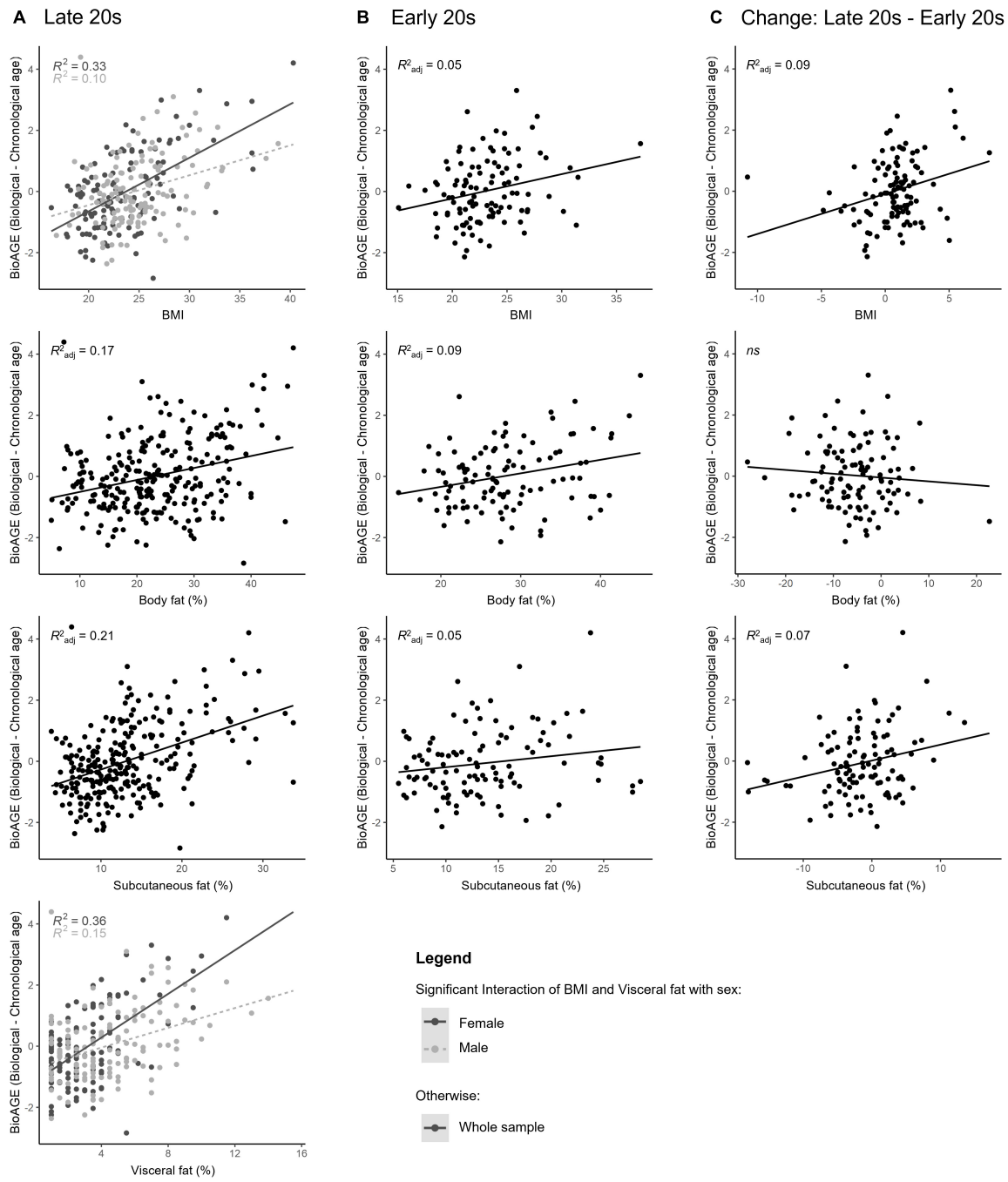

FIGURE 2
BMI and body fat in young adulthood as predictors of biological aging. Accelerated biological aging in the late 20s was predicted not only by BMI and body fat measured in the late 20s **(A)**, but also by BMI and body fat in the early 20s **(B)**, as well as the change in BMI and body fat over the 5-year period between the measurements **(C)**.

birth length explained 3.7% of the variance [$F(122,1) = 6.49$, $p = 0.012$], early puberty onset explained additional 5.2% [$F(121,1) = 8.00$, $p = 0.005$], and visceral fat another 12.1% [$F(119,2) = 10.28$, $p < 0.001$]. For every cm of birth length, BioAGE decreased by 0.119 years ($\beta = -0.236$, $p = 0.012$). Participants with early puberty onset had on average 0.56 years more advanced BioAGE than those with late puberty onset ($\beta = -0.244$, $p = 0.005$). For every% of visceral fat, BioAGE increased by 0.167 years ($\beta = 0.361$, $p = 0.006$). The effect of subcutaneous fat did not reach significance in the multiple regression ($p = 0.737$). Complete statistics with all regressors are in **Supplementary Table 4A**.

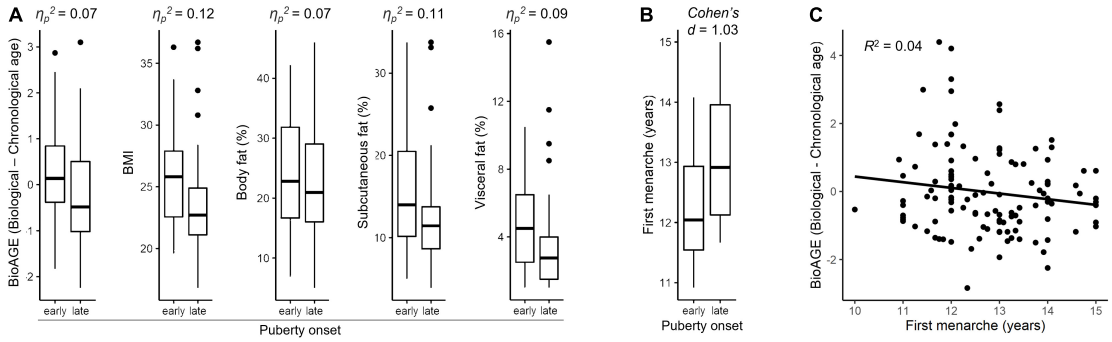

FIGURE 3
Pubertal timing. Differences between early and late onset of puberty based on secondary sexual characteristics in relation to accelerated biological aging, BMI, and fat measures in late 20s within whole sample **(A)** and in relation to age of first menarche in women **(B)**. Accelerated biological aging (BioAGE) in women predicted by earlier age of first menarche **(C)**.

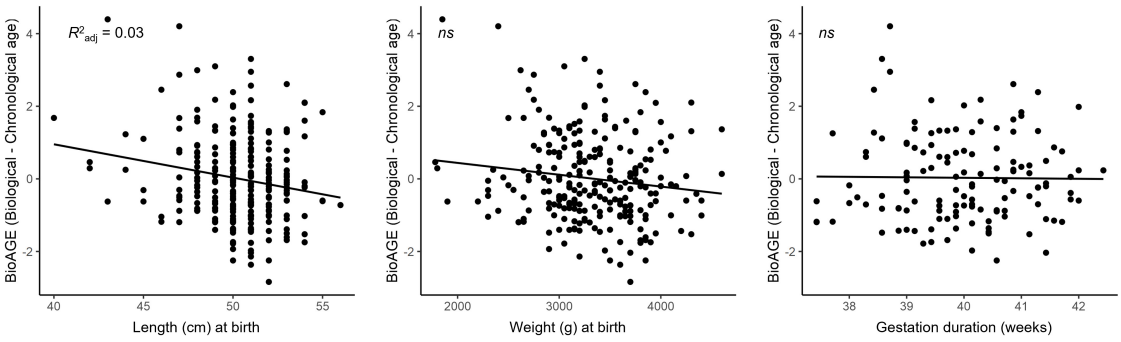

FIGURE 4
Accelerated biological aging in the late 20s was predicted by birth length but not birth weight or duration of gestation.

Similar multiple regression in women, where we could use the age of menarche as a more accurate measure of puberty timing, showed that the whole model explained even 30% of the variance [R^2_{adj} = 0.30, F(4,108) = 13.01, p > 0.001]. While birth length explained 6.9% of the variance [F(111,1) = 9.26, p = 0.003], adding year of first menarche explained additional 0.9% although not significant [F(110,1) = 2.11, p = 0.149], and body fat another 22.2% [F(1008,2) = 18.47, p < 0.001]. For every cm of birth length, BioAGE decreased by 0.137 years (β = −0.28, p < 0.003). For every% of visceral fat, BioAGE increased by 0.247 years (β = 0.445, p < 0.001). The effect of first menarche (p = 0.149) and subcutaneous fat (p = 0.634) did not reach significance in the multiple regression. Complete statistics with all regressors are in **Supplementary Table 4B**.

4. Discussion

We studied biological aging in young adults from the ELSPAC-CZ prenatal birth cohort and demonstrated that accelerated biological aging in young adulthood was associated with higher BMI as well as higher overall, subcutaneous, and visceral body fat, with visceral fat showing the strongest association. Moreover, we showed that the effects of BMI and body fat on biological aging are stable–present in both early and late 20s–and reach a greater effect size in women as compared to men. Most importantly, we demonstrated that reduction of BMI over the 5-year period between the measurements was associated with decelerated biological aging, suggesting that reducing weight over a relatively short period of time during adulthood can possibly slow down the pace of biological aging.

These findings extend previous prospective cohort studies, which linked higher BMI (10–12) and body fat (38) with increased mortality. They also support research by others reporting associations between higher BMI and accelerated epigenetic aging (39–43). While the mechanisms explaining the relationships between higher BMI and accelerated epigenetic aging remain to be clarified, the associations suggest the existence of a shared developmental mechanism (16).

The relationships between accelerated biological aging and higher BMI in both the late and early 20s demonstrate the

stability of the effect. But interestingly, a reduction of BMI over the 5-year period predicted lower BioAGE, suggesting that we might be able to slow down the speed of our biological aging by relatively accessible management options. This is in agreement with previous research suggesting dieting (44) and caloric restriction (15) as means to increase lifespan. Consistently, exercise was found to affect epigenetic changes in DNA methylation (45), histone modification (46), chromatin modifications (47), and non-coding RNAs (48) that are associated with aging (16). Further research is needed to assess the link between exercise and BioAGE.

Early puberty onset was another key predictor of accelerated biological aging, particularly in women. This is in agreement with previous studies that found a relationship between earlier menarche and accelerated epigenetic aging in women (5, 23). While Binder et al. (23) reported a relationship between epigenetic aging and menarche but not breast development, we found the effects of both menarche and breast development (together with pubic hair development). These divergent findings might be attributed to differences in methodology: first, our measure of aging is composed of wider selection of biomarkers; second, compared to the onset of breast development used by Binder et al. (23), we measured the degree of development at the age of 11.

We found only limited evidence for the hypothesized early life origins of biological aging. In particular, newborns who were shorter (but not lighter or younger) at birth were aging faster in their late 20s. This might be related to the fact that all our participants fell within the healthy range and the low variance in gestational age and birth weight might not have allowed us to detect any significant relationships with biological aging in young adulthood.

Finally, combining predictors of BioAGE from birth to adulthood allowed us to explain up to 21% of the variance in the whole sample and up to 30% of the variance in the women' group. Interestingly, visceral but not subcutaneous fat was a significant predictor of BioAGE. While both types of fat have been associated with increased morbidity (49, 50), there are indications that visceral fat is a more relevant predictor of cardiometabolic diseases than subcutaneous fat (51–53). Our findings suggest that higher levels of visceral fat might have important health consequences not only for the risk of cardiometabolic diseases but also aging and that high levels of visceral fat have particular negative impact on women. The puberty timing measured by secondary sexual characteristics was another significant predictor of accelerated aging in both the whole sample as well as women only. However, the timing of puberty measured by the first menarche did not constitute a significant predictor of BioAGE in women, when birth length and measures of visceral and subcutaneous fat in adulthood were accounted for. It must be noted that the effect of the first menarche was rather small even when considered alone and its lack of effect in the multiple regression model might be attributed to the limited sample size.

Overall, our findings support the Life History Theory, according to which early pubertal maturation can be accounted for accelerated biological aging (21). The rationale behind the theory is that the adaptation of an organism to early live adversity is reflected in accelerated aging. This leads to earlier pubertal maturation which increases the organism's chance of reproduction before dying. However, the payoff for the earlier pubertal maturation is decreased health in adulthood which is associated with aging, leading to increased morbidity and premature mortality.

Our study has several limitations that need to be acknowledged. First, the sample size is considerably small, which can be, at least in part, attributed to the longitudinal design of our study. Second, members of our prenatal birth cohort were not born preterm and had a healthy birth weight, limiting the possibility to study the impact of birth outcomes. Third, while our study uses longitudinal data for the predictors, the blood sample to estimate biological age was collected only at a single point at the late 20s. Further research is needed to assess the stability of the biological age gap (BioAGE) across the lifespan. Fourth, potential confounders such as lifestyle and dietary behavior might have affected the results and should be considered by future studies. Finally, this is a correlational study and therefore does not allow us to prove causal relationships between BioAGE and its predictors.

In conclusion, using longitudinal data on the ELSPAC-CZ prenatal birth cohort, we demonstrated that birth length, puberty timing, and visceral fat predict biological aging in young adulthood. In particular, the results of our study provide comprehensive support for the Life History Theory, suggesting that early life adversity might trigger accelerated aging, which in turn leads to earlier pubertal timing but decreasing fitness in adulthood, reflected by higher visceral fat and BMI. Moreover, we discovered that a reduction of BMI in young adulthood might slow down biological aging.

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 European Longitudinal Study of Pregnancy and Childhood (ELSPAC-CZ) Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MJ: investigation, formal analysis, and writing the original draft. LZ: investigation. PP: resources. LA: resources. MB: review and editing. KM: conceptualization, methodology, supervision, funding acquisition, review, and editing. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1100237/full#supplementary-material

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The effect of 12 weeks of combined training on hepatic fat content and metabolic flexibility of individuals with non-alcoholic fatty liver disease: Protocol of an open-label, single-center randomized control trial

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Introduction: Metabolic flexibility (MetF) is the capacity of an organism to oxidate substrate according to substrate availability or demand. The mismatch of substrate availability and oxidation may cause ectopic fat accumulation in the muscle and the liver. The objectives of the study are to examine the effect of 12 weeks of combined exercise on hepatic fat reduction and investigate metabolites related to MetF before and after the high-fat diet between individuals with NAFLD and healthy control with an active lifestyle.

Methods: This study is an open-label, single-center trial randomized controlled clinical study plus a cross-sectional comparison between individuals with NAFLD and healthy control. Individuals with NAFLD were allocated into two groups receiving resistance training (RT) combined with high-intensity interval training (HIIT) or moderate-intensity continuous training (MICT). Anthropometric indicators, clinical blood markers about glucose, lipid metabolism, and hepatic fat content (HFC) were assessed before and after the intervention. The metabolomics was also used to investigate the discrepant metabolites and mechanisms related to MetF.

Discussion: Metabolic flexibility reflects the capacity of an organism to switch the oxidation substrates flexibly, which is associated with ectopic fat accumulation. Our study aimed to explore the discrepant metabolites related to MetF before and after a high-fat diet between individuals with NAFLD and healthy control. In addition, the study also examined the effectiveness of RT combined with HIIT or MICT on hepatic fat reduction and quantificationally analyzed the metabolites related to MetF before and after the intervention. Our results provided a perspective on fatty liver-associated metabolic inactivity.

Trial registration ClinicalTrials.gov: ChiCTR2200055110; Registered 31 December 2021, http://www.chictr.org.cn/index.aspx.

KEYWORDS

NAFLD, metabolic flexibility, exercise intervention, combined training, randomized controlled trial, hepatic fat content

1. Introduction

The capacity of an organism to oxidate substrates according to the substrate availability or demand was described as metabolic flexibility (MetF) (1, 2). Metabolic inflexibility occurs in individuals with obesity or other metabolic diseases, which is manifested by a lowering of respiratory quotient (ΔRQ) before and after the euglycemic-hyperinsulinemic clamp (EHC) (1). MetF has been considered an indicator of metabolic health (3) since a progressive loss of MetF may be the cause of obesity-related comorbidities (4, 5).

Non-alcoholic fatty liver disease (NAFLD) is characterized by excess fat accumulation in the liver without excess alcohol intake (6). It covered approximately 25% of people worldwide (7) and 29.2% of people in China (8). Recently, NAFLD was termed as a metabolic (dysfunction)-associated fatty liver disease to reflect the disease feature (9, 10). Metabolic inflexibility is characterized by the mismatch of substrate oxidation and availability, which may be the etiology of insulin resistance (1). Thus, individuals with impaired MetF would form a set to favor the fat accumulating in the liver when they have a chronic high-fat diet or the increasing lipolysis of insulin-resistant adipose tissue (11, 12). However, there is little evidence about the causality between MetF and NAFLD. The cross-sectional study demonstrated that individuals with NAFLD show a typical feature of impaired MetF. Croci et al. reported that adults with NAFLD have lower ΔRQ (fasting and after stimulation of EHC) compared to healthy controls (13), and a similar result was also found in adolescents with NAFLD (14). In addition, even among obese individuals, those with NAFLD showed lower MetF than those without (15), and the hepatic and whole-body fat oxidation is reduced with the increase in hepatic fat content (13). Gastaldelli considered that the reduction of MetF in individuals with NALFD is a protective mechanism against excess FFA (16), and hyperglycemia will occur when the compensation mechanism is defective for the substrate overflow (16, 17). Thus, the response to substrate flux may play an important role in the development of NAFLD. Rudwill et al. found that metabolic inflexibility to a high-fat diet was preceded by whole-body glucose intolerance (18). Beyage et al. compared the different stimulations for MetF assessment and found that metabolic inflexibility to a high-fat diet is the only significant predictor for weight gain (5). In addition, Galgani et al. suggested that a high-fat diet is an advisable stimulating method for assessing MetF to lipid (19). Similarly, Fritzen et al. considered that the impaired response to FFA flux is a characteristic of obesity, and methods for improved FFA oxidation may be helpful for weight loss (20). However, little is known about MetF to a high-fat diet in individuals with NALFD, and there are few reports about the different metabolites related to MetF to a high-fat diet between individuals with NAFLD and in people with a healthy lifestyle.

Exercise intervention is the first-line method for treating metabolic disease, which is also an effective method for improving MetF (21, 22). The beneficial effects of exercise on hepatic fat reduction may depend substantially on the energy deficit (23), and a meta-analysis has demonstrated that high-volume exercise was superior to low-volume high-intensity exercise (24). However, a recent meta-analysis concluded that the effect of high-intensity interval training (HIIT) and moderate-intensity continuous training (MICT) on hepatic fat reduction was comparable (25). Furthermore, Hashida et al. found that resistance training (RT) improves NAFLD with less energy (26). Therefore, in addition to the energy deficit caused by exercise, some other pathways are mediating the association between exercise intervention and hepatic fat reduction. Both aerobic and resistance training are beneficial for hepatic fat reduction, and they all have their unique health benefits, such as improving cardiorespiratory fitness or muscle fitness. Combined training may be the ideal modality to gain the maximum health benefit, and Morze and colleagues reported that combined training may be the best modality for improving obesity (27), but little evidence about the effect of this exercise modality on hepatic fat content is available (28). The current physical activity guideline recommended that individuals with metabolic disease should perform both RT and moderate or vigorous physical activity (29), which could be implemented effectively by combined exercise. However, little is known about whether RT combined with HIIT would generate a similar effect on hepatic fat reduction.

Based on the aforementioned background about the MetF and NAFLD, some issues need to be explored. Thus, the objectives of our study are to examine the discrepant metabolites related to MetF to a high-fat diet in individuals with NAFLD and healthy control. In addition, we will compare the effect of different combined exercises on hepatic fat reduction and also analyze the metabolites related to MetF quantitively before and after the exercise intervention. Our results will provide evidence for clinical practice in choosing different combined exercises, and the results also provide a perspective on fatty liver-associated metabolic inflexibility.

2. Methods and analysis

2.1. Study design

This is an open-label, single-center trial randomized controlled clinical study. This study was approved by the ethics committee of Shanghai University of Sport (Number: 102772021RT08), and this trial has been registered in the Chinese Clinical Trial Registry (Number: ChiCTR2200055110, Registered 31 December 2021). This study recruited individuals with NAFLD and healthy individuals with active lifestyles. A cross-sectional comparison of MetF and related metabolites was conducted between individuals with NAFLD and the healthy control group. Subjects with NAFLD will be assigned into resistance training (RT) plus moderate-intensity continuous training (MICT) or high-intensity interval training (HIIT), and the control group in a 1:1:1 ratio. The subjects in the exercise group undergo a 12-week supervised exercise intervention based on their previous physical activity and diet habits, and the control group was asked for maintaining their previous lifestyles (Figure 1).

The assessment included two visits. First, participants who signed informed consent were invited for gathering the anthropometric information, and the exercise risk scan was performed by Pre-Activity Readiness Questionnaire plus (PARQ+) (30). Whereafter, the hepatic fat content (HFC) and cardiorespiratory fitness were assessed.

On the second visit, subjects were asked to arrive at the laboratory at 7:00–7:15 am. All subjects were advised to refrain from intensive physical activity, caffeine intake, and taking medicines for 24 h before each visit. The physical activity was investigated by the International Physical Activity Questionnaire short (IPAQ-short) (31, 32) and the 3-day diet before the test was also recorded by a photograph. Participants consumed a high-fat diet (consisting of 44% CHO, 41% fat, and 15% protein) within 15 min of collecting the fasting blood

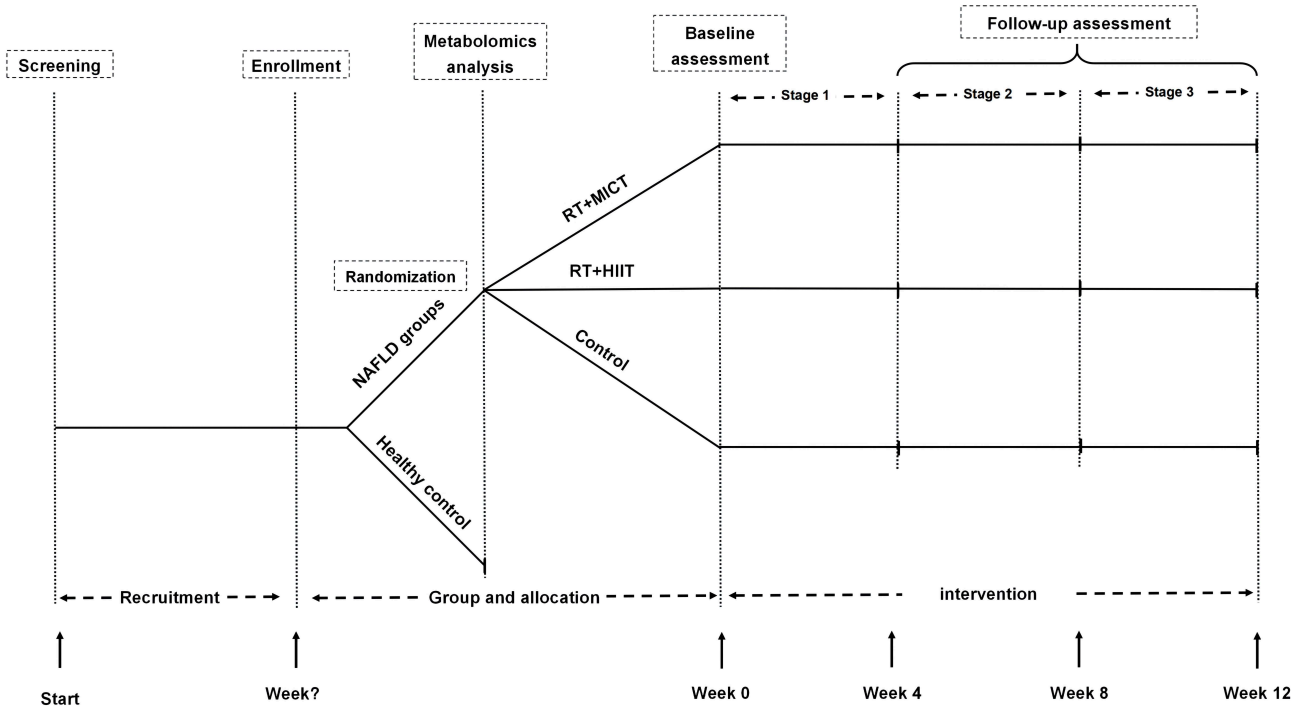

FIGURE 1
Flow diagram. RT, resistance training; HIIT, high-intensity interval training; MICT, moderate-intensity continuous training; NAFLD, non-alcoholic fatty liver disease.

sample (18). Blood samples were collected at 30, 60, 90, 120, and 180 min. Respiratory quotient (RQ) was evaluated by an indirect calorimeter (ParvoMedics TrueOne 2,400 Metabolic Measurement System) before and 90 min after a high-fat diet. The substrate oxidation was calculated by the Frayn equation as follows (33):

$$Fat\ oxidation\ (g/min) = 1.67VO2\ (L/min) - 1.67VCO2\ (L/min)$$

$$Carbohydrate\ oxidation\ (g/min) = 4.55VO2\ (L/min) - 3.21VCO2(L/min)$$

2.2. Participants

Individuals who satisfied the following criteria were included in this study: (1) men or women aged 18–45 years who were overweight, obese ($24 \leq$ BMI < 35), or had central obesity (waist circumference $\geq$ 90 cm for men and $\geq$ 85 cm for women); (2) those who were diagnosed with NAFLD by ultrasonography and had ongoing or recent alcohol consumption of < 30 g ($\sim$10 g of alcohol per one drink unit) for men and < 20 g for women on average per week (7); (3) those who had no chronic cardiovascular disease or other diseases that prevent participation in exercise (assessed by doctors); (4) those who had stable drug consumption for the past 3 months (kind and dose); and (5) those who took no regular exercise in the past year (< 3 times/week and 30 min/time). The individuals were excluded if they (1) were being treated using insulin; (2) had unstable body weight (change $\geq$5 kg); (3) had uncontrollable blood pressure or glucose levels, or rapidly progressing disease; and (4) were unable to exercise due to any reasons.

2.3. Randomization and allocation

Eligible participants were assigned in a 1:1:1 ratio to undergo a 12-week intervention in the RT+MICT group ($n = 18$), RT+HIIT ($n = 18$) group, and the control group ($n = 18$), respectively. Computer-based randomization (www.radomization.com) was used with a block randomization design, and the block was defined by age (18–25, 26–30, 31–35, 36–40, or 41–45) and sex (male or female). Before the intervention, the allocation was concealed in opaque envelopes and drawn by the participants.

2.4. Intervention

After all the assessments, participants in the two intervention groups spent a week acclimating, which consisted of teaching the correct movements, assessing the one-repetition maximum (1RM) of each movement, and acclimatizing at 50–60% 1RM. For safety reasons, the 1-RM load was estimated at 10 RM according to the conversion table (34), and the contracting and relaxing course lasted 3–4 s to recruit more muscle fiber and prevent injury. The control group received usual care without additional exercise guides and intervention, and they were promised to obtain the same intervention after 12 weeks to increase compliance. Two intervention groups performed a total of 36 sessions of RT+MICT or RT+HIIT in the next 12 weeks, and each session consisted of 5 min of warm-up and stretching; (2) 30 min of resistance training and 30 min of MICT or 15 min of HIIT; and (3) 5 min of cool down. RT was performed on fixed strength training machine (Life Fitness, Illinois, US), consisting of 1–2 sets with 10–15RM for 10 movements,

including chest press, shoulder press, seated pull-down, row, leg curl, leg press, leg extension, glute, abdominal curl, and prone raise. The MICT and HIIT were performed on a treadmill, elliptical machine, cycle ergometer, or rowing machine. MICT was performed at 40–60% heart rate reserve (HRR) for 30 min, and the HIIT protocol consisted of 3× 3 min of high-intensity intervals at 70–90% HRR, interspersed with 2 min of active recovery. Table 1 visualizes the intervention protocol. The exercise intensity of MICT and HIIT was monitored by Polar OH1 with a pad, which could display the heart rate in real-time.

All instructors received uniform training before the intervention, including the whole intervention process (Table 1), movement standard (e.g., when doing leg extension, the calf straight is counted), and how to fill in the training record.

2.5. Outcome assessment

2.5.1. Primary outcome

The primary outcome of this study is the hepatic fat content, which is assessed by Siemens 3T Magnetom Prisma scanner (Siemens Medical Solutions, Erlangen, Germany).

2.5.2. Secondary outcomes

Secondary outcomes include anthropometric indicators (body weight, body mass index, and the circumference of the waist and the hip), body composition (body fat, body fat percentage, lean body mass, abdominal subcutaneous, and visceral fat), physical fitness (grip strength and peak oxygen uptake), clinical blood markers (glucose, insulin, non-esterified fatty acid, blood lipid profile, and liver enzyme), metabolic flexibility (the change of respiratory quotient before and after the high-fat diet), and target and untargeted metabolomics, which explore the discrepant metabolites related to MetF between individuals with NAFLD and healthy control and quantitively analyze the change of these metabolites before and after the exercise intervention. In addition, physical activity, dietary habits, and sleep were investigated by questionnaires. The details of the assessing method are in the Supplementary material. The schedule of study assessment is presented in the Table 2.

TABLE 1 Details of the exercise protocol.

Stage	Exercise protocol
Stage 1	**RT**: 1 Set, 15 RM, 10 Movements **MICT**: 45–55% HRR for 30 min with treadmill, ergometer or elliptical **HIIT**: 40% HRR ∗ 2 min/ 70% HRR ∗ 3 min
Stage 2	**RT**: 1 Set, 10 RM, 10 Movements **MICT**: 55–60% HRR for 30 min with treadmill, ergometer or elliptical **HIIT**: 40% HRR ∗ 2 min / 80% HRR ∗ 3 min
Stage 3	**RT**: 2 Sets, 10 RM, 10 Movements **MICT**: 55–60% HRR for 30 min with treadmill, ergometer or elliptical **HIIT**: 40% HRR ∗ 2 min/ 90% HRR ∗ 3 min

The exercise intervention is divided into three stages, and each stage contains 4 weeks. RT, resistance training; MICT, moderate-intensity continuous training; HIIT, high-intensity interval training.

2.6. Concomitant adherences and procedures

To increase adherence, we organized the participants into collaborative groups according to the time of participation, and one instructor was assigned to each training group. The instructors notified them *via* WeChat before the agreed training time. In addition, we provided training feedback regularly and gave a reward according to the phased attendance each month. The attendance time was recorded in a spreadsheet, and the adherence was calculated as attendance time divided by total training times. The time points and reasons for dropping out and withdrawing were also recorded.

2.7. Sample size estimation

The sample size calculation was based on the results of a previous study (35), and we calculated the effect size as 0.8 based on accessible data. The effect size was estimated conservatively, with an effect size of 0.4 in our study. At least 42 participants were needed to provide 95% statistical power with a two-tail 0.05 significance level when using the one-way ANOVA and to keep the ratio 1:1:1 in the three groups. With an attrition rate of 20%, a total of 54 participants need to be recruited (G*power 3.1). The calculating process in G*power is in the Supplementary material. At least 10 healthy active individuals were recruited for metabolomic analysis, and this sample size was enough for metabolomic analysis (36).

2.8. Statistical analysis

The continuous variables (ΔRQ, blood pressure, glucose, etc.) were presented as means and standard deviations, and discrete variables were presented as numbers and percentages (e.g., sex). The Shapiro–Wilk test was used to test the normality of the continuous variables. For the continuous data to fit the normal distribution, the Student's *t*-test was used to compare the difference between the two samples (two groups in baseline, or before and after intervention), or the Wilcoxon rank sum test was used, and the Chi-squared test was used for categorical data. The covariance analysis was used to examine the difference between clinical and metabolomics data, with the baseline level as the covariate. In addition, the intention-to-treat analysis was conducted by a linear mixed-effect model (LMM), which considers the missing data as randomized and does not impute the missing data (37). The sensitivity analysis is performed to verify the robustness of the results. Age, sex, and diabetic or not were included in the LMM when comparing the main clinical indicators, and we included the baseline variables that differed between the dropout participants and those who completed the study as additional covariates in the LMM of the primary analysis. This method helped in decreasing the bias and provided a more realistic data analysis. A two-tailed test was conducted, and a $p < 0.05$ was regarded as a statistically significant difference. The data analysis was performed by the JMP pro version 16 (SAS Institute Inc., Cary, NC). For metabolomics analysis, the raw data were transformed to centroid mode and mass-corrected before being analyzed using the XCMS platform. The preprocessed data were imported into excel for normalization, and a two-dimensional matrix was formed. A principal component analysis

TABLE 2 Schedule of study assessment.

Variables	Screening visit	Baseline assessment	Week 4	Week 8	Week 12
Informed consent	•				
Drugs investigation	•	•	•	•	•
Physical activity	•	•			•
Dietary investigation		•	•	•	•
Anthropometrics	•	•	•	•	•
Body composition		•	•	•	•
Physical fitness		•	•	•	•
Hepatic fat content		•			•
Abdominal visceral and subcutaneous fat		•			•
Clinical blood markers		•			•
Metabolic flexibility		•			•
Metabolomics		•			•

and an orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using the SIMCA 13.0 software (Umetrics AB, Malmo, Sweden). The identification of discrepant metabolites was according to the standard by variable weight (VIP)>1 and $p < 0.05$ in the OPLS-DA model. The correlation between discrepant metabolites and MetF was analyzed using Spearman's correlation and partial correlation analysis, and the change of the discrepant metabolites before and after interventions was analyzed by the Student's *t*-test.

3. Discussion

NAFLD is a chronic liver disease that affects approximately 25% of the population worldwide (7), and metabolic inflexibility may be the factor for the occurrence of NAFLD (8). Our study aimed to analyze the discrepant metabolites related to MetF when receiving a high-fat diet between individuals with NAFLD and healthy control with cardiorespiratory fitness, which has been found associated with MetF. In addition, we also compared the effect of different combinations of RT plus HIIT or MICT on hepatic fat reduction and examined the association between the changes of hepatic fat and the discrepant metabolites before and after the 12-week exercise intervention. In addition to examining the effect of different exercise combination on hepatic fat reduction, our study also provided an alternative perspective for NAFLD treatment, which improved the substrate metabolism rather than making an energy deficit.

3.1. Exercise, metabolic flexibility, and non-alcoholic fatty liver disease

Until now, there are no specific drugs for treating NAFLD, but exercise is the first-line method for treating this disease and preventing the progress of non-alcoholic fatty liver (22). On the one hand, exercise decreases the HFC by increasing energy consumption. The meta-analysis demonstrated that the effectiveness of exercise on hepatic fat reduction is mediated by weight loss (23). On the other hand, exercise decreases the HFC and may not be mediated by energy deficit completely because Hallsworth et al. have found that resistance training decreases the HFC without weight loss (38). An impaired MetF in response to a high-fat diet will cause more weight gain in future (5), and the high-fat diet will cause more hepatic fat accumulation and metabolic inflexibility (39). In addition, physical inactivity is one of the impaired capacities in response to high-fat diet stimulation of healthy individuals, followed by a decrease in insulin sensitivity (18). Therefore, the metabolic inflexibility to a high-fat diet caused by physical inactivity may be one of the etiologies of NAFLD. Exercise intervention is an effective method for increasing metabolic flexibility, whose core is the mitochondrial function (40). Thus, exercise intervention decreased hepatic fat reduction, which may be mediated by metabolic flexibility improvement partially.

Combined exercise also termed concurrent exercise is an effective method for meeting the physical activity guideline (41). Although different exercise modalities have specific adaptions, such as resistance exercise increasing muscle strength and mass and aerobic exercise increasing the capacity to intake and utilize oxygen, both resistance exercise and aerobic exercise are effective for improving metabolic health. Bacchi et al. reported that 40 patients with T2DM were randomly assigned to aerobic training or resistance training groups; aerobic training improved the peak oxygen consumption and resistance training increased the muscle strength, but they demonstrated a similar effect on HbA1c decreasing (42). Furthermore, Hashida and colleagues systematically reviewed the effectiveness of these two-exercise modalities on hepatic fat reduction and reported that resistance training decreases hepatic fat similar to aerobic training with less energy consumption (26). However, there may be some interference effect in training adaption when performing resistance training and aerobic training concurrently (41, 43), due to the distinct training adaption, while it may be more effective in improving the metabolic health of individuals with metabolic dysfunction (27, 44) and untrained individuals (45, 46). HIIT is an exercise modality characterized by repeated bouts of high-intensity effort interspersed with recovery periods (47). It has been demonstrated that HIIT could decrease abdominal fat (48) and cause similar effects on body composition (49) and hepatic fat content with MICT (25) but with less time and energy consumption. As described in the introduction, exercise decreases hepatic fat may be independent of energy consumption partially. Although many investigations have examined the effectiveness of a single exercise modality on hepatic fat reduction, few studies have compared

the different combinations of resistance training plus moderate-intensity continuous training or high-intensity interval training (28, 50, 51). In the current physical activity guidelines, individuals with chronic diseases need to perform moderate-intensity aerobic physical activity or vigorous-intensity aerobic physical activity combined with muscle-strengthening activity. Thus, the study provided some evidence about the adaption of different exercise combinations with a randomized control design.

However, the biggest challenge of our study was to motivate the volunteers with a sedentary lifestyle formerly adhere to exercise. For this issue, we took some strategies for behavior change, such as regular notice before each exercise session, setting a personal goal, and assessing completion regularly. In addition, a regular evaluation was conducted and an award was presented for a higher attendance rate. On the contrary, supervised training was important. First, the subjects in our study were untrained people, so they did not know how to start exercising. Exercise, especially resistance exercise, had a high injury risk without correct movement, and they would not recruit the correct muscle without specialized support. Furthermore, the subjects in our study are at moderate-to-high exercise risk; hence, they need specialized personnel to supervise their training.

3.2. Metabolomics and non-alcoholic fatty liver disease

Metabolomics is a method to examine the small molecules and metabolic products, such as amino acids, fatty acids, and carbohydrates, which have been applied widely to explore the marker for diagnosis and pathophysiology (52). Dietary triglycerides cover 15% of the total fat accumulated in the liver in individuals with NAFLD (53). Thus, exploring the discrepant metabolites in fasting and after a high-fat diet stimulation among individuals with NALFD and healthy control with good cardiorespiratory fitness may help in understanding the pathophysiology of NAFLD. Individuals with good cardiorespiratory fitness are a good model for understanding the mitochondrial capacity on substrate metabolism since cardiorespiratory fitness is the reflection of mitochondrial capacity (54). Although elite endurance athletes are superior metabolic models, it is not realistic for our participants to reach this level. Thus, recruiting individuals with good CRF as the control is suitable. Yu et al. reported that the pathway of the tricarboxylic acid cycle, primary bile acid biosynthesis, and linoleic acid metabolism was significantly different at fasting and after the mixed-meal challenge, but they did not correlate these pathways to physiological function (MetF) (55). However, few studies have explored the association between HFC and postprandial metabolomes (56). In addition, whether there is some correlation between training effectiveness and postprandial metabolic features like gut microbiotas is still unclear (57). We also quantitively analyzed the change of discrepant metabolites before and after the exercise intervention, which helped us understand the effectiveness of exercise on substrate metabolism. In addition, it also contributed to the development of new drugs or treatment methods.

Summarily, if our study implements successfully, it not only provides evidence for combined training in hepatic fat reduction but also provides an alternative perspective for exercise to improve metabolic health.

3.3. Limitation

We did not use the oral glucose tolerance test (OGTT) or euglycemic-hyperinsulinemic clamp (EHC) as the stimulating method for assessing metabolic flexibility (MetF), which are the standard methods for measuring the insulin sensitivity and have the reference standard. However, there is no standard assessing method for MetF. In addition, the study from Galgani et al. and Fritzen et al. emphasized the importance of a high-fat diet in assessing MetF (19, 20). Thus, we chose the high-fat diet as the stimulating method for MetF assessment. Second, our study did not control the diet strictly, which limits the effectiveness of our intervention on weight loss. However, it is challenging to limit energy intake and kinds of diets. Our study adds an exercise intervention to daily life with regular diets and drug use and asks the subjects to maintain their everyday life. Thus, our study will provide evidence of the effectiveness of exercise intervention in real-world conditions. Third, our intervention of HIIT and MICT is not energy-matched. In daily life, individuals would not participate exercise with a precise energy monitor. In other words, the limitation for individuals conducting a training plan is not energy consumption but time. Thus, we design the HIIT protocol with a half-time of MICT, rather than half of the energy consumption of MICT.

Ethics statement

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

Author contributions

WH, WR, JL, and JZhu: study design and manuscript drafting. CH: recruitment, sample collection, and writing. JL and JZhu: project administration, design, data collection, data analysis, writing, and supervision. XD, HZ, JM, and JZha: daily administration of volunteers and performing the training. YL and TW: data collection, data management, and data analysis. All authors revised the final version of the protocol manuscript, read, and approved the final manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1065188/full#supplementary-material

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Interactions between genetic and lifestyle factors on cardiometabolic disease-related outcomes in Latin American and Caribbean populations: A systematic review

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Introduction: The prevalence of cardiometabolic diseases has increased in Latin American and the Caribbean populations (LACP). To identify gene-lifestyle interactions that modify the risk of cardiometabolic diseases in LACP, a systematic search using 11 search engines was conducted up to May 2022.

Methods: Eligible studies were observational and interventional studies in either English, Spanish, or Portuguese. A total of 26,171 publications were screened for title and abstract; of these, 101 potential studies were evaluated for eligibility, and 74 articles were included in this study following full-text screening and risk of bias assessment. The Appraisal tool for Cross-Sectional Studies (AXIS) and the Risk Of Bias In Non-Randomized Studies—of Interventions (ROBINS-I) assessment tool were used to assess the methodological quality and risk of bias of the included studies.

Results: We identified 122 significant interactions between genetic and lifestyle factors on cardiometabolic traits and the vast majority of studies come from Brazil (29), Mexico (15) and Costa Rica (12) with FTO, APOE, and TCF7L2 being the most studied genes. The results of the gene-lifestyle interactions suggest effects which are population-, gender-, and ethnic-specific. Most of the gene-lifestyle interactions were conducted once, necessitating replication to reinforce these results.

Discussion: The findings of this review indicate that 27 out of 33 LACP have not conducted gene-lifestyle interaction studies and only five studies have been undertaken in low-socioeconomic settings. Most of the studies were cross-sectional, indicating a need for longitudinal/prospective studies. Future gene-lifestyle interaction studies will need to replicate primary research of already studied genetic variants to enable comparison, and to explore the interactions between genetic and other lifestyle factors such as those conditioned by socioeconomic factors and

the built environment. The protocol has been registered on PROSPERO, number CRD42022308488.

Systematic review registration: https://clinicaltrials.gov, identifier CRD42022308488.

KEYWORDS

systematic review, nutrigenetics, Latin American and Caribbean, genetics, gene-lifestyle interaction, dietary intake, physical activity

1. Introduction

Cardiometabolic diseases such as hypertension and type 2 diabetes (T2D) are accountable for most non-communicable disease (NCD) deaths and impose an economic burden on low- and middle-income countries (1). In Latin American and the Caribbean populations (LACP), the prevalence of hypertension, T2D and obesity is 47, 22, and above 20%, respectively (2, 3). The etiology of cardiometabolic diseases is multifactorial where studies have demonstrated an interaction between the environment, genetic, behavioral, physiological, and socioeconomic factors (4–9). These intertwined mechanisms interact, modifying the risk of developing cardiometabolic diseases. Genetic variations or single nucleotide polymorphisms (SNPs) may modify the susceptibility to cardiometabolic diseases conditioned by the exposure to lifestyle factors (4, 5). Genome-wide association studies have identified several genetic loci associated with cardiometabolic traits but most of these studies have been performed in Caucasian populations (10–15). Similarly, majority of nutrigenetic studies have been performed in Western countries and the findings might not be applicable to low-income countries due to variations in allele frequencies, dietary pattern, and environmental factors (5, 16).

Factors such as changes in patterns of food consumption, the process of urbanization, increased health and socioeconomic disparities, underfinanced healthcare systems, lower levels of income and productivity, and the rise in sedentary lifestyle have led to an increase in NCDs (17–21). Moreover, studies have shown that metabolic responses to lifestyle factors such as diet and physical activity vary between ethnicities due to genetic heterogeneity (4, 5, 22, 23), and hence we sought to determine which lifestyle factors are interacting with genetic variants in different LACP with regards to cardiometabolic disease traits. The discovery of gene-lifestyle interactions in LACP will help to identify population subgroups that will respond to lifestyle interventions.

The influence of gene-lifestyle interactions on obesity, T2D and cardiovascular diseases (CVDs) has been broadly studied, and there is evidence that the genetic risk of cardiometabolic traits can be modified (4, 5, 24–27). However, to our knowledge, no previous systematic reviews have been conducted regarding the interactions of genetic and lifestyle factors on cardiometabolic disease traits in LACP. Thus, the objective of this systematic review was to identify studies examining the interactions between genetic variants and lifestyle factors such as diet, nutrient intake, nutritional status, physical activity, socioeconomic factors, and the built environment on obesity, CVDs, and T2D-related traits in LACP.

2. Methods

2.1. Inclusion and exclusion criteria

Eligible for inclusion were articles that explored the interaction between genetic variations and lifestyle factors on cardiometabolic disease traits in LACP. All cardiometabolic diseases and traits were considered including CVDs, cerebrovascular diseases such as stroke, blood lipid levels, obesity-related traits such as body mass index (BMI) and T2D-related traits such as fasting glucose. The eligible articles included observational and dietary intervention studies and were in either English, Spanish, or Portuguese. Articles that did not explore gene-lifestyle interactions or were not based on LACP were excluded.

2.2. Information sources and search strategy

A literature search was conducted in MEDLINE (*via* PubMed and EBSCO Host), Web of Science, ScienceDirect, SciELO, SCOPUS, Taylor & Francis Online, Cochrane library, LILACS (Latin American and Caribbean Health Sciences Literature), IBECS, Google Scholar, and ERIC (Education Resources Information Center *via* EBSCO Host) search engines until the 25th of May 2022. To reach literature saturation, the researchers conducted independent search strings (**Supplementary Table 1**), and the included publications were searched through to identify potential articles in reference lists. We followed the Peer Review of Electronic Search Strategies (PRESS) guideline (28) and the literature search was limited to human participants and had no dates of publication restrictions. The protocol was registered on PROSPERO, number CRD42022308488.

2.3. Study selection, synthesis methods, effect measures, and data collection process

Duplicate articles were removed using Rayyan software (29), titles and abstracts were blindly screened to assess against the pre-established inclusion criteria, followed by full-text screening and discussion until consensus between E.F.V. and R.W. All the data required to assess the eligibility of the studies was available, hence study investigators were not contacted to obtain or confirm the data. The reviewers ensured consistency across the data that needed to

be extracted, and a narrative synthesis was conducted to collate the data, including populations, lifestyle factors, study designs, genetic variations, cardiometabolic disease traits, and *P*-values for gene-lifestyle interactions on obesity, diabetes and CVD traits. *P*-values for gene-lifestyle interactions were used as indicators of the relationship between the exposure (genetic and lifestyle factors) and the outcome (cardiometabolic traits). *P*-values < 0.05 were considered statistically significant. $P_{interaction}$ refers to the *P*-value for the interaction between the genetic variant and dietary/lifestyle factors on cardiometabolic traits. To synthesize the findings, we categorized the outcomes into four categories: obesity, diabetes, CVD, and overall cardiometabolic risk. We then coded the exposures considering major themes; proteins, carbohydrates, fats, and fiber as well as plasma fatty acids, polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA), breastfeeding, smoking, alcohol, coffee, and lifestyle (if the exposure was multiple, including factors embracing diet, physical activity, smoking, and/or socioeconomic status, education), macronutrients (when the exposures included at least proteins, carbohydrates, fats, and fiber), and micronutrients (when the exposure referred to minerals or vitamins). The final graphical representation of the interaction between the genetic variations, and the coded lifestyle factors on the clustered outcomes was a heat map, where the intensity of the color corresponds to the *P*-values of the gene-lifestyle interactions (**Figures 1–4**). All heat maps were produced using the ggplot2 package (30) in R software with RStudio environment (31). A meta-analysis could not be conducted due to the wide range of dietary factors, genetic variants and cardiometabolic traits investigated by the included studies, in addition to heterogeneity in the methods used.

2.4. Data items

Data was extracted in **Table 1** and the main outcomes were diabetes, obesity, CVD, and their related traits including lipid levels, blood pressure and anthropometric measurements.

2.5. Risk of bias and certainty of assessment

To evaluate the methodological quality and risk of bias (RoB) of cross-sectional studies we used the Appraisal tool for Cross-Sectional Studies (AXIS) (32) (**Supplementary Tables 2, 3**). Cohort studies, case-control studies, and non-randomized trials were assessed by using the RoB in Non-randomized Studies—of Interventions (ROBINS-I) assessment tool (32, 33) (**Supplementary Table 4**). Risk of bias due to missing results was assessed using the AXIS RoB (questions 12–14) and the ROBINS-I assessment [part 5 (questions 5.1–5.4)]. The current article adheres to the recommendations of the Synthesis without Meta-analysis (SWiM) in Systematic Reviews: Reporting Guideline (34).

3. Results

3.1. Study selection and characteristics

The search string results had an output of 29,092 articles and from these, 101 articles were identified as potential studies. After the full-text screening, 27 articles were excluded for the following reasons: six studies were not based on LACP (35–40), five studies aimed to identify the effect of genomic ancestry (41–45), six studies focused only on genetic associations (46–51), eight studies did not include cardiometabolic diseases (52–59), and two studies investigated gene x phenotype interactions (60, 61) as shown in **Figure 5**. Finally, after excluding the irrelevant articles based on the exclusion criteria, 74 studies were included in this systematic review as shown in **Table 1**.

3.2. Gene-lifestyle interactions in LACP

The 74 studies conducted in LACP encompass ethnicities from Argentina, Colombia, Chile, Costa Rica, Mexico, Brazil, and LACP diaspora, including Dominicans, Puerto Ricans, Mexicans, and other Hispanic ethnicities residing in the United States of America (USA). Most of the studies are focused on four countries: Brazil (29), Mexico (15), Costa Rica (12), and Puerto Ricans in Boston (10). The studies have identified 122 significant gene-lifestyle interactions on cardiometabolic traits ($p < 0.05$), as shown in **Table 1**. The results are stratified by country to enable identification of ethnic-specific gene-lifestyle interactions and to present a structured mapping of the research gaps for a multidisciplinary audience.

3.3. Gene x lifestyle interactions in Brazilians

3.3.1. Interaction between dietary fat intake and genetic variants on CVD traits

Interaction between dietary fat intake and genetic variants on CVD-related traits was examined by five Brazilian studies (62–66). In a cross-sectional study of 567 participants (62), a significant interaction was reported between olive oil intake and Apolipoprotein E (*APOE*) genotype on low-density lipoprotein cholesterol (LDL) ($P_{interaction} = 0.028$), where a high intake of olive oil ($\geq$ once a week) was associated with lower LDL levels in men carrying the "ε*2*" allele but had no effect in men without the "ε*2*" allele. In this study (62), a high polyunsaturated fatty acid (PUFA) intake (> twice a week) was associated with increased LDL levels in carriers of the "ε*4*" allele, but this was not observed in participants without the "ε*4*" allele ($P_{interaction} = 0.04$). A reduction in triglyceride levels in response to a high PUFA intake was also observed in carriers of the "ε*2*" allele but not in participants without the "ε*2*" allele ($P_{interaction} = 0.04$). A high PUFA intake was also associated with increased high-density lipoprotein cholesterol (HDL) concentration in participants without the "ε*4*" allele and reduced HDL levels in carriers of the "ε*4*" allele ($P_{interaction} = 0.018$) (62). In contrast, a cross-sectional study of 252 Brazilian women (63) observed increased triglyceride and very-low density lipoprotein cholesterol (VLDL) in response to a low PUFA or a high fat diet intake in carriers of the "ε*4*" allele of *APOE*, but not in non-carriers ($P_{interaction} < 0.05$ for both). The findings of the first study (62) indicate that, PUFA intake might be beneficial in increasing HDL levels in individuals without the "ε*4*" allele, while in those with the "ε*4*" allele, PUFA intake might contribute to a rise in triglyceride and LDL levels which is associated with higher risk of CVDs (67). Nonetheless, the findings of the second study (63) suggest a detrimental effect of low PUFA intake in carriers of the "ε*4*"

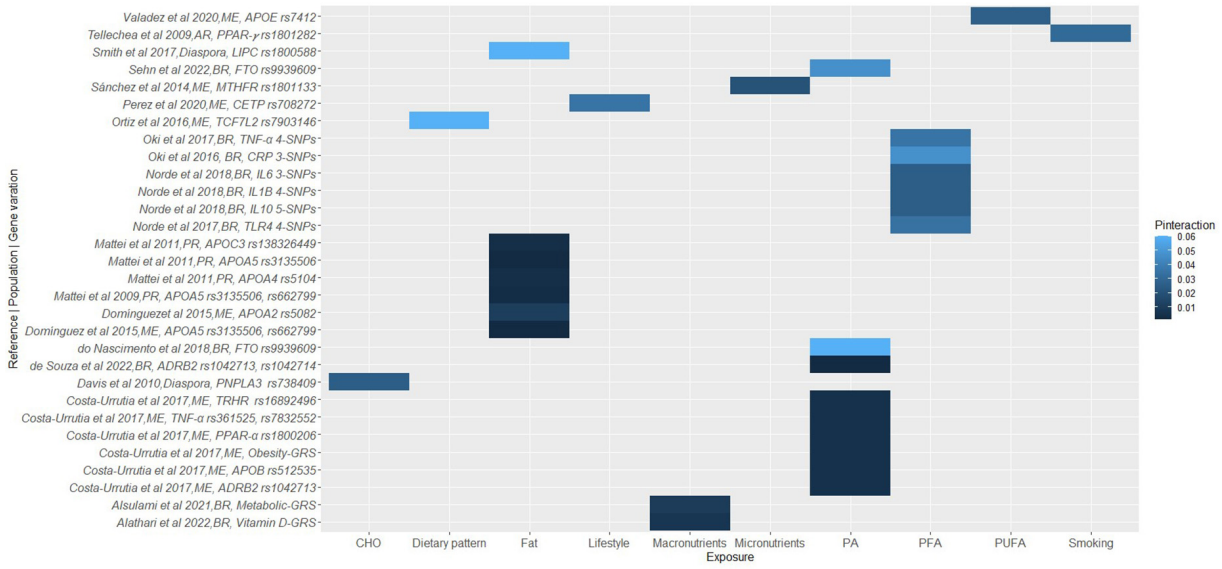

FIGURE 1
A heat map showing the findings for gene-lifestyle interactions on overall cardiometabolic disease risk. Alsulami et al. (72), Metabolic-GRS = *TCF7L2 (rs12255372, rs7903146); MC4R (rs17782313, rs2229616); PPAR γ (rs1801282); FTO (rs8050136); CDKN2A/2B (rs10811661); KCNQ1 (rs2237892); CAPN10 (rs5030952);* Alathari et al. (73), Vitamin D-GRS = *VDR (rs2228570, rs7975232), DHCR7 (rs12785878), CYP2R1(rs12794714), CYP24A1(rs6013897), GC (rs2282679), FTO (rs8050136, rs10163409), TCF7L2 (rs12255372, rs7903146), MC4R (rs17782313), KCNQ1 (rs2237895, rs2237892), CDKN2A (rs10811661), PPAR γ (rs1801282), CAPN10 (rs5030952);* Costa-Urrutia et al. (118), Obesity-GRS = *ABCA1 (rs2230806, rs9282541); ADIPOQ (rs2241766); ADRB2 (rs1042713); AGT (rs699); APOA4 (rs675); APOB (rs512535); APOE (rs405509); CAPN10 (rs2975760, rs2975762, rs3792267); FTO (rs1121980, rs9939609); HNF4 (rs745975); LIPC (rs1800588); LPL (rs320); PPAR-α (rs1800206); PPAR-γ (rs1801282); SCARB1 (rs1084674); TCF7L2 (rs7903146); TNF (rs361525); TRHR (rs1689249, rs7832552)*; Norde et al. (79), 5-SNPs = *IL10 rs1554286, rs1800871, rs1800872, rs1800890, rs3024490*; Oki et al. (78), 4-SNPs = *TNF-α rs1799724, rs1800629, rs361525, rs1799964;* Norde et al. (76), 4-SNPs = *TLR4 rs11536889, rs4986790, rs4986791, rs5030728;* Oki et al. (77), 3-SNPs = *CRP rs1205, rs1417938, rs2808630;* Norde et al. (79), 4-SNPs = *IL1B rs16944, rs1143623, rs1143627, rs1143643; 3-SNPs = rs1800795, rs1800796, rs1800797;* BR, Brazilian; ME, Mexican; PR, Puerto Rican; AR, Argentinian.

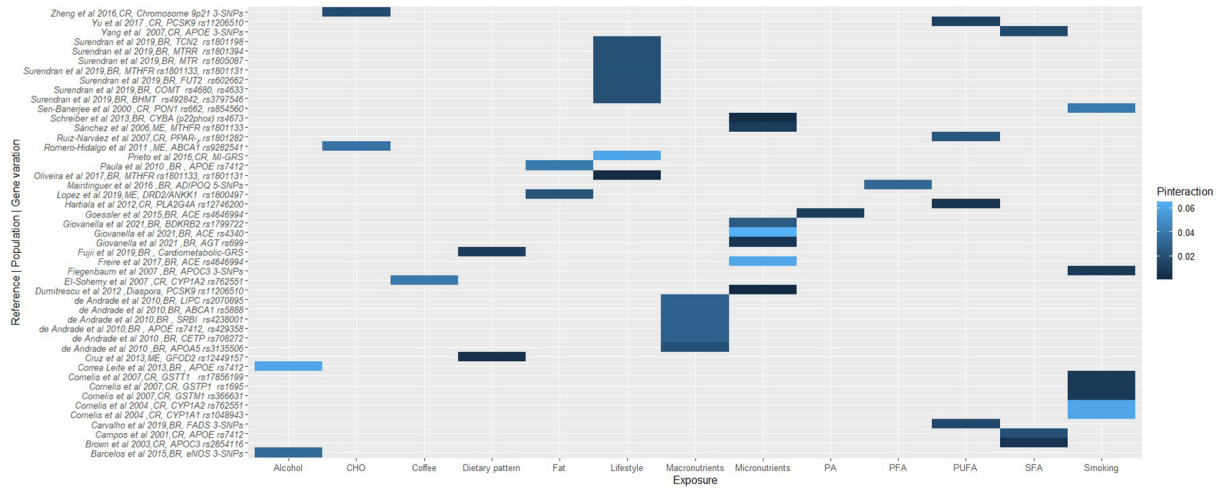

FIGURE 2
A heat map showing the findings for gene-lifestyle interactions on cardiovascular disease traits. Sotos-Prieto et al. (144), MI-GRS = *CDKN2A/2B (rs4977574, rs10757274, rs2383206, rs1333049); CELSR2-PSRC1-SORT1 (rs646776, rs599839); CXCL12(rs501120, rs1746048); HNF1A, C12orf43 (rs2259816); MRAS (rs9818870); SLC22A3 (rs2048327); LPAL2 (rs3127599); LPA (rs7767084, rs10755578);* Fujii et al. (64), Cardiometabolic-GRS = *APOA5 (rs662799); APOB (rs693, rs1367117); LDLR (rs688, rs5925); LIPC (rs2070895, rs1800588);* Brown et al. (125), 3-SNPs = *APOE rs7412, rs449647, rs429358*; Fiegenbaum et al. (92), 3-SNPs = APOC3 rs2854116, rs2854117, rs5128; Maintinguer Norde et al. (75), 5-SNPs = ADIPOQ rs2241766, rs16861209, rs17300539, rs266729, rs1501299; Carvalho et al. (65), 3-SNPs = FADS rs174575, rs174561, rs3834458; Barcelos et al. (88), 3-SNPs = eNOS rs2070744, rs1799983, rs61722009; Zheng et al. (135), 3-SNPs = Chromosome 9p21 rs4977574, rs2383206, rs1333049. BR, Brazilian; CR, Costa Rican.

allele. The differences in the findings could be attributed to the small sample sizes and the fact that, the second study (63) was conducted in women unlike the first study (62). PUFA is a ligand for peroxisome proliferator-activated receptors (PPARs) which are involved in regulating several lipid-pathway genes and it has been suggested that, increased consumption of PUFA might promote the expression of APOE and hepatic uptake of "ε*4*"-containing VLDL particles (68, 69).

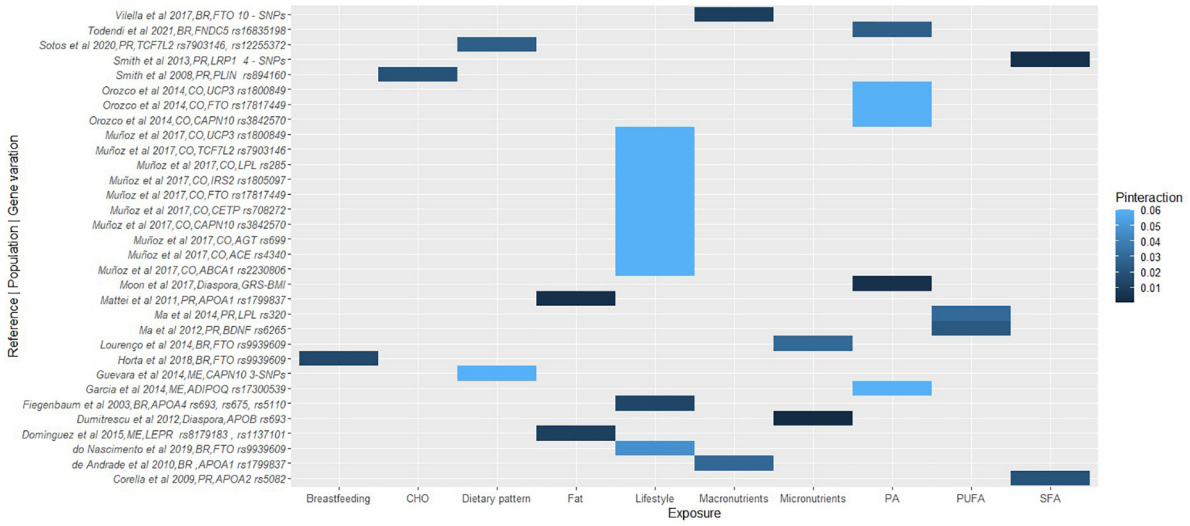

FIGURE 3
A heat map showing the findings for gene-lifestyle interactions on obesity traits. Vilella et al. (80), 10-SNPs = *FTO rs79149291, rs62048379, rs115530394, rs75066479, rs2003583, rs115662052, rs114019148, rs62034079, rs1123817, rs16952663;* Smith et al. (146), 4-SNPs = *LRP1 rs1799986, rs1799986, rs1800191, rs715948*; Cao et al. (107), 3-SNPs = *CAPN10 rs5030952, rs3792267, rs2975762*. BR, Brazilian; PR, Puerto Rican; ME, Mexican; CO, Colombian.

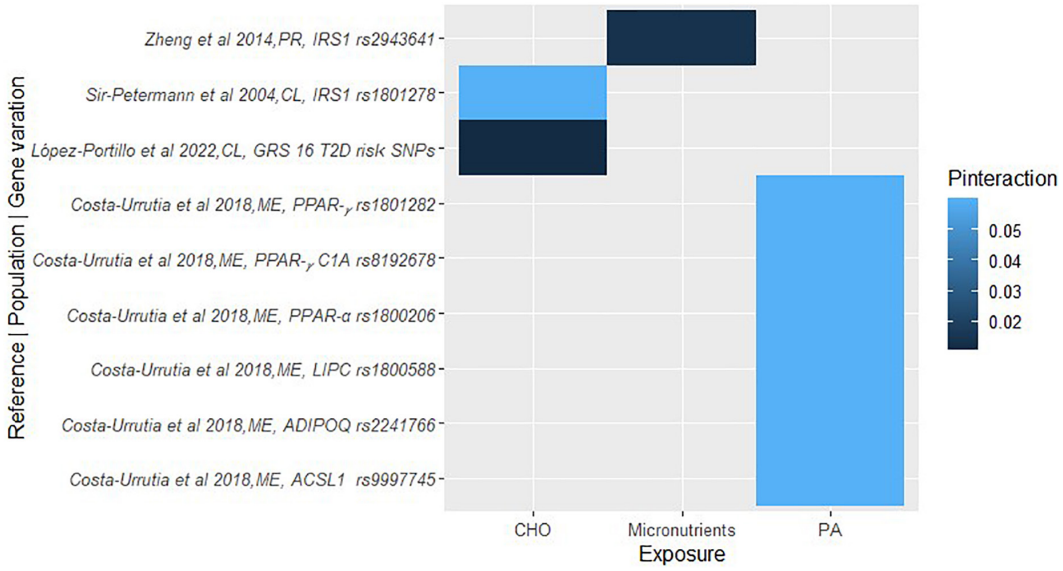

FIGURE 4
A heat map showing the findings for gene-lifestyle interactions on diabetes traits. López-Portillo et al. (161), GRS-16 Type 2 Diabetes (T2D) risk SNPs = *MTNR1B (rs10830963); TCF7L2 (rs7903146); CDKAL1 (rs7756992); ADCY5 (rs11717195); ANK1 (rs516946); BCAR1 (rs7202877); CDC123 (rs11257655); DUSP9 (rs5945326); GRB14 (rs3923113); RASGRP1 (rs7403531); TLE4 (rs17791513); TLE1 (rs2796441); ZBED3 (rs6878122).*

Furthermore, a cross-sectional study of 228 Brazilian participants from the Health Survey of São Paulo (HS-SP) (64) observed significant interactions between a GRS based on seven SNPs (**Table 1**) and the Brazilian Healthy Eating Index Revised (BHEI-R) on the risk of dyslipidaemia. Participants with a higher GRS (5–8) had a lower odds ratio for dyslipidaemia with an intake of BHEI-R oil component above the median ($P_{interaction} = 0.019$); while those with a GRS > 9 had a lower odds ratio for dyslipidaemia with an intake of BHEI-R solid fats, alcoholic beverages and added sugars (SoFAAS) component below the median ($P_{interaction} < 0.001$). Similarly, a cross-sectional study involving 250 pregnant women (65) observed significant interactions between fatty acid desaturase (*FADS*) SNPs (rs174561 and rs3834458) and dietary α-linolenic acid (ALA) and linoleic/α-linolenic acid ratio (LA/ALA) on plasma concentrations of omega-3 (n-3) PUFAs. It was reported that, in women with high ALA intake, plasma ALA concentrations were higher in homozygotes for the minor allele ($p < 0.05$), compared to carriers of the major allele (MM and Mm) of rs174561 and rs3834458. However, the *P*-values given in the study ($p = 0.004$ for rs174561 and $p = 0.028$ for rs3834458) seem to represent associations stratified by genotype, instead of interactions. FADS are involved in the synthesis of PUFA and their activation is linked to inflammation and coronary artery disease (70, 71), and these findings suggest that SNPs which alter the activation of FADS might affect plasma concentration of PUFA. In

another cross-sectional study of 113 adolescents from the Obesity, Lifestyle and Diabetes in Brazil (BOLD) study (66), no significant interactions were reported between seven genes involved in the one-carbon metabolism pathway (**Table 1**) and fat intake on lipid-related traits.

3.3.2. Interaction between dietary fat intake and genetic variants on glycemic traits

Interaction between dietary fat intake and genetic variants on glycemic traits was investigated by two cross-sectional studies (72, 73) using data from the BOLD study. In the first study which consisted of 200 participants (72), a high total fat intake [37.98% of total energy intake (TEI)/day] was shown to interact with a 10-SNP metabolic-GRS (**Table 1**), where individuals with 5 or more risk alleles had increased homeostasis model assessment estimate of insulin secretion (HOMA-B) ($P_{interaction} = 0.016$), fasting insulin ($P_{interaction} = 0.017$), body fat mass ($P_{interaction} = 0.009$), and decreased insulin:glucose ratio ($P_{interaction} = 0.01$), but the interaction did not influence homeostasis model assessment estimate of insulin resistance (HOMA-IR), glycated hemoglobin (HbA1c), or waist circumference (WC). Similarly, the second BOLD study (73) which also examined the interaction between dietary fat intake and a 10-SNP metabolic-GRS did not find significant interactions between the GRS and dietary fat intake on fasting glucose, fasting insulin or HbA1c (**Table 1**). The mechanisms through which dietary fat intake influence glycemic traits are unclear, although a sustained increase in blood glucose levels following a high fat meal has been reported (74).

3.3.3. Interaction between plasma fatty acid profile and genetic variants on systemic inflammation

Five Brazilian cross-sectional studies (75–79) investigated the interaction between plasma fatty acids and genetic variants on systemic inflammation, using data from the HS-SP. The first study (75) consisted of 262 adults, and significant interactions were identified between plasma n-3 and adiponectin (*ADIPOQ*) SNP rs2241766 ($P_{interaction} = 0.019$); arachidonic acid and *ADIPOQ* rs16861209 ($P_{interaction} = 0.044$); docosapentaenoic acid and *ADIPOQ* rs16861209 ($P_{interaction} = 0.037$); and SFA and *ADIPOQ* rs17300539 ($P_{interaction} = 0.019$) on the risk of systemic inflammation. Carriers of the "G" allele of rs2241766 had a reduced odds ratio of having inflammatory biomarkers when plasma n-3 levels were above the median, while participants with the "CC" genotype of rs16861209 had a lower odds ratio of having inflammatory biomarkers in the 50th percentile of plasma arachidonic acid and docosapentaenoic acid. Moreover, carriers of the "A" allele of rs17300539 had a higher odds ratio of having inflammatory biomarkers in the upper 50th percentile of plasma SFA compared to those with the "GG" genotype (75). In the second study (76), which consisted of 262 participants, an interaction was also observed between plasma arachidonic acid/eicosapentaenoic acid ratio and toll-like receptor 4 (*TLR4*) SNP rs11536889, in which individuals with the "C" allele had an increased odds ratio of having inflammatory biomarkers at the higher percentile of arachidonic acid/eicosapentaenoic acid ratio ($P_{interaction} = 0.034$). Similarly, the third study consisting of 262 participants (77) identified a significant interaction between plasma palmitoleic acid and C-reactive protein (*CRP*) SNP rs1417938, where individuals with the "AA" genotype had a higher odds ratio of having inflammatory biomarkers with a plasma palmitoleic acid above the median ($P_{interaction} = 0.047$).

In line with these findings, an increasing risk of having inflammatory biomarkers in response to increasing plasma SFA was observed in carriers of the "A" allele of tumor necrosis factor-α (*TNF*-α) SNP rs180062 (−308G/A) ($P_{interaction} = 0.041$); while a decreasing risk with increasing plasma stearic acid was found in participants with the "GG" genotype ($P_{interaction} = 0.046$), in a sample of 281 participants from the HS-SP (78). Furthermore, a decreasing risk of metabolic syndrome (MetS) was observed in response to increasing plasma stearic acid levels in "A" allele carriers of interleukin 1 beta (*IL1B*) SNP rs16944 ($P_{interaction} = 0.043$), and in response to increasing plasma arachidonic acid levels in those with the "GG" genotype of interleukin 10 (*IL10*) SNP rs1800896 ($P_{interaction} = 0.007$), in a sample of 301 participants from the HS-SP (79). However, no significant interactions were identified between total SFA, myristic acid, palmitic acid, stearic acid and *ADIPOQ* SNPs rs1501299 and rs266729; *TLR4* SNPs rs11536889 and rs5030728; and *CRP* SNP rs1205 on inflammatory biomarkers in three of the studies (75, 78, 79). Plasma fatty acid profile is considered an indicator of dietary fatty acid intake (75) and these findings suggest that plasma fatty acid profile can interact with SNPs of several genes and modify the risk of systemic inflammation which is linked to cardiometabolic diseases such as type 2 diabetes and CVDs (75).

3.3.4. Interaction between carbohydrate intake and genetic variants on cardiometabolic traits

Three Brazilian cross-sectional studies (66, 72, 73) investigated the interactions between carbohydrate intake and genetic variants on cardiometabolic traits, using data from the BOLD study. In the first study which consisted of 113 participants (66), a total carbohydrate intake of 47.7% TEI was associated with a significantly increased homocysteine concentration ($P_{interaction} = 0.031$) in carriers of the "*AA*" genotype of fucosyltransferase 2 (*FUT2*) SNP rs602662. Carbohydrate intake also interacted with Catechol-O-Methyltransferase (*COMT*) SNP rs4680, increasing oxidized-LDL more in carriers of "AA" than "GG" genotype ($P_{interaction} = 0.005$) (66). Notwithstanding, after applying Bonferroni correction for multiple testing, none of the interactions were considered significant (66). Moreover, the other two studies (72) which consisted of 200 participants and (73) which consisted of 187 participants, from the BOLD study, did not identify significant interactions between carbohydrate intake and a metabolic-GRS based on 10 SNPs (**Table 1**) on cardiometabolic traits.

3.3.5. Interaction between protein intake and genetic variants on cardiometabolic traits

Three studies (66, 73, 80) investigated the interaction between protein intake and genetic variants on cardiometabolic traits, two of which (66, 73) used data from the BOLD study. A cross-sectional study of 1191 overweight and normal weight children (80) observed a significantly increased BMI ($p = 0.01$) among participants carrying the "*T*" allele of *FTO* SNP rs79149291 with a protein intake above 12.7% TEI/day (80). Similarly, in the BOLD study discussed above (66), those with a protein intake of 16.99% TEI who were carriers of the "*AA*" genotype of *FUT2* SNP rs602662 ($P_{interaction} = 0.007$) had increased homocysteine levels (66). However, in the other BOLD study (73), there were no interactions between protein intake and a GRS based on 10 SNPs (**Table 1**) on obesity or diabetes traits.

TABLE 1 Summary table of gene-lifestyle interactions and study characteristics.

Gene and SNP	Population and sample size	Study design	Dietary/lifestyle factor	Outcome	$P_{interaction}$*	References
FTO						
rs9939609	Brazilian $n = 1,088$	LS	Plasma vitamin D	BMI	0.02–0.04	Lourenço et al. (84)
rs9939609	Brazilian $n = 1,215$	C-S	Screen time	Cardiometabolic risk score	0.047	Sehn et al. (96)
rs9939609	Brazilian $n = 432$	C-C	Physical activity intervention	TC, HDL, LDL, TG, glucose, insulin, HOMA-IR, QUICKI	NS	do Nascimento et al. (98)
rs9939609	Brazilian $n = 3,701$	P-C	Breastfeeding	BMI, overweight, fat mass, lean mass, WC, visceral, and subcutaneous abdominal fat thickness	0.01–0.02	Horta et al. (102)
rs9939609	Brazilian $n = 434$	C-C	hypocaloric diet, physical exercise program	BMI, WC, AC	0.047	do Nascimento et al. (99)
rs17817449	Colombian $n = 212/212$	C-C	Physical activity	BMI	NS	Orozco et al. (163)
rs17817449	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
rs79149291, rs62048379, rs115530394, rs75066479, rs2003583, rs115662052, rs114019148, rs62034079, rs1123817, rs16952663	Brazilian $n = 1,191$	C-S	Carbohydrate, protein, total fat, MUFA, PUFA:SFA intake	Overweight/ obesity	0.01	Vilella et al. (80)
APOE						
rs7412, rs429358	Brazilian $n = 567$	C-S	Olive oil, PUFA, sucrose, soluble and insoluble fiber	LDL, TG, TC	0.018–0.04	de Andrade et al. (62)
rs7412	Brazilian $n = 252$	C-S	Total fat, PUFA: SFA	LDL, TG, VLDL	<0.05	Paula et al. (63)
rs7412	Brazilian $n = 851$	P-C	Alcohol intake	SBP, DBP	NS	Correa Leite et al. (89)
rs7412	Mexican $n = 224$	C-S	MUFA intake, n-3:n-6	TC, Non-HDL, LDL, HbA1c	0.016–0.035	Torres-Valadez et al. (103)
rs7412	Costa Rican $n = 420$	C-S	SFA	TG, TC, VLDL, LDL, HDL, Apo A1, Apo B, LDL particle size	0.02–0.03	Campos et al. (124)
rs7412, rs429358, rs449647	Costa Rican $n = 1,927/1,927$	C-C	SFA	TC, HDL, LDL, TG, MI	0.0157	Yang et al. (125)
APOA5						
rs3135506	Brazilian $n = 567$	C-S	Olive oil, PUFA, sucrose, soluble and insoluble fiber	LDL, TG, TC	0.018–0.04	de Andrade et al. (62)
rs3135506, rs662799	Mexican $n = 100/100$	C-C	SFA, total fat	TC, TG, LDL, HDL, obesity	0.001–0.02	Domínguez-Reyes et al. (105)
rs3135506, rs662799	Puerto Rican $n = 802$	LS	Total fat	WC, serum glucose, SBP, DBP, HDL, LDL, TC, VLDL	0.002–0.032	Mattei et al. (152)
APOA5						
rs3135506	Puerto Rican $n = 821$	LS	Total fat	WC, SBP, DBP	0.001–0.005	Mattei et al. (147)
MTHFR						
rs1801133, rs1801131	Brazilian $n = 3,803$	C-S	Physical activity, alcohol intake, and blood folate	Homocysteine	<0.001–0.002	Oliveira et al. (85)
rs1801133, rs1801131	Brazilian $n = 113$	C-S	Fat, protein, carbohydrate intake, physical activity	Vitamin B12, homocysteine, folic acid, HDL, LDL, TG, oxidized LDL	0.005–0.034	Surendran et al. (66)

(Continued)

TABLE 1 (Continued)

Gene and SNP	Population and sample size	Study design	Dietary/lifestyle factor	Outcome	$P_{interaction}$*	References
rs1801133	Mexican $n = 996$ (women); 231 (new-borns)	P-C	Folate and Vitamin B12	Weight, length and BMI of new-born	0.02	Torres-Sánchez et al. (115)
rs1801133	Mexican $n = 130$	C-S	Vitamin B12, alcohol intake	Plasma Folate, total homocysteine	0.01	Torres-Sánchez et al. (116)
ACE						
rs4340	Brazilian $n = 335$	C-S	Sodium, potassium, calcium, magnesium	SBP, DBP	0.004–0.009	Giovanella et al. (81)
rs4340	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
rs4646994	Brazilian $n = 234$	C-C	Sodium	Hypertension	NS	Freire et al. (82)
ACE						
rs4646994	Brazilian $n = 34$	RCT	Physical activity	SBP, DBP	0.02 –0.002	Goessler et al. (97)
TCF7L2						
rs7903146	Mexican $n = 137$	P-LS	Two diets: Nopal tortilla and whole grain bread	Weight, BMI, WC, HC, WHR, glucose, HbA1c, TG, TC, HDL, LDL, insulin, HOMA-B, HOMA-IR, GLP-1	NS	López-Ortiz et al. (185)
rs7903146	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
rs7903146, rs12255372, rs7903146, rs12255372	Puerto Rican $n = 1,120$	C-S	Mediterranean diet score	BMI, WC, weight	0.014–0.036	Sotos-Prieto et al. (150)
ABCA1						
rs5888	Brazilian $n = 567$	C-S	Olive oil, PUFA, sucrose, soluble and insoluble fiber	LDL, TG, TC	0.018–0.04	de Andrade et al. (62)
rs9282541	Mexican $n = 3,591$	C-S	Carbohydrate	HDL	0.037	Romero-Hidalgo et al. (112)
rs2230806	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
LIPC						
rs2070895	Brazilian $n = 567$	C-S	Olive oil, PUFA, sucrose, soluble and insoluble fiber	LDL, TG, TC	0.018–0.04	de Andrade et al. (62)
rs1800588	Mexican $n = 167/398$	C-C	Maximal oxygen consumption (VO2 max), muscle endurance (ME)	pre-diabetes (fasting glucose concentrations)	NS	Costa-Urrutia et al. (119)
rs1800588	Dominican/Puerto Rican, other Caribbean Hispanics $n = 41$	RCT	High fat diet	HDL, LDL, TC, TG, glucose	NS	Smith et al. (153)
APOC3						
rs2854116, rs2854117, rs5128	Brazilian $n = 673$	C-S	Smoking	TG	0.009	Fiegenbaum et al. (92)
rs2854116, T-625del	Costa Rican $n = 336$	C-S	SFA	TG, TC, LDL, HDL, Apo B, LDL diameter	0.0004 –0.01	Brown et al. (126)
rs138326449	Puerto Rican $n = 821$	LS	Total fat	WC, SBP, DBP	0.001–0.005	Mattei et al. (147)
CETP						
rs708272	Brazilian $n = 567$	C-S	Olive oil, PUFA, sucrose, soluble and insoluble fiber	LDL, TG, TC	0.018–0.04	de Andrade et al. (62)

(Continued)

TABLE 1 (Continued)

Gene and SNP	Population and sample size	Study design	Dietary/lifestyle factor	Outcome	$P_{interaction}$*	References
rs708272	Mexican $n = 215$	C-S	Sucrose intake, physical activity	TC, LDL, TG, HDL, TG:HDL, BMI, WC	0.033–0.037	Campos-Perez et al. (113)
CETP						
rs708272	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
ADIPOQ						
rs2241766, rs16861209, rs17300539, rs266729, rs1501299	Brazilian $n = 262$	C-S	Plasma fatty acids (14:0, 16:0, 16:1 n-7, 18:0, 18:1, 18:2 n-6, 18:3 n-3, 20:3 n-6, AA, EPA, DPA, DHA, SFA, MUFA, n-6, n-3, PUFA, n-3 HUFA, SCD-16, SCD-18, D5D, D6D)	Systemic Inflammation	0.019–0.044	Maintinguer Norde et al. (75)
rs17300539	Mexican $n = 394$	C-S	MUFA, physical activity	adiponectin level	NS	Garcia-Garcia et al. (120)
rs2241766	Mexican $n = 167/398$	C-C	VO2 max, ME	pre-diabetes (fasting glucose concentrations)	NS	Costa-Urrutia et al. (119)
PPAR-γ						
rs1801282	Mexican $n = 167/398$	C-C	VO2 max, ME	pre-diabetes (fasting glucose concentrations)	NS	Costa-Urrutia et al. (119)
rs1801282	Costa Rican $n = 1,805/1,805$	C-C	PUFA intake	MI, PUFA in adipose tissue	0.016 –0.03	Ruiz-Narváez et al. (127)
PPAR-γ						
rs1801282	Argentina $n = 572$	C-S	Smoking status	MetS, fasting plasma glucose, SBP, DBP, WC, HDL, TG, fasting insulin, loginsulin, HOMA-IR, LogHOMA-IR, QUICKI	0.031	Tellechea et al. (165)
PPAR-γ C1A						
rs8192678	Mexican $n = 167/398$	C-C	VO2 max, ME	pre-diabetes (fasting glucose concentrations)	NS	Costa-Urrutia et al. (119)
PPAR-α						
rs1800206	Mexican $n = 167/398$	C-C	VO2 max, ME	pre-diabetes (fasting glucose concentrations)	NS	Costa-Urrutia et al. (119)
rs1800206	Mexican $n = 608$	C-C	VO2 max, ME	BMI, WC, fat mass, pre-DM	0.001–0.007	Costa-Urrutia et al. (118)
APOA4						
rs693, rs675, rs5110	Brazilian $n = 391$	C-S	Smoking, alcohol intake, physical activity	BMI, WC	0.007–0.02	Fiegenbaum et al. (91)
rs5104	Puerto Rican $n = 821$	LS	Total fat	WC, SBP, DBP	0.001–0.005	Mattei et al. (147)
IRS1						
rs2943641	Puerto Rican $n = 1,144$	LS	25(OH)D	HOMA-IR	0.004–0.023	Zheng et al. (159)
rs1801278	Chile $n = 243$	NRCT	3-day unrestricted diet containing 300 g/d of carbohydrate, an overnight fast of 10 h and 75 g glucose	Fasting glucose, fasting insulin, fasting HOMA-IR, insulinogenic index, insulin sensitivity index composite	NS	Sir-Petermann et al. (162)
IRS2						
rs1805097	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)

(Continued)

TABLE 1 (Continued)

Gene and SNP	Population and sample size	Study design	Dietary/lifestyle factor	Outcome	$P_{interaction}$*	References
PON1						
rs662	Mexican $n = 206$	C-S	Urinary 1-hydroxypyrene	Serum asymmetric dimethylarginine (ADMA)	0.02	Ochoa-Martínez et al. (121)
rs662	Mexican $n = 185$	C-S	Urinary arsenic levels	ADMA, fatty acid-binding protein 4, micro-RNAs	< 0.001 – < 0.010	Ochoa-Martínez et al. (122)
rs662, rs854560	Costa Rican $n = 492/518$	C-C	Smoking status	MI	0.04	Sen-Banerjee et al. (138)
AGT						
rs699	Brazilian $n = 335$	C-S	Sodium, potassium, calcium, magnesium	SBP, DBP	0.004–0.009	Giovanella et al. (81)
rs699	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
ADRB2						
rs1042713, rs1042714	Brazilian $n = 197$	P-C	Physical exercise intervention	Body fat, AC, BMI, DBP, SBP, TC, HDL, LDL, TG, glucose, insulin, HOMA-IR, QUICK, TG-glucose index	0.001	de Souza et al. (94)
rs1042713	Mexican $n = 608$	C-C	VO2 max, ME	BMI, WC, fat mass, pre-DM	0.001–0.007	Costa-Urrutia et al. (118)
TNF-α						
rs1799724, rs1800629, rs361525, rs1799964	Brazilian $n = 281$	C-S	Plasma fatty acids (C14:0, C16:0, C18:0, C16:1, C18:1, n-6, C18:2, C20:3, C20:4, n-3, C18:3, C20:5, C22:5, C22:6, n-3 HUFA, SCD-16, SCD-18, D5D, D6D, n-6:n-3, SFA, MUFA, PUFA)	Systemic inflammation	0.026 –0.044	Oki et al. (78)
TNF-α						
rs361525, rs7832552	Mexican $n = 608$	C-C	VO2 max, ME	BMI, WC, fat mass, pre-diabetes	0.001–0.007	Costa-Urrutia et al. (118)
CAPN10						
rs5030952, rs3792267, rs2975762	Mexican $n = 31$	P-C	Low SFA diet, soy protein, soluble fiber	TC, TG, HDL, LDL	NS	Guevara-Cruz et al. (107)
rs3842570	Colombian $n = 212/212$	C-C	Physical activity	BMI	NS	Orozco et al. (163)
rs3842570	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
PCSK9						
rs11206510	Costa Rican $n = 1,932/2,055$	C-C	LC n-3 PUFA, EPA, DPA, DHA	MI	0.012	Yu et al. (128)
rs11206510	Mexican American $n = 1,734$	C-S	Serum Vitamin A	LDL	7.65×10^{-5}	Dumitrescu et al. (158)
CYP1A2						
rs762551	Costa Rican $n = 2,014/2,014$	C-C	Coffee intake	MI	0.04	El-Sohemy et al. (136)
rs762551	Costa Rican $n = 873/932$	C-C	Smoking	MI	NS	Cornelis et al. (139)
CYP1A1						
rs1048943	Costa Rican $n = 873/932$	C-C	Smoking	MI	NS	Cornelis et al. (139)

(Continued)

TABLE 1 (Continued)

Gene and SNP	Population and sample size	Study design	Dietary/lifestyle factor	Outcome	$P_{interaction}$*	References
APOA2						
rs5082	Mexican $n = 100/100$	C-C	SFA, total fat	TC, TG, LDL, HDL, obesity	0.001–0.02	Domínguez-Reyes et al. (105)
rs5082	Puerto Rican $n = 930$	C-S	SFA	BMI	0.02	Corella et al. (145)
APOA1						
rs1799837	Puerto Rican $n = 821$	LS	Total fat	WC, SBP, DBP	0.001–0.005	Mattei et al. (147)
rs1799837	Brazilian $n = 567$	C-S	Olive oil, PUFA, sucrose, soluble and insoluble fiber	LDL, TG, TC	0.018–0.04	de Andrade et al. (62)
APOB						
rs512535	Mexican $n = 608$	C-C	VO2 max, ME	BMI, WC, fat mass, pre-DM	0.001–0.007	Costa-Urrutia et al. (118)
rs693	Mexican American $n = 1,734$	C-S	Serum Vitamin E	LDL	8.94×10^{-7}	Dumitrescu et al. (158)
LPL						
rs320	Puerto Rican $n = 1,171$	LS	Low PUFA, n-3 PUFA, n-6 PUFA intake	BMI, WC	0.02–0.04	Ma et al. (148)
rs285	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
UCP3						
rs1800849	Colombian $n = 212/212$	C-C	Physical activity	BMI	NS	Orozco et al. (163)
rs1800849	Colombian $n = 1,081$	C-S	Socioeconomic stratum, maternal education year, maternal breastfeeding	BMI	NS	Muñoz et al. (164)
TLR4 *rs11536889, rs4986790, rs4986791, rs5030728*	Brazilian $n = 262$	C-S	Systemic Inflammation	0.034	Norde et al. (76)	Systemic inflammation
BDKRB2 *rs1799722*	Brazilian $n = 335$	C-S	Sodium, potassium, calcium, magnesium	SBP, DBP	0.004–0.009	Giovanella et al. (81)
FADS *rs174575, rs174561, rs3834458*	Brazilian $n = 250$	C-S	α-linolenic acid, linoleic:α-linolenic acid ratio.	Plasma concentration of PUFA	0.004–0.028	Carvalho et al. (65)
CYBA (p22phox) *rs4673*	Brazilian $n = 1,298$	C-S	Urinary sodium	SBP, DBP, hypertension	<0.001–0.004	Schreiber et al. (83)
eNOS *rs2070744, rs1799983, rs61722009*	Brazilian $n = 113$	C-S	Alcohol intake	SBP, DBP, nitrite levels in plasma	0.033	Barcelos et al. (88)
FNDC5 *rs16835198*	Brazilian $n = 1,701$	C-S	Cardiorespiratory fitness, lower limb strength	WC, BMI	0.007–0.044	Todendi et al. (95)
LEPR rs8179183, rs1137101	Mexican $n = 100/100$	C-C	SFA, total fat intake	TC, TG, LDL, HDL, obesity	0.001–0.02	Domínguez-Reyes et al. (105)
ACSL1 rs9997745	Mexican $n = 167/398$	C-C	VO2 max, ME	pre-diabetes	NS	Costa-Urrutia et al. (119)
TRHR rs16892496	Mexican $n = 608$	C-C	VO2 max, ME	BMI, WC, fat mass, pre-diabetes	0.001–0.007	Costa-Urrutia et al. (118)
DRD2/ANKK1 rs1800497	Mexican $n = 175$	C-S	Maltose, total fat, MUFA, dietary cholesterol	**TG**	0.001 –0.041	Ramos-Lopez et al. (104)
GFOD2 rs12449157	Mexican $n = 41$	P-C	Low SFA diet, soy protein and soluble fiber	TC, LDL, HDL, TG	0.002–0.006	Guevara-Cruz et al. (106)
PLA2G4A *rs12746200*	Costa Rican $n = 1,936/2,035$	C-C	n-6 PUFA intake	MI	0.005	Hartiala et al. (129)

(Continued)

TABLE 1 (Continued)

Gene and SNP	Population and sample size	Study design	Dietary/lifestyle factor	Outcome	$P_{interaction}$*	References
CRP *rs1205, rs1417938, rs2808630*	Brazilian $n = 262$	C-S	Plasma fatty acids (Myristic acid, Palmitic acid, Stearic acid, C16:1, C18:1, n-6, C18:2, C20:3, C20:4, n-3, C18:3, C20:5, C22:5, C22:6, n-3 HUFA, SFA, MUFA, PUFA, SCD-16, SCD-18, D5D, D6D, n-6/n-3)	Systemic Inflammation	0.047	Oki et al. (77)
GSTM1 *rs366631* ***GSTP1*** *rs1695* ***GSTT1*** *rs17856199*	Costa Rican $n = 2,042/2,042$	C-C	Cruciferous vegetables, smoking	MI	0.008	Cornelis et al. (137)
IL1B *rs16944, rs1143623, rs1143627, rs1143643* ***IL6*** *rs1800795, rs1800796, rs1800797* ***IL10*** *rs1554286, rs1800871, rs1800872, rs1800890, rs3024490*	Brazilian $n = 301$	C-S	Plasma fatty acid (C14:0, C16:0, C16:1 n-9, C18:0, C18:1 n-9, C18:2 n-6, C18:3 n-3, AA, EPA, DHA, n-6, n-3); desaturates activity (SCD-16, SCD-18, D6D, D5D)	MetS	0.007–0.043	Norde et al. (79)
MTR *rs1805087* ***MTRR*** *rs1801394* ***TCN2*** *rs1801198* ***COMT*** *rs4680, rs4633* ***BHMT*** *rs492842, rs3797546* ***FUT2*** *rs602662*	Brazilian $n = 113$	C-S	Fat, protein, carbohydrate intake, physical activity	Vitamin B12, homocysteine, folic acid, HDL, LDL, triglycerides, oxidized LDL	0.005–0.034	Surendran et al. (66)
GSTM1 *rs366631* ***GSTP1*** *rs1695* ***GSTT1*** *rs17856199*	Costa Rican $n = 2,042/2,042$	C-C	Cruciferous vegetables, smoking	MI	0.008	Cornelis et al. (137)
LRP1 *rs1799986, rs1799986, rs1800191, rs715948*	Puerto Rican $n = 676$	P-C	SFA, palmitic acid (C16:0), stearic acid (C18:0), butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0)	BMI, WC, HC	0.002–0.004	Smith et al. (146)
PLIN *rs894160*	Puerto Rican $n = 920$	LS	Complex carbohydrate, total carbohydrate, simple sugars	WC, HC, BMI	0.004–0.035	Smith et al. (155)
Chromosome 9p21 *rs4977574, rs4977574, rs2383206, rs1333049*	Costa Rican $n = 1,560/1,751$	C-C	Sugar sweetened beverages, fruit juice	MI	0.005–0.03	Zheng et al. (135)
BDNF *rs6265*	Puerto Rican $n = 1,340$	LS	PUFA, n-3: n-6, food intake	BMI, WC, HC	0.002–0.043	Ma et al. (149)
PNPLA3 *rs738409*	Hispanic ancestry $n = 153$	C-S	Carbohydrate, sugar	Hepatic fat	0.01–0.04	Davis et al. (156)
SRBI *rs4238001*	Brazilian $n = 567$	C-S	Olive oil, PUFA, sucrose, soluble and insoluble fiber	LDL, TG, TC	0.018–0.04	de Andrade et al. (62)
GRS*:TCF7L2 (rs12255372, rs7903146); MC4R (rs17782313, rs2229616); PPARγ (rs1801282); FTO (rs8050136); CDKN2A/2B (rs10811661); KCNQ1 (rs2237892); CAPN10 (rs5030952)*	Brazilian $n = 200$	C-S	Total fat, SFA, PUFA, MUFA, carbohydrate, protein	HbA1c, HOMA-IR, HOMA-B, fasting glucose, fasting insulin, insulin:glucose, body fat mass, BMI, WC	0.002–0.017	Alsulami et al. (72)
GRS: *VDR (rs2228570, rs7975232), DHCR7 (rs12785878), CYP2R1(rs12794714), CYP24A1(rs6013897), GC (rs2282679), FTO (rs8050136, rs10163409), TCF7L2 (rs12255372, rs7903146), MC4R (rs17782313), KCNQ1 (rs2237895, rs2237892), CDKN2A (rs10811661), PPARγ (rs1801282), CAPN10 (rs5030952)*	Brazilian $n = 187$	C-S	Carbohydrate, protein, fat and fiber	BMI, WC, body fat, glucose, HbA1c, fasting insulin	0.006	Alathari et al. (73)

(Continued)

TABLE 1 (Continued)

Gene and SNP	Population and sample size	Study design	Dietary/lifestyle factor	Outcome	$P_{interaction}$*	References
GRS: *ABCA1 (rs2230806, rs9282541); ADIPOQ (rs2241766); ADRB2 (rs1042713); AGT (rs699); APOA4 (rs675); APOB (rs512535); APOE (rs405509); CAPN10 (rs2975760, rs2975762, rs3792267); FTO (rs1121980, rs9939609); HNF4 (rs745975); LIPC (rs1800588); LPL (rs320); PPAR-α (rs1800206); PPAR-γ (rs1801282); SCARB1 (rs1084674); TCF7L2 (rs7903146); TNF (rs361525); TRHR (rs1689249, rs7832552)*	Mexican $n = 608$	C-C	VO2 max, ME	BMI, WC, fat mass, pre-diabetes	0.001–0.007	Costa-Urrutia et al. (118)
GRS: *CDKN2A/2B (rs4977574, rs10757274, rs2383206, rs1333049); CELSR2-PSRC1-SORT1 (rs646776, rs599839); CXCL12(rs501120, rs1746048); HNF1A, C12orf43 (rs2259816); MRAS (rs9818870); SLC22A3 (rs2048327); LPAL2 (rs3127599); LPA (rs7767084, rs10755578)*	Costa Rican $n = 1{,}534/1{,}534$	C-C	Lifestyle cardiovascular risk score (unhealthy diet, physical inactivity, smoking, elevated waist:hip ratio, high alcohol intake, low socioeconomic status.)	MI	NS	Sotos-Prieto et al. (144)
GRS based on 97 BMI associated SNPs	Puerto Rican, Mexicans, Dominicans, Cuban, Central American, South American $n = 9{,}645$	P-C	Total physical activity, physical activity at a moderate to vigorous intensity, sedentary behavior	BMI, fat mass, fat mass index, fat percentage, WC Fat-free mass	0.001 –0.005	Moon et al. (160)
GRS: *MTNR1B* (*rs10830963*); *TCF7L2* (*rs7903146*); *CDKAL1* (*rs7756992*); *ADCY5* (*rs11717195*); *ANK1* (*rs516946*); *BCAR1* (*rs7202877*); *CDC123* (*rs11257655*); *DUSP9* (*rs5945326*); *GRB14* (*rs3923113*); *RASGRP1* (*rs7403531*); *TLE4* (*rs17791513*); *TLE1* (*rs2796441*); *ZBED3* (*rs6878122*)	Chile $n = 2{,}828$	P-C	Sugar sweetened beverages intake	Fasting glucose	0.001–0.02	López-Portillo et al. (161)
GRS: *APOA5 (rs662799); APOB (rs693, rs1367117); LDLR (rs688, rs5925); LIPC (rs2070895, rs1800588)*	Brazilian $n = 228$	C-S	Brazilian Healthy Eating Index Revised	Dyslipidaemia	0.001 –0.019	Fujii et al. (64)

APOE, Apolipoprotein E; *APOA*, Apolipoprotein A; *ApoB*, Apolipoprotein B; *SRBI*, scavenger receptor class B member 1; *ABCA1*, ATP binding cassette subfamily A member 1; *CETP*, cholesteryl ester transfer protein; *APOC3*, Apolipoprotein C; *ADIPOQ*, adiponectin; *TLR4*, toll like receptor 4; *FTO*, alpha-ketoglutarate dependent dioxygenase; *CRP*, C-reactive protein; GRS, genetic risk score; *MTHFR*, methylenetetrahydrofolate reductase; *FADS*, fatty acid desaturase; *TNF*, tumor necrosis factor; *ADRB*, adrenoceptor beta; *ACE*, angiotensin I converting enzyme; *AGT*, angiotensinogen; *BDKRB*, bradykinin receptor; *eNOS*, endothelial nitric oxide synthase; *CYBA*, cytochrome B-245 alpha chain; *IL*, interleukin; *FNDC5*, fibronectin type III domain containing 5; GRS, genetic risk score; *VDR*, vitamin D receptor; *DHCR7*, 7-dehydrocholesterol reductase; *CYP2R1*, cytochrome P450 family 2 subfamily R member 1; *CYP24A1*, cytochrome P450 family 24 subfamily A member 1; *GC*, group-specific component; *TCF7L2*, transcription factor 7 like 2; *MC4R*, melanocortin-4-receptor; *KCNQ1*, potassium voltage-gated channel subfamily Q member 1; *CDKN*, cyclin dependent kinase inhibitor; *PPAR*, peroxisome proliferator activated receptor; *CAPN*, Calpain; *MTR*, methionine synthase; *MTRR*, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; *TCN2*, transcobalamin 2; *COMT*, catechol-O-methyltransferase; *BHMT*, betaine-homocysteine S-methyltransferase; *FUT2* fucosyltransferase 2; *LEPR*, leptin receptor; *TRHR*, thyrotropin releasing hormone receptor; *LIPC*, hepatic lipase; *ACSL*, acyl-CoA synthetase long chain family member 1; *GFOD2*, Glucose-Fructose Oxidoreductase Domain Containing 2; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PON1* Paraoxonase 1; *CYP1A2*, cytochrome P450 family 1 subfamily A member 2; *PLA2G4A*, phospholipase A2 group IVA; *GSTM1*, glutathione S-transferase Mu 1; *GSTP1*, glutathione S-transferase Pi 1; *GSTT1*, glutathione S-transferase theta 1; *CYP1A1*, cytochrome P450 family 1 subfamily A member 1; *CELSR2*, Cadherin EGF LAG seven-pass G-type receptor 2; *PSRC1*, proline and serine rich coiled-coil 1; *SORT1*, sortilin 1; *CXCL12*, C-X-C motif chemokine ligand 12; *HNF1A*, hepatocyte nuclear factor 1; *MRAS*, muscle RAS oncogene homolog; *SLC22A3*, solute carrier family 22 member 3; *LPAL2*, lipoprotein(A) like 2, pseudogene; *LPA*, lipoprotein(A); *IRS*, insulin receptor substrate; *MTNR1B*, melatonin receptor 1B; *CDKAL1*, CDK5 regulatory subunit-associated protein 1-like 1; *ADCY5*, adenylyl cyclase type V; *ANK1*, ankyrin-1; *BCAR1*, breast cancer anti-estrogen resistance protein 1; *CDC123*, cell division cycle 123; *DUSP9*, dual specificity phosphatase 9; *GRB14*, growth factor receptor bound protein 14; *RASGRP1*, RAS guanyl-releasing protein 1; *TLE*, transducin-like enhancer protein; *ZBED3*, zinc finger BED-Type containing 3; *UCP3*, uncoupling protein 3; *LPL*, lipoprotein lipase; MetS, metabolic syndrome; SBP, systolic blood pressure; DBP, diastolic blood pressure; WC, waist circumference; BMI, body mass index; TG, triglycerides. HDL, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment estimate of insulin resistance; QUICKI, quantitative insulin-sensitivity check index; AUC, area under the curve; TC, total cholesterol; VLDL, very-low density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol. MI, myocardial infarction; PUFA, polyunsaturated fatty acid. MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; n-3, omega-3; LC, long-chain; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; C-S, C-S, cross-sectional; RCT, randomized controlled trial; NRCT, non-randomized controlled trial; P-C, prospective cohort; LS, longitudinal study; C-C, case-control; NS, not significant. *Only significant $P_{interaction}$ values are given.

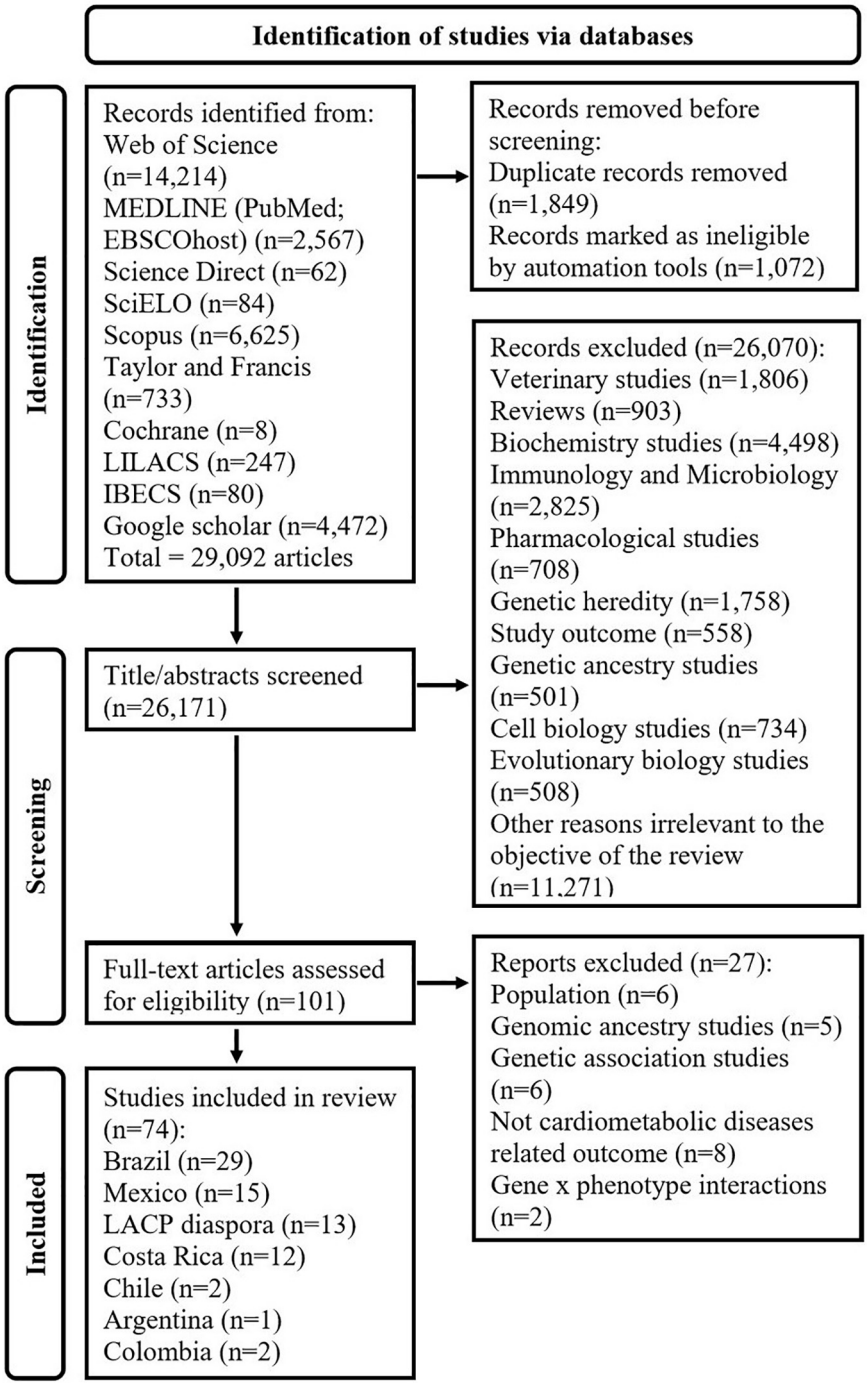

FIGURE 5
Flow chart showing the exclusion criteria and selection of studies. Literature search was conducted in MEDLINE (*via* PubMed and EBSCO Host), Web of Science, ScienceDirect, SciELO, SCOPUS, Taylor & Francis Online, Cochrane library, LILACS (Latin American and Caribbean Health Sciences Literature), IBECS, Google Scholar, and ERIC (Education Resources Information Center *via* EBSCO Host) search engines until the 25th of May 2022.

3.3.6. Interactions between micronutrients and genetic variants on cardiometabolic traits

The interaction between micronutrients and genetic variants on cardiometabolic traits was examined by five Brazilian studies (81–85). A cross-sectional study of 335 healthy young adults (81), observed a pronounced increase in systolic blood pressure (SBP) ($P_{interaction}$ = 0.016) among carriers of the "*G*" allele of Angiotensinogen (*AGT*) SNP rs699 with a higher plasma magnesium (209.3 mg). Similarly, among those with a high calcium intake (573.3 mg), carriers of the "*T*" allele of Bradykinin Receptor B2 (*BDKRB2*) SNP rs1799722 had significantly higher SBP ($P_{interaction}$ = 0.015) and diastolic BP (DBP) ($P_{interaction}$ = 0.014) than carriers of the "*CC*" genotype (81). In line with these findings, a case-control study of 234 elderly people (82) reported an interaction between sodium intake and angiotensin-converting enzyme (*ACE*) SNP rs4646994 on the risk of hypertension, where carriers of the "*I/I*" genotype with a high sodium intake (>2 g/day) had an increased risk of hypertension ($P_{interaction}$ = 0.007). Furthermore, in

a cross-sectional study of 1298 healthy adults (83), those carrying the "*T*" allele of Cytochrome B-245 Alpha Chain (*CYBA*) (*p22phox*) with more than 86.5 mEq sodium per 12 h of urine collection, had increased SBP ($P_{interaction} < 0.001$) and DBP ($P_{interaction} = 0.011$). Sodium is known to increase BP by reducing vasodilation (86), while dietary calcium is believed to stabilize intracellular calcium in smooth muscles, thereby reducing vasoconstriction and BP (87). Additionally, the "*A*" allele of *AGT* SNP rs699 is thought to be a risk factor for elevated SBP, possibly due to its association with a rise in plasma AGT levels (60, 81), and the findings of the study discussed above (81) indicate that, the protective effect of the "*G*" allele might be lost in the presence of higher plasma magnesium.

Similarly, in a longitudinal study of 1088 children with a follow up of 4.6 years (84), those with a deficit of plasma vitamin D (<75 nmol/L) and carriers of the risk allele ("*A*") of *FTO* SNP rs9939609 had increased BMI ($P_{interaction} = 0.033$). However, a cross-sectional study examining folate intake in 5914 healthy adults (85) did not identify interactions between folate intake and *MTHFR* SNP rs1801133 on homocysteine concentrations.

3.3.7. Interactions between alcohol intake and genetic variants on cardiometabolic traits

Three Brazilian studies (85, 88, 89) examined the interaction between alcohol intake and genetic variants on cardiometabolic traits. In a cross-sectional study of 113 participants (88), a significant interaction was observed between alcohol intake and endothelial nitric oxide synthase (*eNOS*) SNP rs2070744 (−786 T/C) on plasma nitrite levels. Individuals carrying the "C" allele who consumed alcohol had lower plasma nitrite levels ($P_{interaction} = 0.033$). However, there were no significant interactions between alcohol intake and rs2070744 on BP (88). Similarly, in a cross-sectional study of 3,803 participants from the Pelotas Birth Cohort (85), an interaction was identified between alcohol intake and *MTHFR* SNP rs1801133 (C677T), in which men with the "677TT" genotype who consumed ≥ 15 g of alcohol per day had the highest homocysteine concentration ($P_{interaction} = 0.002$); but the interaction was not observed in women. Moreover, a prospective cohort study of 964 postmenopausal women (89), reported no interactions between alcohol intake and *APOE* genotype on lipid traits. A rise in homocysteine concentration is attributed to a deficiency in B vitamins and folate, and SNPs of *MTHFR* might affect homocysteine concentration by impairing folate metabolism (90). However, it is unclear how alcohol intake modifies the activity of MTHFR, and the finding of the study (85) suggests a sex-specific response.

3.3.8. Interactions between smoking and genetic variants on cardiometabolic traits

Two studies (91, 92) investigated the interaction between smoking and genetic variants on cardiometabolic traits in Brazilians. In a cross-sectional study of 391 participants (91), smoking interacted with *APOA-IV* SNPs rs693 (*Xba*I), rs675 (Thr347Ser) and rs5110 (Gln360His), increasing BMI in individuals with the "X*2" ($P_{interaction} = 0.007$) and "347Ser" ($P_{interaction} = 0.02$) alleles. However, men with the "360His" allele who were non-smokers had a larger WC than homozygotes for the "Gln" allele ($P_{interaction} = 0.018$) (91). Similarly, in a cross-sectional study of 673 overweight adults (403 women and 270 men) (92), carriers of the "S2" allele of *APOC3* SNP rs5128 had increased triglycerides and the effect was more pronounced in women who smoked than in non-smokers ($P_{interaction} = 0.009$). Serum APOC3 concentration has been shown to be positively associated with triglyceride levels, and smoking has been reported to lower the concentration of APOC3 but only in women without central obesity (93), indicating a sex-specific response which is influenced by obesity traits.

3.3.9. Interactions between physical activity and genetic variants on cardiometabolic traits

Interactions between physical activity and genetic variants on cardiometabolic traits were investigated by nine Brazilian studies (66, 85, 91, 94–99). In a longitudinal study of 197 overweight or obese children (94), a physical exercise program (3 sessions/week for 12 weeks) interacted with adrenoceptor beta 2 (*ADRB2*) SNP rs1042714, decreasing triglyceride levels and triglyceride-glucose index ($P_{interaction} = 0.001$ for both) more in carriers of the "Glu27Glu" genotype than those carrying the "Gln27" allele. A cross-sectional study of 1701 children and adolescents (95) also reported higher BMI and WC in individuals with the "TT" genotype of fibronectin type III domain containing 5 (*FNDC5*) SNP rs16835198 compared to carriers of the "G" allele only in those with lower levels of cardiorespiratory fitness (CRF) ($P_{interaction} = 0.038$ and $P_{interaction} = 0.007$ for WC and BMI, respectively); and lower limb strength ($P_{interaction} = 0.040$ and $P_{interaction} = 0.044$ for WC and BMI, respectively). Physical activity has been proposed to alter the expression of certain genes (100), and the findings of these studies indicate that, the effect of physical activity on lipid, glycemic and anthropometric traits might be influenced by SNPs of *ADRB2* and *FNDC5* genes.

Similarly, a sedentary behavior (a screen time of > 378 min/day) was shown to increase cardiometabolic risk score in carriers of "AA" genotype of *FTO* SNP rs9939609 with a low CRF but not in those with a high CRF in a cross-sectional study of 1,215 children and adolescents ($P_{interaction} = 0.047$) (96). Along this line, a randomized controlled trial of 34 participants (97) reported that, a 45-min walk on a treadmill at moderate intensity resulted in a reduction in SBP ($P_{interaction} = 0.02$) and DBP ($P_{interaction} < 0.01$) in carriers of the "I" allele of *ACE* SNP rs4646994 compared with a non-exercise control session, but the reduction was not observed in participants with "DD" genotype. However, five studies (66, 85, 91, 98, 99) did not identify significant interactions between physical activity and genetic variants on cardiometabolic traits as shown in **Table 1**.

3.3.10. Other gene-diet interactions in Brazilians

In the BOLD study consisting of 113 participants (66), a total fat intake of 25.36% TEI interacted with Betaine-Homocysteine S-Methyltransferase (*BHMT*) SNP rs492842, increasing vitamin B12 concentrations ($P_{interaction} = 0.034$) in participants with the "TT" genotype. A case-control interventional study of 126 obese women (101) also reported that, a hypocaloric diet (< 600 kcal/day) for 7 weeks was associated with a decreased abdominal circumference ($P_{interaction} = 0.04$) among carriers of the "*A*" allele of *FTO* SNP rs9939609. Furthermore, in a prospective cohort study of 3,701 women, breastfeeding (> 6 months duration) interacted with *FTO* SNP rs9939609, decreasing BMI ($P_{interaction} = 0.03$), fat mass ($P_{interactin} = 0.03$), and WC ($P_{interaction} = 0.04$) in carriers of the "*A*" allele (102).

In summary, research in Brazil stands out in comparison to the rest of the gene-lifestyle research in LACP for being the most abundant; twenty-nine studies investigated gene x lifestyle interactions in the Brazilian population as shown in **Table 1**, covering

a wide range of cardiometabolic traits. Dietary fat intake and plasma fatty acid profile were the most frequently investigated dietary factors examined by seven and five studies, respectively, although all the studies examining plasma fatty acid profile used data from the HS-SP. Carbohydrate intake was examined by only three studies, all of which used data from the BOLD study. Similarly, protein intake was investigated by only three studies, two of which used data from the BOLD study. Physical activity was the most frequently examined lifestyle factor, followed by smoking and alcohol intake. Breastfeeding was examined by only one study (102), and lifestyle factors such as socioeconomic status, level of education, and the effect of rural and urban environments were not investigated. Only one study was conducted in rural settings (88), but it was not focused on interaction of the rural environment with genetic variants. The *FTO* SNP *rs9939609* was the most studied, being explored by five studies (84, 85, 96, 98, 99). Overall, the findings call for further research into lifestyle factors such as socioeconomic status, level of education and the effect of rural and urban environments as well as other dietary factors such as fruit and vegetable intake.

3.4. Gene x lifestyle interaction in Mexicans

3.4.1. Interaction between dietary fat intake and genetic variants on CVD traits

The interaction between dietary fat intake and genetic variants on CVD-related traits was examined by five Mexican studies (103–107). In a cross-sectional study of 224 participants with T2D (103), interactions between monounsaturated fatty acid (MUFA) intake and *APOE* genotype on blood lipid concentrations were reported. A low MUFA intake (< 10–15% TEI) was found to be associated with higher total cholesterol (TC) ($P_{interaction}$ = 0.016), non-HDL ($P_{interaction}$ = 0.024) and LDL ($P_{interaction}$ = 0.030) only in carriers of the "ε2" allele of *APOE* SNP rs7412. Similarly, interactions between MUFA intake ($P_{interaction}$ = 0.001), total fat intake ($P_{interaction}$ = 0.001), dietary cholesterol intake ($P_{interaction}$ = 0.019) and Dopamine Receptor D2/Ankyrin Repeat and Kinase Domain Containing 1 (*DRD2/ANKK1*) SNP rs1800497, increasing triglyceride levels in carriers of the "A2A2" genotype were observed in a cross-sectional study of 175 Mexican adults with T2D (104). MUFA intake has been linked to decreased triglyceride concentration (108) which is consistent with the findings of the first study (103). However, the findings of the second study (104) imply that MUFA intake might not be beneficial for individuals with the "A2A2" genotype of rs1800497. Both studies were conducted in participants with T2D which is known to affect lipid metabolism (109). Moreover, as highlighted by the authors of the second study (104), the effect of dietary fat intake on triglycerides concentration may be influenced by other factors including physical activity and the level of insulin resistance.

A Mexican case-control study consisting of 100 participants with normal weight and 100 participants with obesity (105) also found significant interactions between SFA intake and leptin receptor (*LEPR*) SNP rs1137101 on TC ($P_{interaction}$ = 0.002) and triglyceride ($P_{interaction}$ = 0.02) levels. It was reported that, a SFA intake of ≥ 12 g/day was associated with a 3.8 times higher risk of hypercholesteroleamia and a 2.4 times higher risk of hypertriglycerideamia compared to an intake of < 12 g/day in participants carrying the "G" allele of rs1137101 (105). An interaction between total fat intake with *LEPR* SNP rs1137101 on TC ($P_{interaction}$ = 0.001) was also reported in this study (105), where a high intake of total fat (≥83 g/d) was associated with a 4.1 times higher risk of hypercholesteroleamia in carriers of the "G" allele of rs1137101. Similarly, in a prospective cohort study involving a dietary intervention in 41 participants with hypercholesterolemia (106), interactions were observed between consumption of a diet low in SFA (<6% TEI/day) in addition to another diet containing 15 g of soluble fiber and 25 g of soy protein for 2 months and Glucose-Fructose Oxidoreductase Domain Containing 2 (*GFOD2*) SNP rs12449157 on TC ($P_{interaction}$ = 0.006) and LDL ($P_{interaction}$ = 0.025). Participants carrying the "G" allele had a larger decrease in TC and LDL in response to the dietary intervention compared to subjects with the "AA" genotype of rs12449157 (106). In this study (106), baseline LDL and TC levels were higher in carriers of the "G" allele, but they responded better to the dietary intervention, which indicates that the genetic risk of dyslipidaemia can be modified by a dietary intervention. However, in another study of 31 Mexican participants with dyslipidaemia (107) from the same cohort as above (106), using the same dietary intervention, no significant interactions were identified between the diet and Calpain 10 (*CAPN10*) SNPs rs5030952, rs2975762, and rs3792267 on lipid traits. It has been reported that SFA of different types and from different food sources might have different effects on cardiometabolic traits (110, 111), however, both studies (106, 107) used the same dietary intervention. Nonetheless, factors such as physical activity have also been reported to influence the effect of dietary fat intake on cardiometabolic traits (104), which could explain the differences in the findings.

3.4.2. Interaction between carbohydrate intake and genetic variants on cardiometabolic traits

Interactions between carbohydrate intake and genetic variants on cardiometabolic traits were examined by three Mexican studies (104, 112, 113). In a cross-sectional study of 3591 adults (112), carbohydrate intake was negatively associated with HDL concentrations in premenopausal women carrying the risk allele ("C") of ATP Binding Cassette Subfamily A Member 1 (*ABCA1*) SNP rs9282541 (*R230C*), but not in those carrying the "R" allele ($P_{interaction}$ = 0.037). In another cross-sectional study of 215 healthy adults (113), a high sucrose intake (>5% TEI) significantly increased TC ($P_{interaction}$ = 0.034) and LDL ($P_{interaction}$ = 0.037) more in participants with "B1B2/B2B2" genotype than those with "B1B1" genotype of cholesteryl ester transfer protein (*CETP)* SNP rs708272. However, the interaction did not influence triglycerides, HDL, BMI nor waist circumference (113). In contrast, the cross-sectional study discussed above (104), reported that the intake of maltose (0.68 ± 0.42 g/day) significantly decreased triglycerides ($P_{interaction}$ = 0.023) in carriers of the "A1"allele of *DRD2/ANKK1* SNP rs1800497. These findings indicate that carbohydrate intake might modulate lipid levels in Mexicans with certain genetic variants, but the mechanism through which carbohydrates affect lipid levels are unclear. Moreover, it has been reported that, the effect of carbohydrates on lipids might be dependent on glycemic index or glycemic load, and highly processed carbohydrates are linked to unfavorable lipid profiles (114).

3.4.3. Interaction between micronutrients and genetic variants on cardiometabolic traits

Two cross-sectional studies examined the interaction between micronutrients and genetic variants on cardiometabolic traits (115,

116). In the first study which consisted of 231 healthy new-borns (115), a deficient maternal vitamin B12 (<2.0 mcg/d) was found to be associated with a smaller size baby at birth in mothers with the "TT" genotype of *MTHFR* SNP rs1801133 ($P_{interaction}$ = 0.02) but a deficient maternal folate (<400 mcg/d) was not associated with anthropometric parameters (weight, length or BMI) of new-borns (115). A low vitamin B12 intake (<2.0 mcg/d) was also associated with increased homocysteine levels ($P_{interaction}$ = 0.01) in carriers of the "TT" genotype of *MTHFR* SNP rs1801133 in a cross-sectional study of 130 healthy women (116). The "TT" genotype of *MTHFR* is associated with decreased enzymatic activity and increased homocysteine concentration (117) and the findings of these studies suggest that increasing the intake of vitamin B12 might improve fetal development in Mexican women with the "TT" genotype.

3.4.4. Interaction between alcohol intake and genetic variants on cardiometabolic traits

The cross-sectional study of 130 healthy women discussed above (116), was the only study which examined alcohol intake and no interaction was found between alcohol intake and *MTHFR* SNP rs1801133 on homocysteine levels which could be due to the fact that 80% of the studied population consumed less than 1 cup/week of alcohol (116).

3.4.5. Interaction between physical activity and genetic variants on cardiometabolic traits

Interactions between physical activity and genetic variants on cardiometabolic traits were investigated by four Mexican studies (113, 118–120). In the cross-sectional study discussed above (113), increased concentration of TC ($P_{interaction}$ = 0.033) was observed in individuals carrying the "B2" allele of *CETP* SNP rs708272 who did not perform physical activity, compared to those with the "B1B1" genotype. However, there were no interactions on TG, HDL, TG:HDL ratio, LDL, BMI or WC (113). Similarly, interactions between physical fitness measured by muscular endurance (ME) and aerobic capacity with genetic variants were observed in a case-control study of 608 physically active adults (118), where higher levels of ME and aerobic capacity were associated with a lower WC in individuals with a high GRS based on 23 SNPs (**Table 1**) ($P_{interaction}$ = 0.0001 for both). In this study (118), a higher risk of obesity was found in older participants (≥ 40 years) with the "AA" genotypes of *APOB* SNP rs512535 ($P_{interaction}$ = 0.004) and tumor necrosis factor (*TNFA*) SNP rs361525 ($P_{interaction}$ = 0.007) with low levels of ME. However, another cross-sectional study of 565 physically active participants (119) did not find significant interactions between physical fitness and six SNPs (*ADIPOQ* rs2241766, *ACSL1* rs9997745, *LIPC* rs1800588, *PPARA* rs1800206, *PPARG* rs1801282 and *PPARGC1A* rs8192678) on glycemic traits. Moreover, the fourth cross-sectional study which consisted of 394 participants (120), did not identify interactions between physical activity and *ADIPOQ* SNP –11391G/A on adiponectin levels.

3.4.6. Other gene-lifestyle interactions in Mexicans

In a cross-sectional study of 206 Mexican women (121), an interaction between polycyclic aromatic hydrocarbons (PAHs) and Paraoxonase 1 (*PON1*) SNP rs661 (Q192R) on serum asymmetric dimethylarginine (ADMA) was observed, where individuals carrying the "R" allele had higher ADMA levels compared to those with the "QQ" genotype in response to higher levels of urinary 1-hydroxypyrene ($P_{interaction}$ = 0.02). Increased levels of ADMA ($p < 0.01$) and fatty acid-binding protein 4 ($p < 0.001$) were also identified in individuals with the "RR" genotype of *PON1* SNP rs661 with higher urinary arsenic levels (>45.0 μg/g of creatinine) in comparison with participants with the "QQ" genotype in a sample of 185 Mexican women (122). The mechanisms of the interaction may be shared in the case of exposure to PAHs as these are also involved in the generation of reactive oxygen species (123).

Overall, different cardiometabolic traits have been investigated in Mexico, where eleven out of fifteen studies found significant gene x lifestyle interactions (103–106, 112, 113, 115, 116, 118, 121, 122) as shown in **Table 1**. Dietary fat intake was the most frequently examined dietary factor, being investigated by five studies (103–107); followed by carbohydrate intake, which was examined by three studies (104, 112, 113). Physical activity was the most frequently examined lifestyle factor, while alcohol intake was investigated by only one study. Lifestyle factors such as smoking, socioeconomic status, level of education and the impact of rural and urban environments were not investigated. Moreover, dietary factors such as consumption of protein, complex carbohydrates, and fruits and vegetables have not been investigated, highlighting a need for further research.

3.5. Gene x lifestyle interaction in Costa Ricans

3.5.1. Interactions between dietary fat intake and genetic variants on CVD-related traits

The interaction between dietary fat intake and genetic variants on CVD-related traits was examined by six Costa Rican studies (124–129). In a cross-sectional study of 420 participants (124), SFA intake interacted with *APOE* genotype and influenced blood lipid concentrations. A higher SFA intake (13.5% energy) was associated with higher levels of very-low density lipoprotein cholesterol (VLDL) ($P_{interaction}$ = 0.03) and lower concentration of HDL ($P_{interaction}$ = 0.02) in carriers of the "ε*2*" allele. However, no significant interactions were identified between SFA intake and *APOE* genotype on lipids in a case-control study involving 1,927 participants with myocardial infarction (MI) and 1,927 matched controls (125). In another cross-sectional study of 336 participants (126), SFA intake was found to interact with *APOC3* genotype and impact on the concentration of TC ($P_{interaction}$ = 0.0004) and LDL ($P_{interaction}$ = 0.01). Homozygotes for the *APOC3-455T-625T* alleles had a 13% increase in TC and a 20% increase in LDL with a high SFA intake (>11% of energy intake), but the interaction was not significant in individuals with the *APOC3-455C-625del* allele (126). In the case-control study discussed above (125), a significant interaction between SFA intake and *APOE* genotype on the risk of MI ($P_{interaction}$ = 0.0157) was also reported, in which carriers of the "ε*4*" allele had a 49% increased risk of MI compared to a 2.2 fold increased risk in those with the "ε*2*" allele in response to a high SFA intake (>11.8% of energy intake).

APOE plays a key role in lipid metabolism, being a main component of triglyceride-rich lipoproteins and HDL, and a ligand for LDL receptor (124, 130) and it is believed that the metabolism of fatty acids is impaired in carriers of the "ε*4*" allele which is considered a risk factor for CVDs (131). However, the above findings indicate that, a high SFA intake is more detrimental to carriers of the "ε*2*" allele

than those carrying the "ε*4*" allele, highlighting the potential role of SFA intake in modifying genetic risk.

In accordance with the findings above, a case-control study of 1805 participants with a first non-fatal MI and 1,805 matched controls (127), reported an interaction between PUFA intake and *PPARγ* SNP rs1801282, influencing the risk of MI ($P_{interaction} = 0.03$). Individuals with the "Pro12/Pro12" genotype had a 34% reduced risk of MI per 5% increment in energy from PUFA compared to a 7% decreased risk in those carrying the "*Ala12*" allele (127). Similarly, a case-control study of 1932 participants with a first non-fatal MI and 2,055 matched controls (128), reported a significant interaction between long-chain omega-3 (LC n-3) PUFA intake and Proprotein Convertase Subtilisin/Kexin Type 9 (*PCSK9*) SNP rs11206510 on the risk of MI ($P_{interaction} = 0.012$), where carriers of the "C" allele had an odds ratio for MI of 0.84 per 0.1% increase in total energy from LC n-3 PUFA, compared to an odds ratio of 1.02 in participants without the "C" allele (128). Along similar lines, a case-control study of 1936 participants with a first non-fatal MI and 2,035 matched controls (129) reported a significant interaction between omega-6 (n-6) PUFA intake and Phospholipase A2 Group IVA (*PLA2G4A*) SNP rs12746200 on the risk of MI ($P_{interaction} = 0.005$), in which participants with the "G" allele had a reduced risk of MI with an intake of n-6 PUFA above the median compared to those with the "AA" genotype. However, there were no significant interactions with n-3 PUFA intake (129).

These findings indicate that the beneficial effect of PUFA intake reported by some studies (101, 132) might be limited in individuals with certain genetic variants. PPARγ is a nuclear receptor which is involved in adipogenesis and plays a role in the metabolism of glucose and fatty acids (133, 134), and the "*Ala12*" allele of PPARγ SNP rs1801282 has been reported to slow down the release of PUFA from adipocytes, which could explain the smaller reduction in the risk of MI in comparison with carriers of the "Pro12/Pro12" genotype (127).

3.5.2. Interaction between other dietary factors and genetic variants on the risk of MI

Interactions between other dietary factors and genetic variants on the risk of MI were examined by three Costa Rican studies (135–137). In a case-control study of 1,560 incident cases of non-fatal MI and 1,751 matched controls (135), sugar sweetened beverage (SSB) intake interacted with a GRS based on 3 SNPs of chromosome 9p21 (rs4977574, rs2383206 and rs1333049), increasing the risk of MI ($P_{interaction} = 0.03$). SSB intake also interacted with rs4977574, increasing the risk of MI in carriers of the "G" allele ($P_{interaction} = 0.005$), but there was no interaction with fruit juice intake (135). In another case-control study of 2,014 participants with a first acute non-fatal MI and 2,014 matched controls (136), an increased risk of MI with increasing coffee intake was observed in carriers of the "C" allele (also known as "slow metabolizers of caffeine") of Cytochrome P450 Family 1 Subfamily A Member 2 (*CYP1A2*) SNP rs762551 compared to carriers of the "AA" genotype ($P_{interaction} = 0.04$). Similarly, in a case-control study consisting of 2,042 participants with a first non-fatal MI and 2042 control subjects (137), cruciferous vegetable intake (0.86 servings/day of half a cup) interacted with Glutathione S-Transferase Theta 1 (*GSTT1*) SNP rs17856199, lowering the risk of MI in carriers of the "*1" allele, but not in individuals with the "*0*0" genotype ($P_{interaction} = 0.006$). These findings indicate that, dietary factors other than fat intake, might also influence the risk of MI in Costa-Ricans with certain genetic variants.

3.5.3. Interaction between smoking and genetic variants on the risk of MI

Interaction between smoking and genetic variants on the risk of MI was investigated by three Costa Rican case-control studies (137–139), two of which found significant interactions (137, 138). In a case-control study of 492 participants with a first non-fatal MI and 518 matched controls (138), an interaction was observed between smoking status and Paraoxonase 1 ($PON1_{192}$) SNP rs661 on the risk of MI ($P_{interaction} = 0.04$), where the $PON1_{192Arg}$ allele was associated with an increased risk of MI only in non-smokers. Similarly, in the case-control study discussed above (137), the combined intake of cruciferous vegetables (>5 servings/day) and smoking (1–10 cigarettes/day) in carriers of the "*1" allele of rs17856199, lowered the risk of MI ($P_{interaction} = 0.008$). However, there were no significant interactions with *GSTM1* or *GSTP1* genotype on the risk of MI (137). Moreover, in the third Costa Rican case-control study which involved 873 participants with a first non-fatal MI and 932 control subjects (139), no significant interactions were observed between smoking and *CYP1A1* SNP rs1048943 or *CYP1A2* SNP rs762551 on the risk of MI. Smoking has been linked to increased risk of MI (140, 141) although the mechanisms are unclear. Smoking is also believed to impair the activity of PON1, which is linked to increased risk of CVDs (142, 143), but this is not supported by the findings of the studies above, suggesting that Costa Ricans with certain genetic variants might respond differently to smoking.

3.5.4. Other gene-lifestyle interactions in Costa Ricans

One case-control study consisting of 1534 participants with a first non-fatal MI and 1,534 matched controls (144), investigated the interaction between a lifestyle cardiovascular risk score comprising of physical activity, smoking, alcohol consumption, waist-to-hip ratio, and socioeconomic status; and a GRS based on 14 SNPs (**Table 1**) on the risk of MI, and no significant interactions were identified.

The research in Costa Rica has mainly focused on CVD traits in adults, with an emphasis on the risk of MI, and dietary fat intake has been the most frequently examined exposure. Socioeconomic status was examined by one study (144), and lifestyle factors such as educational level, the effect of rural and urban environments as well as dietary factors such as consumption of protein, fiber and complex carbohydrates have not been explored, highlighting a need for further research.

3.6. Gene x lifestyle interaction in LACP diaspora

3.6.1. Interaction between dietary fat intake and genetic variants on anthropometric traits

Interaction between dietary fat intake and genetic variants on anthropometric traits were investigated by six studies (145–150), all of which used data from the Boston Puerto Rican Health Study (BPRHS). In a cross-sectional study of 930 Puerto Ricans from the BPRHS (145), a high intake of SFA (≥22 g/day) was associated with a 7.9% higher BMI in individuals with the "CC" genotype of *APOA2* SNP rs5082 than those carrying the "T" allele ($P_{interaction} = 0.003$); but the SNP had no effect on BMI when SFA intake was low (<22 g/day). This study also observed that, among individuals with a high SFA intake (≥22 g/d), those with the "CC" genotype had a

higher risk of obesity than participants carrying the "T" allele of the SNP rs5082 [Odds ratio (OR) = 1.84; 95% confidence interval (CI) = 1.38–2.47; $P < 0.0001$). A similar finding was reported in a prospective cohort study of 920 participants from the BPRHS (146), where a high intake of SFA (≥ 9.3% of total energy) was linked to higher BMI ($P_{interaction} = 0.006$), WC ($P_{interaction} = 0.02$), and hip circumference (HC) ($P_{interaction} = 0.002$) in participants carrying the minor allele ("T") of LDL receptor related protein 1 (*LRP1*) SNP rs1799986 compared to individuals with the "CC" genotype; but the SNP had no effect on anthropometric traits when SFA intake was low (<9.3% of total energy). The "CC" genotype of *APOA2* rs5082 is believed to affect body fat distribution by lowering plasma concentration of APOA2 and these findings indicate that, a low SFA intake might attenuate this genetic risk (145, 151).

An interaction of total fat intake with *APOA1*-75 on WC was also reported in a longitudinal study of 821 participants of the BPRHS (147), in which individuals carrying two copies of the major allele had a lower WC with a low total fat intake than those carrying the minor allele ($P_{interaction} = 0.005$). A longitudinal study performed in 1,171 participants (333 men and 838 women) of the BPRHS (148) also observed that, women with the "TT" genotype of lipoprotein lipase (*LPL)* SNP rs320 had lower BMI ($P_{interaction} = 0.002$) and WC ($P_{interaction} = 0.001$) with a high intake of PUFA but this was not observed in minor allele ("G") carriers and there were no significant interactions in men. In contrast, another longitudinal study of 1,340 participants (395 men and 945 women) of the BPRHS (149) found that, men with the "GG" genotype of brain derived neurotrophic factor (*BDNF)* SNP rs6265 had higher BMI ($P_{interaction} = 0.042$), WC ($P_{interaction} = 0.018$), and HC ($P_{interaction} = 0.009$) with a low PUFA intake (<8.76% of energy) than those carrying the "A" allele but no difference was observed when PUFA intake was high (≥8.76% of energy) and the interaction was not observed in women. Interaction between Mediterranean diet with *TCF7L2* SNP rs7903146 on obesity-related traits was also observed in a cross-section study of 1,120 Puerto Ricans of the BPRHS (150), where carriers of the "T" allele had lower WC (99.2 ± 0.9 vs. 102.2 ± 0.9 cm; $P_{interaction} = 0.026$) and weight (77.3 ± 1.0 vs. 80.9 ± 1.0 kg; $P_{interaction} = 0.024$) with a high Mediterranean diet score than individuals with "CC" genotype. However, there were no significant differences between the genotypes when the Mediterranean diet score was low. The findings suggest that a high intake of PUFA and Mediterranean diet might be beneficial in reducing the genetic risk of obesity-related traits in a sex-specific manner and call for further research into the mechanisms involved.

3.6.2. Interaction between dietary fat intake and genetic variants on CVD traits

Interaction between total fat intake and genetic variants on CVD traits were reported by three studies (147, 152, 153). In a longitudinal study of 802 participants of the BPRHS (152), a significant interaction was observed between total fat intake and *APOA5* SNP -1131T < C on plasma triglycerides ($P_{interaction} = 0.032$), where a high total fat intake (≥31% of total energy) was associated with a higher plasma triglyceride concentration in individuals with the "1131C" allele, although no difference between the genotypes was observed when total fat intake was low. This study (152) also observed an interaction between *APOA5* SNP S19W with total fat intake on SBP ($P_{interaction} = 0.002$) and DBP ($P_{interaction} = 0.007$), where participants with the minor allele ("G") had a higher SBP with a low total fat intake (< 31% of total energy), and a lower SBP with a high total fat intake in comparison with individuals with the "CC" genotype. The study on 821 participants of the BPRHS discussed above (147), also reported significant interactions between total fat intake and *APOC3* -640 on DBP ($P_{interaction} = 0.003$), *APOA4* N147S and *APOA5* S19W on SBP ($P_{interaction} = 0.001$ and $P_{interaction} = 0.002$, respectively). It was observed that, homozygous for the major allele of *APOA1*-75, *APOA4* N147S and *APOA5* S19W had lower SBP with a low intake of total fat (< 31% of total energy) than those carrying the minor allele; while heterozygous for *APOC3* -640 had lower DBP with a high total fat intake (≥ 31% from energy) (147). However, a randomized crossover trial involving 41 adults from Dominican, Puerto Rican and other Caribbean Hispanic origins (153), did not find significant interactions between a high fat diet and hepatic lipase (*LIPC*) SNP rs1800588 on HDL, LDL, TC or plasma glucose concentrations. A high intake of total fat has been associated with an unfavorable lipid profile and high blood pressure (154) and the above findings indicate that, this association might be influenced by variants of several genes.

3.6.3. Interaction between carbohydrate intake and genetic variants on cardiometabolic traits

Two studies investigated the interaction between carbohydrate intake and genetic variants on cardiometabolic traits (155, 156). In a longitudinal study involving 920 participants of the BPRHS (155), a significant interaction was observed between Perilipin 1 (*PLIN 1*) SNP 1,482 G > A and complex carbohydrate intake on WC ($P_{interaction} = 0.002$), where individuals carrying the "A" allele had a higher WC with a low intake of complex carbohydrate (<144 g/day) and a lower WC with a high intake of complex carbohydrate (≥144 g/day) than those with the "GG" genotype. Similarly, a cross-sectional study of 153 children descendent from Hispanic ancestry (156), identified significant interaction between carbohydrate intake (211.4 g/day) and total sugar intake (96.1 g/day), increasing hepatic fat fraction in carriers of the "GG" genotype of Patatin like phospholipase domain containing 3 (*PNPLA3*) SNP rs738409 ($P_{interaction} = 0.04$ and $P_{interaction} = 0.01$, respectively), but the interaction was not observed in individuals carrying the "C" allele. It has been reported that, body weight might be influenced by the type of carbohydrate consumed (157) which is supported by the findings of these studies, but the results also indicate that genetic variants might also play a role.

3.6.4. Interaction between micronutrient intake and genetic variants on cardiometabolic traits

The interaction between micronutrient intake and genetic variants on cardiometabolic traits was investigated by two studies (158, 159). A cross-sectional study involving 1,734 Mexican Americans (158) reported a significant interaction between vitamin E and *APOB* SNP rs693 on LDL ($P_{interaction} = 8.94 \times 10\text{-}7$), and between vitamin A and *PCSK9* SNP rs11206510 on LDL ($P_{interaction} = 7.65 \times 10\text{-}5$), but the direction of the interactions is unclear. Similarly, in the longitudinal study of 1,144 Puerto Ricans of the BPRHS discussed above (159), a significant interaction between vitamin D status and *IRS1* rs2943641 on the risk of T2D was identified in women in which minor allele homozygotes ("TT") had a lower risk of T2D compared with "C" allele carriers only when 25(OH)D was higher than the median [>17 ng/mL (42.4

nmol/L)] ($P_{interaction}$ = 0.007), but the interaction was not observed in men. The findings of these studies indicate that micronutrients might modulate the association between genetic variants and lipid and glycemic traits, but further studies are needed to replicate and elucidate the mechanisms involved.

3.6.5. Interaction between physical activity and genetic variants on cardiometabolic traits

Only one study (160) examined the interaction between physical activity and genetic variants on cardiometabolic traits. This study (160) was a prospective cohort study of 9,645 adult Puerto Ricans, Mexicans, Dominicans, Cuban, Central American, and South American from the Hispanic Community Health Study/Study of Latinos (HCHS/SOL) cohort, USA, and a positive association was observed between a GRS based on 97 SNPs (**Table 1**) and BMI, but the effect of the GRS was stronger in the first tertile of moderate to vigorous physical activity compared to the third tertile ($P_{interaction}$ = 0.005). Significant interactions following the same pattern were observed for fat mass ($P_{interaction}$ = 0.003), fat percentage ($P_{interaction}$ = 0.003) and fat mass index ($P_{interaction}$ = 0.002) (160).

In summary, research in LACP diaspora has mainly focused on Puerto Ricans residing in USA and most of this evidence (10 out of 13 studies) comes from the same study (BPRHS). Dietary fat intake has been the most frequently studied, with carbohydrate intake being examined by only two studies. Similarly, physical activity was investigated by only one study and lifestyle factors such as socioeconomic status, level of education, and the effect of rural and urban environments have not been explored.

3.7. Gene x lifestyle interactions in Chileans

3.7.1. Interaction between carbohydrate intake and genetic variants on glycemic traits

Two gene-diet interaction studies were reported in Chileans (161, 162). The first study (161) was a cross-sectional study of 2828 healthy Chilean adults, and a significant interaction was observed between consumption of SSB and a weighted genetic risk score (wGRS) based on 16 T2D risk SNPs (**Table 1**) on log-fasting glucose ($P_{interaction}$ = 0.02), where the strongest effect was observed between the highest SSB intake (≥2 servings/day of 330 ml) and the highest wGRS. In this study (161), SSB intake also interacted with additive effects of Transcription Factor 7 Like 2 (*TCF7L2*) SNP rs7903146 ($P_{interaction}$ = 0.002) and with the "G/G" genotype of Melatonin Receptor 1B (*MTNR1B*) SNP rs10830963 ($P_{interaction}$ = 0.001), increasing log-fasting glucose levels. The second Chilean study (162) was a non-randomized controlled trial performed in 97 healthy women and 147 women with polycystic ovary syndrome, and there were no reported interactions between a high glycemic carbohydrate intake (75 g of glucose) during an oral glucose tolerance test and Insulin Receptor Substrate 1 (*IRS-1*) SNP rs1801278 on glycemic traits. In Chile, research has been limited to diabetes traits as outcomes and simple carbohydrates as exposure, reflecting a need for further research into other dietary and lifestyle factors such as socioeconomic status, level of education and the effect of rural and urban environments.

3.8. Gene x lifestyle interactions in Colombians

Two gene-lifestyle interaction studies were conducted in Colombians (163, 164). The first study (163) was a case-control study involving 212 normal weight, 112 overweight and 100 obese teenagers and no significant interactions were observed between physical activity and three SNPs (Uncoupling Protein 3 (*UCP3*) rs1800849, *FTO* rs17817449, and *CAPN10* rs3842570) on excess weight. However, sub-group analysis showed that, a sedentary lifestyle was associated with an increased risk of excess weight only in those with the "GG" or 'TT' genotype of *FTO* rs17817449 (p = 0.0005); and 'CC' genotype of *UCP3* rs1800849 (p = 0.0032) (163). It was also observed that, even with an active lifestyle [1.6–1.9 metabolic equivalent task (MET) minute/day], individuals with the "II" genotype of *CAPN10* rs3842570 had a higher risk of excess body weight compared to those carrying the "D" allele (p = 0.0212) (163). The second study which was also a cross-sectional study involved 1,081 Colombian teenagers (164), and there were no interactions between lifestyle factors (socioeconomic stratum, level of education and maternal breastfeeding) and ten SNPs on BMI (**Table 1**). As both studies (163, 164) were conducted in teenagers and focused on obesity traits, there is a need for further research into other cardiometabolic traits in the wider Colombian population.

3.9. Gene x lifestyle interactions in Argentinians

Only one study (165) was conducted in Argentinians, and this was a cross-sectional study consisting of 572 healthy Argentinian men. This study (165) reported a significant interaction between smoking status and *PPARγ* SNP rs1801282 on the risk of MetS ($P_{interaction}$ = 0.031) where among the non-smokers, carriers of the "*Pro/Ala*" genotype (p = 0.0059) and the "*Ala12*" allele (p = 0.009) had a higher risk of MetS than non-carriers. It is unclear whether there were significant interactions between smoking status and rs1801282 genotype on the other outcomes investigated in the study (165) (**Table 1**), since the p-values given are for associations stratified by smoking status. The study adjusted for BMI and age only, but the pathophysiological mechanism of MetS is multifactorial (166), and hence other factors should be considered simultaneously. There have been no studies in Argentina examining the interactions of genetic variants with dietary factors, physical activity, or other lifestyle factors apart from smoking status.

4. Summary of the findings of commonly investigated interactions across the countries

The most commonly investigated interactions in LACP related to dietary fat intake and genetic variants on blood lipids. A high intake of olive oil was associated with lower LDL in Brazilian men with the "ε*2*" allele of *APOE* (62), while a low MUFA intake was linked to higher TC, non-HDL and LDL in Mexicans carrying the "ε*2*" allele of *APOE* (103). In contrast, increased TG concentration in response to a high MUFA intake was observed in Mexicans who were homozygotes

for the A2 allele of *DRD2/ANKK1* SNP rs1800497. A high PUFA intake was also associated with increased concentration of LDL in Brazilian carriers of the "ε*4*" allele, and reduced concentration of TG in those carrying the "ε*2*" allele of *APOE* (62). However, a low PUFA intake was linked to increased TG and VLDL concentration in Brazilian women with the "ε*4*" allele of *APOE* (63).

Furthermore, a high SFA intake was associated with higher VLDL and lower HDL concentrations in Costa Rican carriers of the "ε*2*" allele of *APOE* (124), but no significant interactions were identified between SFA intake and *APOE* genotype on blood lipids in a Costa-Rican case-control study involving participants with MI (125). However, a high SFA intake was linked to increased concentrations of TC and LDL in Costa Ricans who were homozygotes for the *APOC3-455T-625T* alleles (126). Similarly, a high SFA intake was associated with increased TC and TG concentrations in Mexicans with the "G" allele of *LEPR* SNP rs1137101 (105); while a low SFA intake was linked to a decrease in TC and LDL concentrations in Mexicans with the "G" allele of *GFOD2* SNP rs12449157 (106).

The inconsistencies in the findings of the above studies call for further research into the interaction between sub-types of fat and genetic variants on blood lipids. The sources of dietary fat also need to be considered since SFA from different food sources have been reported to have different effects on cardiometabolic traits (111).

5. Discussion

This is the first systematic review to investigate gene-lifestyle interactions on cardiometabolic diseases in LACP, highlighting several gene-lifestyle interactions with effects being significant in Brazilians, Mexicans, Costa Ricans, Chileans, Argentinians, Colombians and LACP diaspora. The most frequently studied genes have been *FTO*, examined in Colombians, Mexicans, and Brazilians, *APOE* explored in Costa Ricans, Mexicans, and Brazilians, and *TCF7L2* investigated in Chileans, Mexicans, Brazilians and LACP diaspora. The concentration of blood lipids such as HDL and LDL was the most widely investigated trait, followed by BMI and WC; MI was examined by 11 studies and one study looked at hepatic fat accumulation, while diseases such as stroke and liver cirrhosis were not investigated. Research has identified gene-lifestyle interactions that describe effects which are population-, gender-, and ethnic-specific. The findings of this review indicate that most of the gene x lifestyle interactions were conducted once, necessitating replication to strengthen the evidence.

Another issue that could affect the results is the accuracy of the methods used to measure exposure variables such as dietary intake and physical activity (167, 168). Some studies used 24-h recall questionnaires and self-reporting methods (64, 77, 81, 112, 144, 158), which might have induced recall bias, inadequate estimations, daily variation bias, and over and underreporting of values (169, 170). Measurement of dietary intake is a crucial part of gene-diet interaction studies as under or overestimation of dietary intake can weaken or reverse the association between dietary factors and cardiometabolic traits (170, 171). Moreover, other studies used food frequency questionnaires with no information on whether they were tested for validity. Genotyping errors can also affect the results of gene-diet interactions by leading to deviations from the true genotype (172, 173).

Sample size has also been highlighted as a key methodological issue in gene-lifestyle interaction studies (167, 168). For complex traits where the main effects of genetic variants are often modest, a large sample size is required to detect small interaction effects (167, 174). Thus, it is important that studies are adequately powered to detect true interactions (168). Nonetheless, most of the studies had small sample sizes and only a few included information on statistical power to detect interactions. There is also the risk of false-positive finding when there is no correction for multiple comparisons (173, 175), but only a few of the studies provided information on correction for multiple comparisons.

Overall, the included studies are majorly cross-sectional, indicating a need for longitudinal/prospective studies. The findings reflect gaps in covering the genetic risks and the socioeconomic variables to which the LACP are exposed; 27 out of 33 LACP have not conducted gene-lifestyle interaction studies yet. Only five studies have been conducted in contexts of low socioeconomic status, and from these, only two studies investigated gene-socioeconomic status interactions (144, 164). Moreover, no studies have examined the impact of rural and urban environments on the genetic predisposition to cardiometabolic diseases, highlighting a gap in knowledge in LACP. The higher number of nutrigenetic studies in Brazil compared to the other countries could be attributed to several factors including existing data on genetic studies (176–181), GWAS done mainly in Brazil (182–184), increased awareness on nutrigenetics in Brazil or more research facilities available in Brazil compared to other LACP. Future gene-lifestyle interaction studies will need to replicate primary research of already studied genetic variants to enable comparison, and to explore the interactions between genetic and other lifestyle factors such as those conditioned by socioeconomic factors and the built environment. Moreover, the molecular mechanisms that underlie the gene-lifestyle interactions identified by this systematic review need to be explored. The strength of this review is the comprehensive search strategy and the inclusion of all dietary/lifestyle exposures and cardiometabolic traits. Another strength is the use of standardized tools to assess the quality of the studies. However, the study has some limitations.

In conclusion, this systematic review has identified several gene-lifestyle interactions on cardiometabolic disease traits in Brazilians, Mexicans, Costa Ricans, Chileans, Argentinians, Colombians and LACP diaspora, highlighting effects which are population-, gender-, and ethnic-specific. However, the lack of replication of most of the gene-lifestyle interactions made it difficult to evaluate the evidence. Moreover, most of the studies were cross-sectional meaning that they preclude causal assumptions hence a temporal relationship cannot be established. Future gene-lifestyle interaction studies will need to replicate primary research of already studied genetic variants to enable comparison, and to explore the interactions between genetic and other lifestyle factors such as those conditioned by socioeconomic factors and the built environment. Moreover, the molecular mechanisms that underlie the gene-lifestyle interactions identified by this systematic review need to be explored.

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 author.

Author contributions

KV: conceptualization, supervision, and project administration. EV, KV, and RW: methodology, validation, investigation, writing—original draft preparation, and resources. EV and RW: software, formal analysis, data curation, and visualization. AS and KV: funding acquisition. All authors contributed to writing—review and editing and read and agreed to the final version of the manuscript.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1067033/full#supplementary-material

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Effect on nutritional status and biomarkers of inflammation and oxidation of an oral nutritional supplement (with or without probiotics) in malnourished hemodialysis patients. A multicenter randomized clinical trial "Renacare Trial"

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Background: Malnutrition in patients undergoing hemodialysis is frequent and associated with a reduction in muscular mass and strength, with an increment in biomarkers of inflammation and oxidation.

Materials and methods: Randomized, multicenter, parallel-group trial in malnourished hemodialysis patients with three groups [(1) control (C) individualized diet, (2) oral nutritional supplement-ONS- + placebo-SU- PL-, and (3) ONS + probiotics-SU-PR]; the trial was open regarding the intake of ONS or individualized diet recommendations, but double-blind for the intake of probiotics. We obtained, at baseline and after 3 and 6 months, anthropometric measurements, handgrip strength, bioelectrical impedance analysis (BIA), dietary records, and routine biochemical parameters. Inflammation and oxidation were determined using ELISA techniques (Versamax and ProcartaPlex multiplex Immunoassay). Results were analyzed by intention to treat.

Results: A total of 31 patients (11 corresponding to group C, 10 to SU-PL, and 10 to SU-PR) completed the 6-months trial. The two groups that took supplements significantly increased their protein calorie, fat (total and n-3), and fiber intake. Weight and fat-free mass (FFM) also increased significantly in the groups on supplements, both at 3 and 6 months, and dynamometry did so in the SU-PL group. At month 3, prealbumin and vitamin D were significantly increased in the SU-TOT (SU-PL + SU-PR) group. No changes were observed regarding levels of phosphorus and potassium in any of the groups. Urea increased significantly at 6 months in the SU-PL group. There were significant changes in some inflammation biomarkers in the

groups on supplements during the intervention (brain-derived neurotrophic factor, bone morphogenetic protein-2, MCP-1, IL-1-beta, IL-10, IL-4, and IL-8). The total antioxidant capacity (TAC) increased significantly in the supplemented patients, with no significant changes observed in isoprostanes.

Conclusion: The specific ONS improved protein-calorie intake, nutritional status (mainly FFM), and some biomarkers of inflammation/oxidation. The addition of probiotics could have a synergistic effect with ONS in such biomarkers.

Clinical trail registration: https://clinicaltrials.gov/ct2/show/, identifier NCT03924089.

KEYWORDS

oral nutritional supplement, hemodialysis, inflammation biomarkers, oxidation biomarkers, malnutrition, probiotics

1. Introduction

Malnutrition, or protein-energy wasting (PEW), is highly prevalent among patients with chronic kidney disease (CKD), especially those undergoing hemodialysis, and is associated with significant morbidity and mortality. The etiology of malnutrition is multifactorial and includes decreased protein-calorie intake due to anorexia and dietary restrictions, inflammation, hypercatabolism, protein loss during dialysis, metabolic acidosis, uremic toxicity, and the presence of comorbidities (1–4). For this reason, it is recommended to evaluate periodically the appetite, dietary intake, and biochemical data, as well as to carry out a nutritional and functional (morphofunctional) assessment and an individualized approach to the diet of patients by expert professionals. When dietary advice is insufficient to achieve protein-calorie intake goals, the use of oral nutritional supplements (ONSs) is the next step to prevent and/or treat malnutrition (1, 3). The use of ONS (standard or specific for CKD) in patients with hemodialysis has shown, in some randomized studies (compared to usual follow-up), that it can increase protein-calorie intake, weight, fat-free mass (FFM), and fat and albumin concentrations, without raising the levels of electrolytes such as phosphorus or potassium (1–9). Furthermore, in retrospective studies, it has been observed that it could reduce hospital admissions and even mortality (10–12). In these patients, mechanisms of inflammation coexist with oxidative stress, which favors cardiovascular complications that could be attenuated with dietary and pharmacological interventions (13).

The Mediterranean diet has been proposed as the dietary pattern of choice for patients with CKD, which could improve endothelial function, inflammation, oxidative stress, and lipid profile, as well as reduce cardiovascular disease incidence (1, 4, 14). Some of its essential components are virgin olive oil, fish (as a source of n-3 fatty acids), and fiber from plant foods. Virgin olive oil is rich in polyphenols, and its consumption has been associated with a decrease in cardiovascular events, diabetes, and other chronic diseases (15). In hemodialysis patients with CKD, the minor polar compounds of extra virgin olive oil seem to exert an antioxidant and anti-inflammatory effect (16). The use of n-3 fatty acid supplements in patients with CKD could decrease markers of inflammation and oxidation (associated with lean mass depletion) (17). The metabolic alterations inherent to uremia and the intake of a Western-type diet could promote intestinal dysbiosis among patients with CKD and may play a key role in disease progression and complications. Dietary patterns such as the Mediterranean could reduce inflammatory processes, including leaky gut and subsequent endotoxemia (18).

In addition, the use of pre- and pro-biotics could have a crucial role in the regulation of the immune system and prevent infectious complications, treat hyperphosphatemia, reduce the levels of solutes that contribute to the uremic syndrome, as well as improve the lipid profile, oxidative stress, and systemic inflammation (19). Although some studies have shown some beneficial results, there is no conclusive rationale for recommending biotic supplements for improving outcomes in patients with CKD (20).

Whey proteins are rich in branched-chain amino acids, leucine, glutamine, and cysteine and are quickly digested; moreover, they favor a greater protein anabolic response than other protein sources. Although this requires still further evidence (21), its use in hemodialysis patients could reduce inflammatory parameters and improve physical function (22–24).

Recently, a new ONS was developed in Spain specifically designed for malnourished (or at risk) hemodialysis patients with a "similar to the Mediterranean diet" pattern (made up of functional nutrients such as extra virgin olive oil, n-3 fatty acids, whey protein, fiber, and antioxidants) that could improve the achievement of dietary as well as nutritional and functional status goals, metabolic changes, and associated inflammation and oxidative stress.

We aimed to study whether the new ONS, associated with probiotics or not, may improve nutritional and functional status and reduce biomarkers of inflammation and oxidation in malnourished hemodialysis patients, compared to individualized diet recommendations.

2. Materials and methods

2.1. Design

Randomized, multicenter, parallel-group trial with three groups, open regarding the intake of ONS or individualized diet recommendations but double-blind for the intake of probiotics. Patients were randomized to one of the following three groups (using a computer-generated random number table):

1: Control (C): received individualized dietary recommendations.
2: ONS + placebo (SU-PL): received ONS + dietary recommendations.
3: ONS + probiotics (SU-PR): received ONS with probiotics + dietary recommendations.

The Renacare® ONS was specifically developed for malnourished hemodialysis patients. It is high in energy (2 kcal/ml) and proteins and enriched with functional nutrients (extra virgin olive oil, omega-3 fatty acids, whey protein, antioxidants, low-glycemic index carbohydrates, fiber, and carnitine). Table 1 shows its composition. The supplement is presented in vanilla flavor, but it includes six additional flavors that can be added to facilitate compliance, acceptance, and individualization.[1]

The individualized nutritional requirements of all patients were estimated based on the recommendations of the International Society of Renal Nutrition and Metabolism. Protein intake targets were more than 1.2 g/kg/day (3). All participants had face-to-face interviews with a dietitian at baseline and after 3 and 6 months. Patients randomized to the ONS groups were recommended to ingest two bricks per day (400 ml) [with a minimum of one daily (200 ml)]. The daily intake of ONS was prospectively recorded in a data collection sheet by the patients. The probiotics and the placebo were supplied in capsules completely indistinguishable by their external appearance (one capsule of 380 g). Each capsule of probiotic contained live bacteria: *Bifidobacterium breve* CNCM I-4035 [1.00E + 09 colony forming units (CFU)], *Bifidobacterium animalis lactis* BPL1 CECT 8145 (3.50E + 09 CFU), and *Lactobacillus paracasei* CNCM I-4034 (5.00E + 08 CFU).

The Research Ethics Committee provincial of Málaga approved the study, and the protocol meets the Ethical Standards of the Declaration of Helsinki. The study was registered with the following code: NCT03924089.

2.2. Inclusion and exclusion criteria

Inclusion criteria comprised adult subjects (> 18 years) undergoing hemodialysis for more than 6 months before inclusion and at least one of the following malnutrition criteria: (a) involuntary weight loss > 5% in 3 months or > 10% in 6 months; (b) serum albumin < 3.5 g/dl or prealbumin < 28 mg/dl; (c) body mass index (BMI) < 23 kg/m^2; (d) muscular mass loss > 5% in 3 months or > 10% in 6 months; and (e) low muscle mass or strength: FFM index (FFMI) lower than 15 kg/m^2 in women or lower than 17 in men or Jamar hand dynamometry in the dominant arm (maximum or mean of three determinations) lower than the fifth percentile of the Spanish population (25). Standard hemodialysis therapy (3 days/week, 240 min, high-flux dialyzer, blood flow > 250 ml/min, and dialysate with bicarbonate buffer with a flow 500 ml/min; Kt/V 1.3) or online hemodiafiltration with high reinfusion rate therapy not being modified in the 3 months before inclusion. Written informed consent was obtained.

Exclusion criteria were not signing the informed consent, type 1 diabetes mellitus or type 2 diabetes mellitus with glycated hemoglobin > 9%, unstable dry weight, limb amputation, significant

TABLE 1 Nutritional composition of the oral nutritional supplement renacare®.

	Units	100 ml	200 ml
Energy value	Kcal/kj	200/837	400/1674
Fats, of which	g	8.7	17
Saturated fatty acids	g	1.6	3.1
Monounsaturated fatty acids	g	5.4	11
Polyunsaturated fatty acids	g	1.8	3.6
Eicosapentaenoic acid (EPA)	mg	231	462
Docosahexaenoic acid (DHA)	mg	144	288
Carbohydrates, of which	g	20.5	41.0
Sugars	g	1.2	2.4
Dietary fiber	g	2.00	4.00
Proteins	g	8.97	17.9
Salt	g	0.15	0.30
Vitamins			
Vitamin A	µg-RE	50	100
Vitamin D	µg	1.35	2.70
Vitamin K	µg	9.8	19.6
Vitamin C	mg	10.0	20
Vitamin B1	mg	0.45	0.90
Vitamin B2	mg	0.50	1.00
Vitamin B6	mg	0.80	1.60
Niacin	mg-NE	4.00	8.00
Folic acid	µg	100	200
Vitamin B12	µg	1.20	2.40
Pantothenic acid	mg	0.85	1.70
Biotin	µg	3.90	7.8
Vitamin E	mg-αTE	3.50	7.00
Minerals			
Sodium	mg	60	120
Chloride	mg	90	180
Potassium	mg	75	150
Calcium	mg	100	200
Phosphorus	mg	30	60
Magnesium	mg	10.0	20.0
Iron	mg	2.00	4.00
Zinc	mg	2.00	4.00
Copper	µg	200	400
Iodine	µg	16.0	32.0
Selenium	µg	7.50	15.0
Manganese	mg	0.28	0.56
Chrome	µg	5.00	10.0
Molybdenum	µg	5.0	10.0
Others			
Coline	mg	42.0	84.0
L-carnitine	mg	120	240

(Continued)

1 https://adventiapharma.com/nutricion-clinica/aromas

TABLE 1 (Continued)

	Units	100 ml	200 ml
Osmolarity	mOsmol/l	390	
Ingredients			
	%		
Carbohydrates			
Low glycemic index maltodextrin	60		
Low glycemic index dextrin	40		
Protein			
Whey	55		
Casein	45		
Fats			
Extra virgin olive oil	75		
Rapeseed oil	10		
Fish oil	12		
Others (lecithin)	3		
Fiber			
FOS	35		
Acacia	35		
Oat fiber	30		

FOS, fructooligosaccharides. https://adventiapharma.com/nutricion-clinica/productos/enteral-oral/bi1-renacare-dialysis/.

edema, active malignancy, hospital admissions in the last 3 months, acute gastrointestinal disease in the 2 weeks before the inclusion, gastrectomy, gastroparesis or abnormal gastric emptying, heart failure grade IV, severe hepatic insufficiency, alcohol or other drugs abuse, participants enrolled in another research study at inclusion, pregnant women, patients who received any ONS in the 4 weeks before the inclusion, receiving enteral tube feeding, galactosemia, fructosemia, or requirement of a no-fiber diet, allergy or hypersensitivity to any ingredient of the ONS, ongoing treatment with glucocorticoids, oral fatty acids omega-3 supplement in the last 4 weeks before inclusion, intradialytic parenteral nutrition in the last 3 months prior to inclusion, or having received any pro- or prebiotics (not as part of the diet) in the last 3 months before inclusion.

2.3. Outcomes

Examinations were performed at baseline and after 3 and 6 months.

2.3.1. Dietary questionnaire

A 5-day prospective dietary questionnaire (including one weekend day) was fulfilled. The data were analyzed using a computer application designed by our group for this purpose (Dietstat®) (26). The composition of the ONS was also included in the database.

A 14-item dietary screening questionnaire was used to assess adherence to the Mediterranean diet. This is a self-administered validated dietary questionnaire used in the PREDIMED trial (Prevención con Dieta Mediterránea). The score ranges from 0 to 14, with higher scores representing greater adherence to the Mediterranean diet (15).

2.3.2. Morphofunctional nutritional assessment

Height and weight were determined with a calibrated stadiometer and scale. Body mass index (BMI) was defined as the weight in kilograms divided by squared height (in meters). "Dry weight" was measured 30 min after the end of dialysis. Mid-arm circumference was obtained with an inextensible tape measure. Skinfold thickness (tricipital) measurements were conducted using a constant pressure lipocalibrator (Holtain Limited) by the same researcher in each hospital. Three measurements were completed, and values were averaged. Mid-arm muscle circumference was calculated as mid-arm circumference minus π times triceps skinfold thickness. Bioelectrical impedance analysis was performed using a tetrapolar 50-kHz bioelectrical impedance analyzer (BIA 101 RJL, Akern Bioresearch, Firenze, Italy). FFMI was calculated (FFM in kg/height in m^2).

Muscle strength was assessed using a dynamometer (Jamar handgrip; Asimow Engineering Co., Los Angeles, CA, USA) prior to the start of dialysis in the dominant hand, this was repeated on three occasions, and the mean was recorded.

The patients performed, prior to the start of dialysis, the short physical performance battery (SPPB) test (consisted of gait speed, a sit-to-stand test performed five times, and balance tests) and was calculated using the previously defined methods (scores ranged between 0 and 12) (27).

2.3.3. Biomarkers

Fasting blood samples were drawn before beginning the dialysis session; plasma and serum were separated into aliquots and stored until analysis at −80°C in the Hospital-IBIMA biobank. One aliquot was analyzed immediately in an autoanalyzer at the laboratories of each hospital to measure C-reactive protein (CRP), triglycerides, cholesterol, creatinine, urea, electrolytes, blood liver function, albumin, prealbumin, and glycated hemoglobin. Vitamin D was analyzed by electrochemiluminescent immunoassay (Modular E-170, Roche Diagnostics). The serum levels of antioxidant biomarkers were determined by enzyme immunoassay techniques following the manufacturer's instructions in Versamax (MTX Lab System, Barcelona, Spain): Cayman's Antioxidant Assay (CAT) (Cayman Chemical Company, MI, USA; Intra-Assay CV = 3.4%; Inter-Assay CV = 3%), 8-isoprostane (Cayman Chemical Company, MI, USA; Intra-Assay CV = 7.6–12%; Inter-Assay CV = 9.7–19.9%). Proinflammatory cytokines and atherosclerosis biomarkers [brain-derived neurotrophic factor (BDNF), bone morphogenetic protein-2 (BMP-2), CD62E (E-selectin), interferon-gamma, interleukin (IL)-1-alpha, IL-1-beta, IL-10, IL-12p70, IL-13, IL-15, IL-17A (CTLA-B), interleukin-1 receptor antagonist, IL-4, IL-6, IL-8 (CXCL8), cytokine-leukemia inhibitory factor (LIF), and monocyte chemoattractant protein-1 (MCP-1), TNF-alpha] were measured in 25 ul of serum with ProcartaPlex Multiplex Immunoassay (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. For VCAM-1 and ICAM-1, we have diluted the sample 100 times. All measurements were performed in duplicate, and the serum concentrations were obtained with a standard curve.

2.3.4. Adherence and side effects

At each visit, the patients filled out questionnaires to assess the presence and intensity of gastrointestinal symptoms in the 30 days prior to the visit on a scale from 0 to 10 (nausea, vomiting, diarrhea,

constipation, reflux, pain, and bloating). In addition to the scheduled visits, the research team made weekly phone calls during the first month and subsequently every 15 days until the end of the study to detect the presence of adverse effects and encourage adherence to diet, supplementation, probiotics, and exercise. A survey was conducted on the acceptance of the supplement and its organoleptic characteristics at months 3 and 6. All patients received individualized physical exercise recommendations based on their SPPB scores.

2.4. Statistical analysis

Data analysis was conducted using the IBM SPSS Statistics Version 26 (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.) program. Quantitative variables were expressed as mean ± SD or median and interquartile range according to normality. In the case of cytokines, log transformation was applied. Normality was assessed by Shapiro–Wilk test.

For the analysis of socio-demographic and basal-clinical characteristics, the chi-square test with Fisher's exact distribution was used for qualitative variables; whereas for quantitative variables, the ANOVA test for independent variables or the Kruskal–Wallis *H*-test was used, according to normality. To compare variables according to the group of study and the modifications along time (at baseline, 3 and 6 months), ANOVA for repeated variables was used, if applicable. Otherwise, the necessary non-parametric techniques were applied: intra-subject Friedman (*post hoc* Wilcoxon) and inter-subject Kruskall–Walis *H* (*post hoc* Mann–Whitney *U*). The level of significance taken into account was 5%; for multiple comparisons (*post hoc*), Bonferroni correction was considered. Data were analyzed as the intention to treat.

The sample size was estimated according to changes in albumin levels in patients on hemodialysis who had received supplements vs. standard treatment (9). Assuming a (bilateral) confidence level of 95% and a potency of 80% to detect differences of at least 0.25 g/dl in albumin concentrations between the C group vs. ONS groups, and with a standard deviation of 0.25 g/dl, it was estimated to treat 17 patients per arm. To prevent 30% dropouts, it was decided to increase the sample to 22 patients per arm (total: 66 patients).

3. Results

From the 220 subjects assessed for eligibility, 59 were randomized. Notably, 31 patients completed the 6-months trial and were analyzed (11 C group, 10 SU-PL, and 10 SU-PR) (**Figure 1**). The causes for consent withdrawal were in the C group, not wanting to continue with visits and individualized diet (n = 4), difficulties in follow-up due to the SARS-CoV-2 pandemic (n = 2), and the decision of their nephrologist (n = 1); in the SU-PL group, transfer to another facility (n = 1), not wanting to continue with visits (n = 2), difficulties in follow-up due to the SARS-CoV-2 pandemic (n = 2), and lack of supplement acceptance (n = 3); and in the SU-PR group, not wanting to continue with visits (n = 2), difficulties in follow-up due to the SARS-CoV-2 pandemic (n = 1), and lack of supplement acceptance (n = 3). The mean intake of supplements during the 6 months of follow-up was 1.5 ± 0.46 in the SU-PL group and 1.55 ± 0.41 in the SU-PR group. There were no significant differences in the digestive symptoms scale between groups (C, SUP-PL, SUP-PR), neither at baseline nor at any of the 6 months of follow-up (**Supplementary Table 1**). Supplement acceptance was high (**Supplementary Table 2**). There was no patient withdrawal due to gastrointestinal side effects. We observed no significant changes regarding gastrointestinal symptoms in patients quitting the study due to consent withdrawal or severe adverse effects (death, admission for transplant) and those who continued in the trial.

There were no basal significant differences between groups regarding age, sex, diabetes, Charlson comorbidity index, or intake of fermented milk or antibiotics during the month prior to inclusion (**Table 2**). Moreover, there were no baseline differences in any of the parameters for the morphofunctional nutritional assessment, dietary intake, biochemical data, or analyzed biomarkers (except for IL-12) (**Tables 3–6**).

3.1. Dietary questionnaire

The groups that took supplements increased significantly their energy, fat (total and n-3), protein, and fiber intake compared to baseline and reached significance with regard to the C group at months 3 and/or 6 (**Table 3**). A significant decrease in the glycemic index in patients on ONS was observed compared to the baseline and the C group. There were no differences regarding the intake of carbohydrates, potassium, phosphorus, or calcium between groups during the intervention. The 14-point PREDIMED scale increased in all groups and for all time periods, reaching statistical significance in the C group at month 3 and supplemented groups at month 6 when compared to baseline (**Table 3**).

3.2. Morphofunctional nutritional assessment

Weight and "dry weight" increased significantly in the SU-PL, SU-PR, and SU-TOT groups at month 6, with respect to baseline and month 3. FFM, FFMI, and "Dry FFM and FFMI" increased significantly in the SU-TOT group at month 6 with respect to baseline, and at month 6 with respect to month 3. Mean hand grip strength increased significantly only in the SU-PL group at month 6, compared to baseline. Fat mass, triceps skinfold, mid-upper arm circumference, and mid-upper arm muscle circumference increased in the groups on ONS, although without reaching significance. There were no differences regarding the score in the SPPB functionality scale (**Table 4**).

3.3. Biomarkers

Prealbumin and 25-OH-Vitamin D3 levels increased significantly in the SU-TOT group at month 3 compared to baseline, and 25-OH-Vitamin D3 levels did so in SU-PR. Albumin showed a tendency toward an increase in patients on ONS that did not reach significance. Total cholesterol increased after 3 months compared to baseline in the SU-PL group, and triglycerides also at month 3 in SU-PR. We observed a decrease in potassium, especially in the SU-PR group, although it was

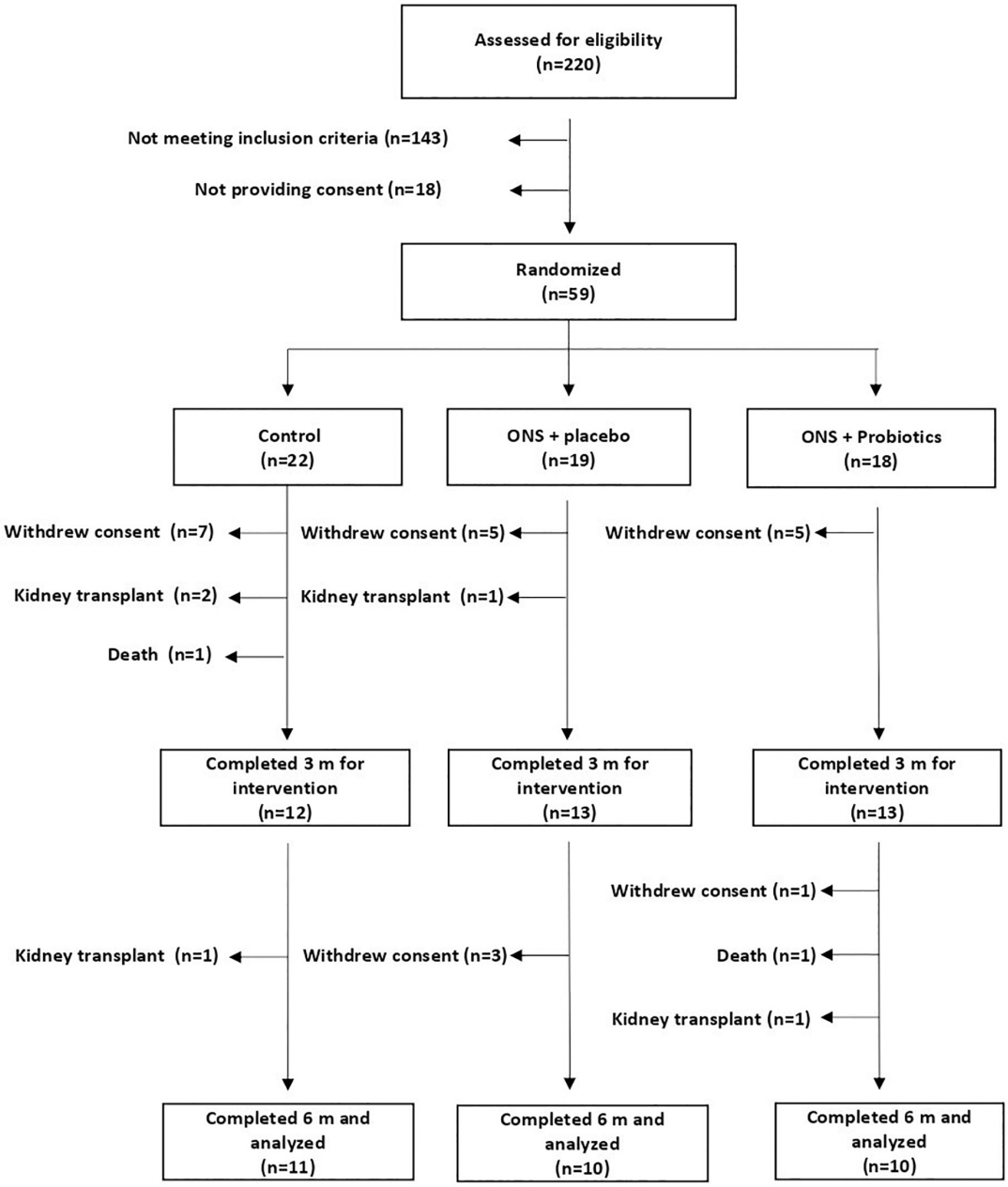

FIGURE 1
Study flowchart. ONS, oral nutritional supplement.

not significant. There were no changes in phosphorus, calcium, creatinine, uric acid, and hemoglobin in any of the groups (Table 5).

Although there was a tendency toward a decrease in most parameters studied, we observed no significant differences in the inflammation markers such as CRP, ICAM-1, VCAM-1, E-selectin, IFN-gamma, IL-1-alpha, IL-12, IL-13, IL-15, IL-17, IL-1RA, LIF, IL-6, and TNF-alpha.

Brain-derived neurotrophic factor levels reached significant differences with respect to baseline at month 3 in the SU-PR group and at month 6 in SU-TOT. BMP-2 levels decreased significantly after 6 months in the SU-TOT group, compared to baseline. MCP-1 levels decreased significantly in the SU-PR and SU-TOT groups between months 3 and 6. IL-1-beta levels decreased significantly with regards to baseline at month 6 in the SU-TOT group and at month 3 in SU-PL. IL-10 levels increased in the groups on ONS at month 3, with this reaching significance in the SU-TOT and SU-PR compared to the C group. IL-4 levels decreased significantly with respect to baseline at month 6 in the SU-PR group. IL-8 decreased significantly with regard to baseline at month 6 and in the SU-TOT group between months 3 and 6.

Total antioxidant capacity (TAC) increased significantly in patients supplemented with ONS, with respect to baseline (SU-PR and SU-TOT), at months 3 and 6. Isoprostanes levels also increased, especially in the control group, although this did not reach statistical differences either over time or between groups (Table 6).

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TABLE 2 Baseline characteristics according to the intervention arm.

	Control (n = 11)	SU-PL (n = 10)	SU-PR (n = 10)	P-values
Age (years) m ± ds	76.3 ± 8.7	65.1 ± 18.4	66 ± 18.5	0.234
Sex, women% (n)	27 (3)	20 (2)	30 (3)	0.999
Diabetes mellitus% (n)	36.4 (4)	40 (4)	30 (3)	0.999
Antibiotic treatment in the last month	9.1 (1)	20 (2)	10 (1)	0.825
Consumption of yogurt or fermented milk in the last month% (n)	63.6 (7)	60 (6)	60 (6)	0.999
Charlson comorbidity Index. m ± ds	4 ± 2.31	5.1 ± 2.02	4.18 ± 2.6	0.262

Data are presented as mean ± standard deviation (m ± ds) or in percentage % (n), SU-PL, supplement group + placebo; SU-PR, supplement group + probiotics.

4. Discussion

In this study, we have observed that supplementation with a new ONS specifically designed for malnourished subjects with CKD on hemodialysis improves dietary intake and nutritional status, as well as some biomarkers of inflammation and oxidation, compared to a group on individualized treatment.

In the groups on ONS, we observed an increase in the total caloric (approximately 250 kcal mean), protein (12 g mean), fat (including n-3 fatty acids), and fiber intake, as well as a decrease in glycemic index and an improvement in adherence to the Mediterranean diet pattern. The purpose of the use of ONS should always be complementing, and never substituting, the intake of natural food. In this study, the addition of ONS to the patients' regular diet helped to achieve the intake recommended in clinical guidelines (1). The increase in energetic intake due to the use of renal-specific protein-energy ONS has also been observed in other randomized studies in patients receiving maintenance hemodialysis (MHD) (7, 28), but not in all of them (1, 5). The new Kidney Disease Outcomes Quality Initiative (KDOQI) clinical practice guideline for nutrition in CKD recommended, for patients on MHD who are metabolically stable, prescribing a dietary protein intake of 1.0–1.2 g/kg body weight per day to maintain a stable nutritional status. Nevertheless, in malnourished (or at-risk) patients with PEW, the recommended intake could be higher (3). In previous randomized studies, no increase in protein intake due to protein-energy supplements had been found (5, 7, 29, 30), although the isolated use of some protein-based supplements did help to achieve an increase (21, 31, 32). The main protein intake in supplemented patients in this study increased to over 1.2 g per kg body weight after the intervention, which, together with the fact that half of the protein in the ONS comes from whey protein, may have contributed to the improvement in body composition we observed. The KDOKI guidelines do not routinely recommend supplementation with n-3 polyunsaturated fatty acids (PUFAs) in order to reduce the risk of mortality or cardiovascular events, although they may be prescribed at pharmacological doses to improve lipid profile. In some studies, the use of n-3 fatty acid supplements in patients with CKD seems to decrease inflammatory and oxidation markers (associated with FFM depletion) (17). In our sample, supplemented patients increased their intake of n-3 PUFA, which may have also contributed to the decrease in biomarkers of inflammation and oxidation. In patients randomized to ONS, there was also a higher intake of fiber and a lower glycemic index; moreover, the score in PREDIMED at 6 months was also higher. All these changes may have improved body composition through different anti-inflammatory and antioxidant mechanisms (4, 14, 16, 18, 33).

Malnutrition in patients with MHD is characterized by changes in body composition, especially a decrease in FFM and muscle strength, and it is associated with high morbidity and mortality (34, 35). In our study, patients taking supplements improved their body composition, with a mean increase of 2.6 kg in dry weight, from which 1.5 kg were FFM. These data are similar to those observed in other clinical trials with energy-based and/or protein-based ONS (1, 22, 29, 36). The increase in only fat mass found in other trials with ONS may not be beneficial for this population as it could increase insulin resistance and systemic inflammation (28). Tomayko et al. demonstrated, after intradialytic supplementation with whey or soy protein, improvements in gait speed and shuttle walk test performance (22); however, in the IHOPE study that combined whey protein + exercise, no significant changes compared to the control group were observed (21). In our study, the increase in FFM was associated with an improvement in hand grip strength only in the SU-PL group, and no changes in functionality, measured by SPPB, were found. Although patients were highly encouraged to perform individualized physical exercise, no supervised specific program was designed to improve the results (37).

The changes described in diet and body composition were associated with an increase in prealbumin that reached significant differences in the groups on ONS at month 3. There was also a non-significant increase in albumin. In other randomized trials comparing the use of protein or energy-protein-based ONS in patients with MHD, there were variable results, with albumin levels tending to increase moderately, especially in malnourished patients compared to control or placebo (1, 9, 36); on the contrary, there were no differences regarding prealbumin (5, 7, 38).

Serum albumin and prealbumin may be considered complementary tools to assess nutritional status and as a predictor of hospitalization and mortality; however, they are influenced by non-nutritional factors, especially the degree of inflammation (1). In our study, we have found significant decreases in several biomarkers of inflammation (BDNF, BMP-2, MCP-1, IL-1-beta, IL-4, and IL-8) in supplemented patients, especially in the group on also probiotics. Apart from being used as a biomarker of inflammation, the BDNF is increased in patients with MHD with sarcopenia and frailty (39, 40), BMP-2 is associated with increased oxidative stress and vascular risk (41), and MCP-1 has been used as a marker of structural kidney damage as well as arteriogenic factor in patients with MHD with cardiovascular disease (42–44). In contrast, there is also an increase in IL-10 levels after 3 months, which behaves as an anti-inflammatory cytokine, and whose decrease is associated with increased morbidity and reduced muscle strength in patients with MHD (45). In other randomized studies, supplementation with energy-protein-based ONS did not produce any change in CRP (7, 21, 22, 28–31, 36) or IL-6 (21, 38) levels; nevertheless, intradialytic supplementation with whey or soy protein reduced serum levels of IL-6 (22).

TABLE 3 Dietary survey.

	Basal				3 months				6 months			
	Control $n = 11$	SU-PL $n = 10$	SU-PR $n = 10$	SU-TOT $n = 20$	Control $n = 11$	SU-PL $n = 10$	SU-PR $n = 10$	SU-TOT $n = 20$	Control $n = 11$	SU-PL $n = 10$	SU-PR $n = 10$	SU-TOT $n = 20$
Energy (kcal) m ± ds	1804 ± 800	1590 ± 347	1731 ± 595	1673 ± 499	1595 ± 436	1746 ± 477	2054 ± 485*	1927 ± 492$	1577 ± 369	1938 ± 490$	1934 ± 387	1939 ± 417*@
Energy/kg (kcal/kg) m ± ds	25 ± 10	26 ± 7	27 ± 12	26 ± 10	23 ± 6	27 ± 9	31 ± 13	29 ± 11	23 ± 8	29 ± 8	28 ± 10	28 ± 9
Protein (g) m ± ds	72 ± 24	69 ± 16	70 ± 17	70 ± 19	67 ± 20	79 ± 14	83 ± 18*	77 ± 19*	69 ± 18	97 ± 24@&	83 ± 18	82 ± 22@&
Protein/kg (g/kg) m ± ds	1 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	1.1 ± 0.3	1 ± 0.4	1.3 ± 0.4	1.2 ± 0.5	1.2 ± 0.4@	1 ± 0.4	1.4 ± 0.4*	1.2 ± 0.4	1.3 ± 0.4@
Total fat (g) m ± ds	74 ± 23	61 ± 17	73 ± 18	68 ± 18	68 ± 15	77 ± 26*	87 ± 22*	83 ± 23$	64 ± 14	83 ± 27*	80 ± 13	82 ± 20*@
Total fat/kg (g/kg) m ± ds	1.05 ± 0.33	1 ± 0.36	1.13 ± 0.5	1.07 ± 0.4	0.96 ± 0.2	1.21 ± 0.5*	1.28 ± 0.5	1.25 ± 0.5*@	0.93 ± 0.3	1.25 ± 0.4*	1.15 ± 0.4	1.19 ± 0.4
SF% m ± ds	29.3 ± 4.8	29.8 ± 5	30.1 ± 7	29.9 ± 5.9	26.5 ± 3.4	24.6 ± 6.2	27.9 ± 9.4	26.4 ± 7.9	29.7 ± 6.4	25.5 ± 4.4	28.6 ± 8.7	27.1 ± 7
MF% m ± ds	54.1 ± 5.9	52.2 ± 6.6	55.2 ± 6.4	54.8 ± 6.5	57.9 ± 4.5	53.5 ± 10.6	53.6 ± 8.4	53.6 ± 9.1	56.7 ± 6.4	58.8 ± 6.2	55.5 ± 8	57.0 ± 7.2
PF% m ± ds;	16.6 ± 4.3	18.0 ± 5.3	14.8 ± 3.2	16.4 ± 4.3	15.6 ± 3.8	21.9 ± 10.7	18 ± 4.2	18.2 ± 6	13.6 ± 1.7	15.7 ± 5.1	16 ± 2.8	15.0 ± 3.3
Carbohydrate (gr) m ± ds	186 ± 75	190 ± 66	198 ± 102	195 ± 87	175 ± 56	184 ± 58	221 ± 66	206 ± 64	174 ± 54	200 ± 66	217 ± 61	210 ± 62
Carbohydrate/kg (g/kg) m ± ds	2.62 ± 1	3.03 ± 1.1	3.03 ± 1.8	3.03 ± 1.5	2.5 ± 0.8	2.86 ± 0.9	3.36 ± 1.7	3.15 ± 1.5	2.54 ± 1	2.98 ± 1	3.15 ± 1.3	3.08 ± 1.2
GI m ± ds	55 ± 5	54 ± 11	47 ± 24	50 ± 19	61 ± 4	44 ± 9@	42 ± 15#	43 ± 12ß	61 ± 6	47 ± 8	44 ± 17@	45 ± 14#
GL m ± ds	103 ± 41	111 ± 42	108.4 ± 83.3	110 ± 68	105 ± 37	77 ± 19	100 ± 32	91 ± 29	105 ± 32	95 ± 43	100 ± 42	98 ± 41
Fiber (g) med (p25; p75)	11 (9; 24)	11 (8; 19)	12 (7; 16)	11 (8; 16)	10 (10; 17)	14 (11; 20)	15 (13; 17)	14 (13; 17)	14 (10; 17)	15 (14; 23)*	17 (15; 20)	17 (15; 20)$
n-3 (g) med (p25; p75)	0.78 (0.57; 1.03)	0.8 (0.6; 1.23)	0.77 (0.55; 0.95)	0.79 (0.57; 1)	1.09 (0.49; 1.22)	1.74 (0.91; 2.73)*	2.14 (1.16; 2.23)@&	1.96 (1.08; 2.25)@&	0.56 (0.45; 1.03)	1.4 (0.87; 2.87)@	1.3 (0.98; 1.63)&	1.33 (0.94; 1.74)#*
EPA (g) med (p25; p75)	0.05 (0.01; 0.13)	0.02 (0.01; 0.04)	0.03 (0.01; 0.08)	0.03 (0.01; 0.07)	0.04 (0.01; 0.21)	0.56 (0.06; 0.93)	0.86 (0.47; 1)#	0.66 (0.47; 0.97)ß+	0.04 (0.01; 0.14)	0.47 (0.02; 0.93)	0.5 (0.46; 0.8)@&	0.49 (0.28; 0.87)#*
DHA (g) med (p25; p75)	0.1 (0.04; 0.18)	0.03 (0.01; 0.11)	0.11 (0.03; 0.21)	0.06 (0.01; 0.18)	0.11 (0.04; 0.3)	0.4 (0.11; 0.58)@*	0.58 (0.29; 0.72)	0.42 (0.24; 0.67)Ω#	0.1 (0.01; 0.3)	0.29 (0.03; 0.58)	0.47 (0.29; 0.58)	0.39 (0.23; 0.58)@
K (mg) m ± ds	2164 ± 1060	2155 ± 1035	1747 ± 710	1915 ± 853	1783 ± 517	1880 ± 237	1583 ± 475	1705 ± 413	1764 ± 350	2416 ± 615	1790 ± 616	2048 ± 675
P (mg) m ± ds	1098 ± 433	1017 ± 316	1056 ± 328	1040 ± 314	1009 ± 306	1042 ± 212	1089 ± 255	1070 ± 232	1047 ± 311	1188 ± 341	1030 ± 323	1095 ± 330
Ca (mg) m ± ds	592 ± 276	612 ± 220	635 ± 332	625 ± 283	520 ± 100	741 ± 357	903 ± 356	836 ± 355	654 ± 260	793 ± 371	734 ± 260	758 ± 301
PREDIMED (total score) m ± ds	7.2 ± 1.4	7.5 ± 1.4	6.9 ± 2.3	7.2 ± 1.9	8.8 ± 1.6$	8.2 ± 1.7	7.6 ± 2.3	7.9 ± 2	8.2 ± 1.6	8.7 ± 1.5*	8 ± 1.9*	8.4 ± 1.7$

Data are presented as mean ± standard deviation (m ± ds) or median and interquartile range: med (p25; p75); SU-PL supplement group + placebo; SU-PR supplement group+ probiotics; SU-TOT: supplement group (SU-PL+SU-PR). Differences between baseline and 3 m or 6 m intragroup: *$p < 0.05$; \$$p < 0.01$; &$p < 0.001$; differences between 3 and 6 months: €$p < 0.05$; Ω$p < 0.01$; + $p < 0.001$; Differences with respect to the control group (at baseline, 3 months, or 6 months): $p < 0.05$; #$p < 0.01$; ßp < 0.001. Kcal, kilocalories; g, gram; SF, saturated fat; MF, Monounsaturated fat; PF, Polyunsatured fat; GI, glycemix index; GL, glycemix load; n-3, omega-3 fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; K, potassium; P, phosphorus; Ca, calcium. Score PREDIMED: "Prevención con Dieta Mediterránea".

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TABLE 4 Morphofunctional nutritional assessment.

	Basal				3 months				6 months			
	Control $n = 11$	SU-PL $n = 10$	SU-PR $n = 10$	SU-TOT $n = 20$	Control $n = 11$	SU-PL $n = 10$	SU-PR $n = 10$	SU-TOT $n = 20$	Control $n = 11$	SU-PL $n = 10$	SU-PR $n = 10$	SU-TOT $n = 20$
Weight (kg) m ± ds	71.7 ± 11	63.2 ± 12.1	70.5 ± 17.9	66.9 ± 15.3	71 ± 9.8	64.2 ± 12.4	72.7 ± 18.7*	68.47 ± 16.05*	71.1 ± 8.8	66.3 ± 11.2*€	73.8 ± 18.5*	70 ± 15.4&€
Dry weight (kg) m ± ds	70.2 ± 10.7	61.9 ± 12.3	68.2 ± 17.5	65 ± 15.1	69.8 ± 9.6	62.9 ± 12.3	69.9 ± 18.5*	66.4 ± 15.7$	69.4 ± 9	64.4 ± 11.4*€	70.9 ± 18.1*	67.6 ± 15.1&€
FFM (kg) m ± ds	48.6 ± 7.9	45.9 ± 6.5	50.7 ± 12.2	48.3 ± 9.8	48.2 ± 7	46.2 ± 7	51.92 ± 13	49.06 ± 10.6	49.63 ± 7.8	48.11 ± 7.2	52.94 ± 12.3	50.53 ± 10.1*€
Dry FFM (kg) m ± ds	47.7 ± 7.6	45 ± 7.6	49.4 ± 12.1	47.2 ± 9.8	47.4 ± 6.9	45.1 ± 7	49.85 ± 12.8	47.5 ± 10.3	48.5 ± 8.3	46.4 ± 7	50.9 ± 12.1	48.7 ± 9.9*€
FM (kg) m ± ds	23.1 ± 7.1	17.2 ± 7.6	19.8 ± 8.4	18.5 ± 7.9	22.8 ± 6.4	18.2 ± 7.2	20.8 ± 7.4	19.5 ± 7.2	20.8 ± 6.5	18.5 ± 7.2	20.7 ± 8.7	19.6 ± 7.8
Dry FM (kg) m ± ds	22.6 ± 7	16.9 ± 7.5	18.8 ± 7.3	17.8 ± 7.3	22.4 ± 6.3	17.8 ± 7.1	20 ± 7.2	18.9 ± 7.1	20.9 ± 7.1	17.9 ± 7.3	19.9 ± 8.4	18.9 ± 7.7
TS (mm) m ± ds	15.5 ± 8.9	11.5 ± 5.7	12.4 ± 7	11.9 ± 6.2	15.2 ± 7.5	12.4 ± 5.3	13.1 ± 5.7	12.7 ± 5.4	14.2 ± 8.8	12.6 ± 6.1	13 ± 7.6	12.8 ± 6.7
FFMI (kg/m^2) m ± ds	16.9 ± 1.3	17 ± 2.2	18 ± 3.4	17.6 ± 2.8	16.8 ± 1.1	16.4 ± 3.2	18.5 ± 3.7	17.4 ± 3.5	17.3 ± 1.5	17.8 ± 2.2	18.9 ± 3.4	18.4 ± 2.8*€
Dry FFMI (kg/m^2) m ± ds	16.6 ± 1.3	16.6 ± 2.3	17.6 ± 3.4	17.1 ± 2.9	16.5 ± 1.1	16.7 ± 2.3	17.8 ± 3.7	17.2 ± 3	16.8 ± 1.6	17.2 ± 2.4	18.2 ± 3.3	17.7 ± 2.9*€
MUAC (cm) m ± ds	27.3 ± 2.9	26 ± 3.5	25.6 ± 4.1	25.8 ± 3.7	27.3 ± 2.6	26.1 ± 3.7	26.7 ± 4.4	26.4 ± 4	26.8 ± 2.4	26.4 ± 4.3	26.4 ± 4.5	26.4 ± 4.3
MUAMC m ± ds	22.9 ± 3.3	22.6 ± 2.9	21.6 ± 3.6	22.1 ± 3.2	22.6 ± 2.7	22.1 ± 2.6	22.6 ± 3.4	22.4 ± 2.9	23.2 ± 2.8	22.8 ± 2.9	22.3 ± 3.7	22.6 ± 3.3
HGS (kg) m ± ds	23.9 ± 9.7	17.7 ± 7.9	17.6 ± 7.1	17.7 ± 7.3	24.4 ± 10.1	20.9 ± 6.4	17.7 ± 6	19.2 ± 6.2	21.1 ± 12.1	23.7 ± 6.5*	17.9 ± 8.2	20.6 ± 7.8
SPPB (total score) m ± ds	6.9 ± 3.4	4.7 ± 3.1	7.1 ± 3.4	5.9 ± 3.4	6.8 ± 2.9	5.4 ± 3.8	6.6 ± 3.7	6 ± 3.4	7.1 ± 3.3	5.4 ± 4	6.9 ± 2.7	6.2 ± 3.4

Data are presented as mean ± standard deviation (m ± ds) or median and interquartile range: med (p25; p75); SU-PL, supplement group + placebo; SU-PR, supplement group + probiotics; SU-TOT, supplement group (SU-PL + SU-PR). Differences between baseline and 3- or 6-months intragroup: *$p < 0.05$; $$p < 0.01$; &$p < 0.001$; differences between 3 and 6 months: €$p < 0.05$. FFM, fat-free mass; FM, fat mass; TS, triceps skinfold; FFMI, fat-free mass index; MUAC, mid-upper arm circumference; MUAMC, mid-upper arm muscle circumference; HGS, handgrip strength.

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TABLE 5 Blood test parameters.

	Basal				3 months				6 months			
	Control *n* = 11	SU-PL *n* = 10	SU-PR *n* = 10	SU-TOT *n* = 20	Control *n* = 11	SU-PL *n* = 10	SU-PR *n* = 10	SU-TOT *n* = 20	Control *n* = 11	SU-PL *n* = 10	SU-PR *n* = 10	SU-TOT *n* = 20
K (mEq/L) m ± ds	4.9 ± 0.6	5.3 ± 0.7	5.4 ± 0.7	5.3 ± 0.7	5.1 ± 0.6	5.3 ± 0.9	5 ± 0.8	5.1 ± 0.9	4.9 ± 0.68	4.9 ± 0.57	4.6 ± 0.6	4.8 ± 0.6
P (mg/dL) m ± ds	4.6 ± 0.8	4.1 ± 0.9	4.1 ± 0.7	4.1 ± 0.8	4.5 ± 1.2	4.2 ± 1.5	3.6 ± 1.2	3.9 ± 1.4	4 ± 1.2	4.4 ± 1.3	3.7 ± 1.4	4.1 ± 1.3
Ca (mg/dL) m ± ds	8.9 ± 0.6	8.6 ± 1.5	9 ± 0.7	8.8 ± 1.2	9 ± 0.6	9.3 ± 0.6	8.9 ± 0.9	9.1 ± 0.8	9.3 ± 0.5	8.9 ± 0.6	9 ± 0.9	8.9 ± 0.8
Creatinine (mg/dL) m ± ds	7.3 ± 32	7.4 ± 1.4	6.1 ± 1.3	6.9 ± 1.5	7.1 ± 1.	7.5 ± 2.2	6.6 ± 0.9	7.1 ± 1.8	6.9 ± 1.5	7.9 ± 2.4	6.2 ± 1.5	7.1 ± 1.8
Urea (mg/dl) m ± ds	110.1 ± 32.2	118.7 ± 30.1	91 ± 25.9	107.6 ± 30.8	113.1 ± 25.5	127.7 ± 40.6	113.6 ± 30.6	118.7 ± 32.8	108.8 ± 23.02	150.1 ± 41.4*	88.7 ± 28.4	118.1 ± 40.7ω
Uric acid (mg/dL) m ± ds	5.5 ± 1.6	6.2 ± 1.5	5.3 ± 1.9	5.8 ± 1.7	4.5 ± 0.3	6 ± 1.4	5.7 ± 1.6	5.9 ± 1.4	4.4 ± 0.8	6.5 ± 2.1	5.1 ± 1	5.9 ± 1.8
Total cholesterol (mg/dL) m ± ds	138.6 ± 28.9	116.1 ± 28.5	138.3 ± 30.7	125.8 ± 30.6	132.1 ± 31.6	132.4 ± 34*	125 ± 25	129.2 ± 29.6	127.8 ± 20.5	116.2 ± 38.8	140.1 ± 20.9	126.7 ± 33.5
LDL cholesterol (mg/dL) m ± ds	72.5 ± 23.8	60.8 ± 19.9	75.6 ± 36.5	68.2 ± 29.4	65.4 ± 27.4	69.6 ± 28	68 ± 40.2	68.8 ± 33.4	58.7 ± 19.4	64.4 ± 33.5	75.4 ± 32.3	69.9 ± 32.3
HDL cholesterol (mg/dL) m ± ds	44.8 ± 14.3	36.3 ± 9.3	47.1 ± 7.2	41.7 ± 9.8	46.2 ± 12.7	37.3 ± 9.5	43.6 ± 5.7*	40.4 ± 8.3	45.9 ± 12.1	36.1 ± 10.3	45.1 ± 6.1	40.6 ± 9.4
Triglycerides (mg/dL) m ± ds	106 ± 36.8	109.4 ± 33.1	92 ± 42.9	102.5 ± 36.9	104.5 ± 46.9	133.2 ± 66.8	143.2 ± 68.5*	137.2 ± 65.2	114.7 ± 59.3	106.6 ± 32.3	120.33 ± 39.6	112.1 ± 34.7
Albumin (g/dL) m ± ds	3.69 ± 0.51	3.5 ± 0.72	3.31 ± 0.42	3.41 ± 0.58	3.66 ± 0.63	3.69 ± 0.74	3.45 ± 0.49	3.57 ± 0.62	3.58 ± 0.69	3.53 ± 0.77	3.32 ± 0.6	3.43 ± 0.68
Prealbumin (mg/dL) m ± ds	25.8 ± 5.2	23.5 ± 4.6	24.2 ± 7.1	23.8 ± 5.7	24.7 ± 3.5	26.9 ± 8.7	26.6 ± 4.8	26.7 ± 6.9*	23.6 ± 3	24 ± 7.4	25 ± 7.7	24.5 ± 7.3
25-OH-vitamin D3 (ng/mL) m ± ds	24.3 ± 9.3	19.7 ± 9.6	19.7 ± 12.5	19.7 ± 11	29.7 ± 14.2	24.5 ± 11.9	30.3 ± 19.6*	27.7 ± 16.4*	25.4 ± 11.9	18.6 ± 12	23.2 ± 13	21. ± 12.4
HbA1c (%) med (p25; p75)	5.4 (4.9; 5.8)	5.2 (4.7; 5.7)	5.3 (5.15; 6.25)	5.3 (5; 5.8)	5.3 (5; 5.7)	5.5 (4.7; 5.9)	5.3 (5.1; 6.9)	5.4 (4.9; 6.3)	5.4 (5.1; 5.8)	5.3 (5.1; 5.6)	5.5 (5.2; 6.5)	5.5 (5.1; 6.3)
Hemoglobin g/dL m ± ds	11.1 ± 1.1	10.8 ± 1.1	10.8 ± 1.3	10.9 ± 1.1	11.5 ± 0.8	11.1 ± 1.0	11.3 ± 1.3	11.3 ± 1.0	11.3 ± 1.1	10.4 ± 1.1	10.8 ± 1.4	10.9 ± 1.2

Data are presented as mean ± standard deviation (m ± ds) or median and interquartile range: med (p25; p75); SU-PL, supplement group + placebo; SU-PR, supplement group + probiotics; SU-TOT, supplement group (SU-PL + SU-PR). Differences between baseline and 3- or 6-months intragroup: *$p < 0.05$. Differences between SU-PR and SU-PL (at baseline, 3 or 6 months): ω$p < 0.05$. K, potassium; P, phosphorus; Ca, calcium; LDL, low-density lipoprotein; HDL, high-density lipoprotein; OH, hydroxy; HbA1c, glycated hemoglobin.

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TABLE 6 Inflammation and oxidation biomarkers.

	Basal				3 months				6 months			
	Control *n* = 11	SU-PL *n* = 10	SU-PR *n* = 10	SU-TOT *n* = 20	Control *n* = 11	SU-PL *n* = 10	SU-PR *n* = 10	SU-TOT *n* = 20	Control *n* = 11	SU-PL *n* = 10	SU-PR *n* = 10	SU-TOT *n* = 20
BDNF med (p25; p75)	1.73 (1.18; 1.9)	1.6 (1.11; 2.14)	1.6 (0.96; 2.12)	1.6 (1.04; 2.13)	1.38 (0.81; 2.01)	1.35 (1.12; 1.85)	1.29 (0.9; 2.17)*	1.35 (0.98; 2.06)	1.59 (0.05; 1.95)	1.1 (0.55; 1.87)	1.38 (0.37; 1.77)	1.29 (0.46; 1.79)*
BMP-2 med (p25; p75)	1.36 (1.19; 1.54)	1.46 (1.28; 1.65)	1.62 (1.24; 1.92)	1.56 (1.26; 1.67)	1.32 (1.12; 1.38)	1.51 (1.23; 1.9)	1.66 (1.31; 2.36)	1.57 (1.27; 2.13)	1.23 (1.12; 1.8)	1.36 (1.21; 1.64)	1.58 (1.12; 2.28)	1.43 (1.15; 1.76)*Ω
MCP-1 m ± ds	1.32 ± 0.22	1.33 ± 0.22	1.38 ± 0.39	1.36 ± 0.31	1.35 ± 0.28	1.31 ± 0.28	1.44 ± 0.42	1.38 ± 0.36	1.25 ± 0.25	1.23 ± 0.16	1.23 ± 0.17€	1.23 ± 0.16 €
CRP (mg/dL) med (p25; p75)	2.9 (0.7; 3.2)	2.9 (0.5; 10.3)	2.95 (2.9; 9.3)	2.9 (0.7; 9.6)	2.9 (0.4; 2.9)	2 (0.5; 10.7)	2.9 (0.4; 2.9)	2.9 (0.9; 10.7)	3.3 (2.2; 5.8)	2.9 (0.4; 17.3)	2.9 (2.6; 17.1)	2.9 (0.4; 17.3)
TNF-α med (p25; p75)	1.4 (1.4; 1.4)	1.41 (1.4; 1.41)	1.4 (1.4; 1.44)	1.41 (1.4; 1.43)	1.4 (1.4; 1.42)	1.4 (1.4; 1.44)	1.43 (1.4; 1.46)	1.41 (1.4; 1.46)	1.4 (1.39; 1.4)	1.4 (1.4; 1.41)	1.4 (1.4; 1.42)	1.4 (1.4; 1.42)
VCAM-1 med (p25; p75)	6.06 (5.9; 6.11)	6 (5.86; 6.23)	6.01 (5.88; 6.27)	6 (5.87; 6.23)	6.06 (5.9; 6.11)	6 (5.86; 6.23)	6.01 (5.88; 6.27)	6.12 (6.01; 6.25)	5.97 (5.91; 6.06)	6.09 (5.81; 6.16)	6.04 (5.92; 6.14)	6.07 (5.87; 6.15)
ICAM-1 med (p25; p75)	5.81 (5.65; 5.89)	5.82 (5.74; 5.99)	5.85 (5.75; 6.04)	5.82 (5.74; 6.02)	5.85 (5.65; 5.91)	5.9 (5.77; 5.99)	5.86 (5.74; 5.97)	5.88 (5.75; 5.98)	5.73 (5.6; 5.87)	5.81 (5.74; 5.94)	5.86 (5.73; 6)	5.81 (5.73; 5.97)
E-selectin med (p25; p75)	3.96 (3.9; 4.07)	4.05 (3.95; 4.24)	4.1 (3.93; 4.16)	4.09 (3.94; 4.16)	3.92 (3.81; 4.07)	4.13 (4.05; 4.17)	4.1 (3.98; 4.16)	4.11 (4.02; 4.16)	3.95 (3.77; 4)	4.08 (4.07; 4.12)	4 (3.9; 4.1)	4.08 (3.95; 4.11)
IFN-γ med (p25; p75)	0.85 (0.82; 0.87)	0.88 (0.85; 0.94)	0.87 (0.82; 0.99)	0.87 (0.85; 0.96)	0.86 (0.82; 0.9)	0.85 (0.85; 0.97)	0.91 (0.85; 1.01)	0.91 (0.85; 1.01)	0.82 (0.81; 0.85)	0.87 (0.85; 0.9)	0.86 (0.82; 0.91)	0.86 (0.82; 0.91)
IL-1 alfa med (p25; p75)	−0.54 (−0.56; −0.42	−0.41 (−0.49; −0.21)	−0.4 (−0.54; −0.14)	−0.41 (−0.51; −0.2)	−0.56 (−0.62; −0.42)	−0.31 (−0.41; −0.18)	−0.33 (−0.54; 0.25)	−0.33 (−0.49; −0.01)	−0.57 (−0.59; −0.27)	−0.38 (−0.39; −0.32)	−0.38 (−0.55; 0.13)	−0.38 (−0.54; −0.27)
IL-1β med (p25; p75)	0.15 (0.07; 0.18)	0.18 (0.18; 0.23)	0.18 (0.13; 0.27)	0.18 (0.13; 0.25)	0.13 (0.13; 0.18)	0.18 (0.13; 0.18)*	0.18 (0.13; 0.31)	0.18 (0.13; 0.27)	0.13 (0.07; 0.13)	0.13 (0.13; 0.18)	0.14 (0.13; 0.18)	0.13 (0.13; 0.18)*
IL-1RA med (p25; p75)	2.55 (2.41; 2.69)	2.37 (2.28; 2.44)	2.43 (2.3; 2.74)	2.37 (2.29; 2.68)	2.41 (2.36; 2.62)	2.5 (2.34; 2.63)	2.44 (2.36; 2.99)	2.47 (2.35; 2.77)	2.35 (2.26; 2.42)	2.4 (2.26; 2.48) €	2.48 (2.38; 2.62)	2.44 (2.27; 2.52)
IL-4 med (p25; p75)	0.66 (0.55; 0.66)	0.7 (0.66; 0.89)	0.84 (0.66; 1.29)	0.74 (0.66; 0.91)	0.66 (0.55; 0.74)	0.7 (0.66; 0.81)	0.81 (0.55; 0.92)	0.72 (0.63; 0.89)	0.55 (0.55; 0.66)	0.68 (0.66; 0.81)	0.66 (0.55; 0.66)*	0.66 (0.6; 0.74)
IL-6 med (p25; p75)	0.62 (0.46; 0.74)	0.79 (0.62; 1.08)	0.75 (0.65; 1.24)	0.77 (0.64; 1.22)	0.65 (0.35; 0.86)	0.89 (0.55; 1.01)	0.88 (0.55; 1.08)	0.88 (0.55; 1.05)	0.68 (0.46; 0.8)	0.72 (0.55; 1.04)	0.87 (0.62; 1.01)	0.77 (0.59; 1.03)
IL-8 med (p25; p75)	0.41 (0.22; 0.68)	0.39 (0.3; 0.93)	0.66 (0.51; 0.69)	0.59 (0.3; 0.93)	0.41 (0.13; 0.59)	0.47 (0.23; 0.86)	0.55 (0.3; 0.82)	0.51 (0.26; 0.86)	0.28 (0.2; 0.44)	0.35 (0.23; 0.41)	0.37 (0.27; 0.52)	0.37 (0.25; 0.52)*€
IL-10 med (p25; p75)	0.12 (0.09; 0.15)	0.17 (0.09; 0.32)	0.3 (0.18; 0.43)	0.23 (0.11; 0.4)	0.11 (0.07; 0.15)	0.17 (0.09; 0.29)	0.3# (0.15; 0.54)	0.23@ (0.12; 0.46)	0.14 (0.09; 0.17)	0.16 (0.06; 0.25)	0.27 (0.06; 0.43)	0.22 (0.06; 0.38)
IL-12 med (p25; p75)	0.89 (0.88; 0.91)	0.91 (0.89; 0.92)	0.91 (0.91; 0.99)@	0.91 (0.89; 0.93)@	0.91 (0.88; 0.91)	0.91 (0.89; 0.91)	0.92 (0.91; 0.96)	0.91 (0.89; 0.93)	0.88 (0.88; 0.89)	0.91 (0.89; 0.93)	0.91 (0.89; 0.93)	0.91 (0.89; 0.93)@
IL-13 med (p25; p75)	0.8 (0.79; 0.8)	0.79 (0.79; 0.8)	0.79 (0.79; 0.8)	0.79 (0.79; 0.8)	0.79 (0.79; 0.79)	0.79 (0.79; 0.79)	0.8 (0.79; 0.81)	0.79 (0.79; 0.8)	0.79 (0.79; 0.79)	0.79 (0.79; 0.8)	0.79 (0.79; 0.8)	0.79 (0.79; 0.8)
IL-15 med (p25; p75)	0.9 (0.88; 0.91)	0.9 (0.9; 0.91)	0.9 (0.9; 0.93)	0.9 (0.9; 0.93)	0.89 (0.88; 0.9)	0.9 (0.88; 0.92)	0.91 (0.9; 0.93)	0.9 (0.89; 0.93)	0.88 (0.87; 0.88)	0.89 (0.88; 0.9)	0.91 (0.87; 0.91)	0.9 (0.88; 0.91)
IL-17A med (p25; p75)	0.42 (0.37; 0.46)	0.44 (0.37; 0.5)	0.37 (0.32; 0.57)	0.4 (0.35; 0.53)	0.37 (0.37; 0.42)	0.4 (0.37; 0.42)	0.37 (0.32; 0.68)	0.37 (0.37; 0.55)	0.4 (0.37; 0.42)	0.4 (0.35; 0.42)	0.35 (0.35; 0.37)	0.37 (0.35; 0.42)
LIF med (p25; p75)	0.21 (0.21; 0.21)	0.19 (0.13; 0.21)	0.25 (0.13; 0.35)	0.21 (0.13; 0.35)	0.21 (0.13; 0.25)	0.25 (0.21; 0.4)	0.25 (0.13; 0.45)	0.25 (0.17; 0.42)	0.21 (0.17; 0.21)	0.23 (0.13; 0.29)	0.25 (0.21; 0.29)	0.23 (0.15; 0.29)
TAC med (p25; p75)	0.37 (0.31; 0.38)	0.33 (0.27; 0.47)	0.35 (0.26; 0.4)	0.33 (0.26; 0.47)	0.36 (0.33; 0.39)	0.46 (0.37; 0.48)	0.41 (0.31; 0.58)*	0.41 (0.31; 0.58)*	0.43 (0.33; 0.5)	0.43 (0.39; 0.48)	0.47 (0.32; 0.62)*	0.43 (0.34; 0.59)*
Isoprostanes med (p25; p75)	0.94 (0.91; 1.22)	1.17 (0.98; 1.54)	1.36 (0.81; 1.56)	1.31 (0.87; 1.56)	1.23 (0.98; 2)	1.55 (1.04; 1.89)	1.13 (0.72; 1.39)	1.34 (0.91; 1.77)	1.51 (1.16; 1.6)	1.44 (0.38; 1.64)	1.42 (0.88; 1.6)	1.42 (0.88; 1.63)

All values shown in this table come from the logarithm of the correspondent parameter. Data are presented as mean ± standard deviation (m ± ds) or median and interquartile range: med (p25; p75). SU-PL, supplement group + placebo; SU-PR, supplement group + probiotics; SU-TOT, supplement group (SU-PL + SU-PR). Differences between baseline and 3- or 6-months intragroup: *$p < 0.05$; differences between 3 and 6 months: €$p < 0.05$; Ω$p < 0.01$; differences with respect to the control group (at baseline, 3 or 6 months): @$p < 0.05$; #$p < 0.01$. BDNF, brain-derived neurotrophic factor; BMP-2, bone morphogenetic protein-2; MCP-1, monocyte chemoattractant protein-1; CRP, C-reactive protein; TNF-α, tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon-gamma; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; LIF, cytokine-leukemia inhibitory factor: TAC. total antioxidant capacity.

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The SU-PR group reached significant differences over time in several biomarkers such as BDNF, MCP-1, IL-10, and TAC, which suggests that probiotics could synergistically act with other active ingredients of the supplement (virgin olive oil, prebiotic fiber, n-3 PUFA, or whey protein, etc) and with the Mediterranean dietary pattern to improve the proinflammatory and oxidative status, and FFM. Despite more studies being necessary to recommend the use of pre- and probiotics in patients with MHD, they could reduce the levels of solutes that contribute to the uremic syndrome, as well as improve the lipid profile, oxidative stress, and systemic inflammation (19, 20).

All these dietary changes in body composition and biomarkers were produced with no increase in the serum levels of phosphorus or potassium (with a clear tendency of the latter toward reduction and achieving normal values in the supplemented groups), in accordance with other randomized studies (1, 36). We also found vitamin D increases after 3 months, similar to the findings of other recent trials (5). These results suggest that this new ONS (with a low content of these electrolytes) can help to achieve nutritional requirements in these patients.

Interestingly, urea levels increased significantly in the SU-PL group but not in the SU-PR. Urea has recently been proposed as a relevant gut-derived toxin that triggers molecular changes leading to insulin resistance and endothelial dysfunction. In CKD models, probiotics from the genus Bifidobacterium have shown reductions in serum urea nitrogen and other uremic toxins levels (46). It is possible that in our study, the probiotics have contributed to the fact that urea levels do not rise despite the increase in protein intake with ONS.

The main limitation of the study (in part due to the SARS-CoV-2 pandemic and the associated difficulties in completing the patients' follow-up) is that, although we did reach the planned sample size (more than 17 subjects per group), this may not be enough for some variables if we consider the high dropout rate. Nonetheless, the power for the variables reaching statistical significance was, in all cases, above 80%. Some statistical significances were only observed after adding the two supplemented groups (SU-TOT), which may be due to an additive effect of the SU-PR group but could also be secondary to the increase in n; the first option cannot be tested as there was no control-PR group. Notwithstanding, no patient withdrew from the trial because of gastrointestinal symptoms (which were similar to the C group during all the interventions), and only six because of lack of supplement acceptance (16% of the total number of patients randomized to ONS). These results are similar (or better) to those from other trials in which adherence was low (7, 47). Acceptance regarding the organoleptic characteristics of the supplement was high, which is in part motivated by the possibility of changing the flavor as, although the supplement is presented in vanilla flavor, it is delivered with six additional flavors that can be added to facilitate compliance, acceptance, and individualization. ONS compliance was self-reported, and no biomarker to evaluate intake was used; in this sense, it would have been better to measure the normalized protein catabolic rate (nPCR) to evaluate protein intake. Finally, no data on the acid–base status were collected, and it could have provided useful information.

As strengths of the study, we highlight the fact that it is a randomized clinical trial (double-blind regarding probiotics intake), the follow-up is in the long term (6 months), its multicentric nature, the measurement of multiple parameters (diet, morphofunctional nutritional assessment, biochemical parameters, and biomarkers of inflammation and oxidation), and the comparison with a C group that followed an individualized diet prescribed by registered dietitians.

5. Conclusion

The new ONS specifically designed for patients with MHD with malnutrition (or at risk) improved caloric-protein intake, nutritional status (especially FFM), and some biomarkers of inflammation and oxidation; the addition of probiotics could act synergistically with the ONS components to improve these biomarkers. This study sets the path for new randomized studies with a higher number of patients and, in the long term, confirms these preliminary results and assesses the efficacy of the new ONS in terms of morbidity.

Data availability statement

The data presented in this study are available on reasonable request from the corresponding authors.

Ethics statement

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

Author contributions

GO contributed to the conceptual design of the research, funding acquisition, and drafted the manuscript. GO, FH, and MP contributed to the interpretation of the data. All authors contributed to the acquisition, analysis of the data, critically revised the manuscript, and agreed to be fully accountable for ensuring the integrity and accuracy of the study.

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Conflict of interest

FH received honoraria support for a presentation from Adventia Pharma. GO received occasional honoraria support for presentations, attending meetings, and travel from Adventia Pharma.

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.2023.1107869/full#supplementary-material

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Association of nonalcoholic fatty liver disease and liver fibrosis detected by transient elastography with serum retinol in American adults

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Background and objective: Retinol is a precursor of vitamin A, which is metabolized and maintained in the liver and is involved in the pathogenesis of the nonalcoholic fatty liver disease (NAFLD) and liver fibrosis. The relationship between NAFLD and liver fibrosis with serum retinol levels remains insufficient and inconclusive. Our study aimed to investigate the correlation between NAFLD, fibrosis, and serum retinol levels in American adults.

Methods: A cross-sectional analysis was conducted using information from the 2017–2018cycle of the National Health and Nutrition Examination Survey (NHANES). The exposure factors were NAFLD and liver fibrosis status detected using transient elastography (TE), and the outcome was serum retinol levels. Weighted multivariate regressions were established to assess the correlation between NAFLD and liver fibrosis and serum retinol levels. Subgroup analyses were also performed.

Results: This study included 3,537 participants. Compared to the group without NAFLD, NAFLD was positively correlated with serum retinol levels (β = 1.28, 95% CI: 0.19, 2.37). In the subgroup analysis, a positive correlation between NAFLD and serum retinol levels was found in people aged < 60years, Mexican Americans, and those with a body mass index (BMI)<25. On the contrary, compared to the group without liver fibrosis, there was a significant negative association between liver fibrosis and serum retinol (β=−3.46, 95% CI: −5.16, −1.75), especially in people aged < 60 years, non-Hispanic white/black individuals, and people with a BMI ≥ 25.

Conclusion: Our study suggests that NAFLD status may be positively associated with serum retinol levels in adult patients, and liver fibrosis may be negatively associated with serum retinol levels. Further studies are required to examine the associations found in our study.

KEYWORDS

NAFLD, liver fibrosis, serum retinol, NHANES, transient elastography

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common chronic liver disease that affects one-third of American adults, resulting in a substantial disease burden (1). NAFLD is a fatty liver disease in which excessive fat is stored in liver cells (2, 3). It is an umbrella term that comprises a continuum of liver conditions that vary in the severity of the injury and resulting fibrosis (4).

NAFLD has a range of pathological changes (5) including isolated steatosis and progressive nonalcoholic steatohepatitis. Approximately 40% of patients with nonalcoholic steatohepatitis eventually develop liver fibrosis, cirrhosis, and hepatocellular carcinoma (6). Because there is no effective treatment for NAFLD and fibrosis, it is necessary to identify the hazardous factors that contribute to disease development.

Dietary vitamin A is absorbed in the small intestine and transported to the liver in the form of retinyl esters, which are then stored in hepatic stellate cells (HSCs) (7). Retinol binds to retinol-binding protein 4 (RBP4) produced by hepatocytes and is secreted into the circulation, where it plays a physiological role. Vitamin A is a key regulator of glucose and lipid metabolism in the liver and adipose tissue (8). It has been suggested that disturbances in vitamin A homeostasis in the liver may contribute to the development of NAFLD (9). Specifically, changes in diet or hormonal signaling can activate HSCs, leading to the loss of the ability of HSCs to store vitamin A. Excess vitamin A metabolism increases lipogenesis, allowing fat to accumulate in hepatocytes, eventually inducing NAFLD. After a long-term chronic liver injury, HSCs transdifferentiate into myofibroblasts and produce an excessive extracellular matrix, leading to liver fibrosis. HSCs lose their stored retinyl esters during this process, eventually leading to a vitamin A deficiency.

Retinol is a precursor of vitamin A, and the serum retinol level is a sensitive marker for the evaluation of vitamin A status (10). However, reports on the epidemiological relationship between NAFLD and liver fibrosis and serum retinol levels are scarce, and the results of studies on serum vitamin A levels in patients with NAFLD or fibrosis are controversial (11–13).

Liver ultrasound transient elastography (TE) is a noninvasive method for estimating liver steatosis and fibrosis (14) and has been used in the general population (15). The National Health and Nutritional Examination Survey (NHANES) first used TE examinations during the 2017–2018 cycle. Here, we aimed to investigate the clinical relevance of serum retinol levels in the setting of NAFLD and liver fibrosis detected by TE in adults and to provide a new perspective on its pathogenesis.

2. Materials and methods

The NHANES is a cross-sectional survey conducted in the United States based on a nationally representative sample. Our study used data from the 2017–2018 NHANES cycle, in which the TE examination was first used.

2.1. Variables

The exposure factors were NAFLD status detected by the control attenuation parameter (CAP) and liver fibrosis status detected by liver stiffness measurement (LSM). CAP and LSM values were implemented on FibroScan®, an instrument that can use the vibration-controlled transient elastography (VCTE) technique, obtained in mobile examination centers (MEC). Qualified VCTE tests required the following three aspects: fasting for at least 3 h, 10 LSM measurements were obtained at least, and an interquartile range (IQR)/median of <30%. Liver steatosis was defined as a median CAP value ≥ 274 dB/m (16) based on a recent study. Significant fibrosis (≥F2) was defined as a median LSM value ≥ 8.0 kPa (17, 18). The outcome was the serum retinol level, detected using a modification of a high-performance liquid chromatography-photodiode array detection method, as recommended by the NHANES website.[1] Specifically, the serum was mixed with an ethanol solution containing an internal standard, retinyl butyrate. After extracting the micronutrients, an aliquot of the filtrate was injected into a C18 reversed-phase column. Quantitative analysis was performed using spectrophotometry. Retinol and retinyl esters were compared with retinyl butyrate at 325 nm.

The covariates included age, race, sex, waist circumference, body mass index (BMI), triglyceride, total cholesterol, alanine aminotransferase (ALT), aspartate transaminase (AST), γ-glutamyl transpeptidase (GGT), total bilirubin, serum albumin, serum creatinine, blood urea nitrogen, recreational physical activity, smoking status, and diabetes status. All data are available on the NHANES's website. The participants were classified as never, former, or current smokers. Participants were considered never smokers if they had smoked <100 cigarettes and former or current smokers based on whether they smoked currently or not, or if they had ever smoked ≥ 100 cigarettes. Diabetes was defined based on the guidelines for the Classification and Diagnosis of Diabetes. HBsAg and anti-HCV antibodies were used to detect the hepatitis B virus (HBV) and hepatitis C virus (HCV), respectively. Alcohol intake data were obtained from the questionnaire according to alcohol consumption in the previous year. Significant alcohol intake was defined as >21 or > 14 standard drinks weekly for men or women (19).

2.2. Study population

In total, 9,254 participants were included in the 2017–2018 NHANES cycle. We excluded the following participants: individuals aged <18 years, individuals whose MEC exam was not available, individuals for whom a transient elastography was not performed

1 wwwn.cdc.gov

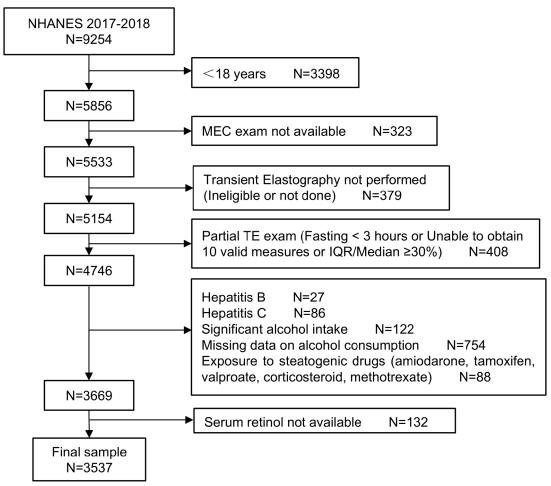

FIGURE 1
Flowchart of the study participants.

(ineligible or not done), individuals for whom only a partial TE exam was performed (fasting < 3 h or incapable of obtaining at least 10 effective measures or IQR/Median ≥ 30%), individuals with Hepatitis B and C, individuals with significant alcohol intake or missing alcohol use data, individuals with exposure to steatogenic drugs (amiodarone, tamoxifen, corticosteroid, valproate, and methotrexate), and individuals whose serum retinol levels were not available. Finally, 3,537 samples were analyzed (Figure 1).

2.3. Statistical analysis

Appropriate sample weights were used for analysis. Data were presented as weighted proportions and weighted mean ± standard error (SE) for different types of covariables. We used weighted chi-square tests and linear regression models to explore differences between patients with NAFLD and fibrosis and those without NAFLD and fibrosis. Multifactor linear regression models were used to assess

TABLE 1 Weighted characteristics of the study sample based on NAFLD and liver fibrosis status.

Characteristic	Total	NAFLD		*p*-value	Liver fibrosis		*p*-value
		No	Yes		No	Yes	
		CAP<274dB/m	CAP≥274dB/m		LSM<8.0kPa	LSM≥8.0kPa	
N	3,669	1983	1,554		3,186	351	
Age (years)	46.92 ± 17.42	44.10 ± 17.61	50.90 ± 16.33	<0.0001	46.57 ± 17.40	50.86 ± 17.17	<0.0001
Gender (%)				<0.0001			<0.0001
Male	49.45	44.99	55.76		48.43	60.86	
Female	50.55	55.01	44.24		51.57	39.14	
Race (%)				<0.0001			0.8006
Mexican American	9.47	6.83	13.20		9.34	10.93	
Other Hispanic	7.07	7.39	6.61		6.98	8.05	
Non-Hispanic white	63.94	65.18	62.18		64.05	62.68	
Non-Hispanic Black	10.41	11.87	8.34		10.51	9.24	
Other Race	9.11	8.73	9.67		9.12	9.10	
BMI	29.61 ± 7.05	26.70 ± 5.62	33.70 ± 6.83	<0.0001	28.95 ± 6.39	36.86 ± 9.55	<0.0001
Waist circumference (cm)	100.19 ± 17.02	92.47 ± 13.94	111.08 ± 14.88	<0.0001	98.59 ± 15.77	117.94 ± 19.97	<0.0001
Total cholesterol (mg/dL)	188.27 ± 40.62	186.40 ± 39.66	190.91 ± 41.79	0.0011	188.69 ± 40.24	183.61 ± 44.38	0.0407
Triglyceride (mg/dL)	112.78 ± 71.82	101.78 ± 42.24	128.32 ± 97.52	<0.0001	111.33 ± 66.43	128.93 ± 114.65	<0.0001
ALT (IU/L)	27.53 ± 18.52	19.23 ± 13.56	27.52 ± 18.57	<0.0001	21.81 ± 15.32	32.20 ± 22.99	<0.0001
AST (IU/L)	21.51 ± 10.93	20.52 ± 9.88	22.92 ± 12.13	<0.0001	21.02 ± 9.68	27.06 ± 19.31	<0.0001
GGT (IU/L)	28.16 ± 36.32	23.09 ± 33.35	35.33 ± 39.03	<0.0001	26.12 ± 27.83	50.90 ± 82.51	<0.0001
Total bilirubin (mg/dL)	0.47 ± 0.28	0.49 ± 0.29	0.45 ± 0.26	<0.0001	0.47 ± 0.28	0.47 ± 0.25	0.9629
Serum albumin (g/dL)	4.11 ± 0.32	4.13 ± 0.31	4.07 ± 0.32	<0.0001	4.11 ± 0.31	4.04 ± 0.36	0.0002
Serum creatinine (mg/dL)	0.88 ± 0.31	0.87 ± 0.24	0.89 ± 0.39	0.0649	0.88 ± 0.30	0.93 ± 0.43	0.0058
Blood urea nitrogen (mg/dL)	14.95 ± 5.06	14.55 ± 4.83	15.51 ± 5.33	<0.0001	14.90 ± 5.03	15.50 ± 5.40	0.0526
Recreational physical activity (%)				<0.0001			<0.0001
Yes	56.89	63.35	47.78		58.24	41.94	
No	43.11	36.65	52.22		41.76	58.06	
Smoking status (%)				0.0002			0.0125
Never	58.01	59.88	55.37		58.53	52.25	
Former	25.45	22.92	29.02		24.80	32.67	
Current	16.54	17.20	15.62		16.67	15.08	
Diabetes (%)				<0.0001			<0.0001
Yes	13.91	5.99	25.08		11.45	41.23	
No	86.09	94.01	74.92		88.55	58.77	
Serum retinol (μg/dL)	54.52 ± 15.66	53.34 ± 15.18	56.19 ± 16.16	<0.0001	54.64 ± 15.64	53.18 ± 15.79	0.1269
CAP (dB/m)	261.97 ± 62.41	219.17 ± 36.08	322.41 ± 36.30	<0.0001	256.78 ± 59.85	319.59 ± 61.31	<0.0001
LSM (kPa)	5.62 ± 4.87	4.82 ± 2.99	6.75 ± 6.50	<0.0001	4.79 ± 1.24	14.81 ± 13.33	<0.0001

Mean ± SD and % for continuous and categorical variables. The *P*-value was calculated by weight linear regression model and weighted chi-square test, respectively.

TABLE 2 Relationship between NAFLD or liver fibrosis status and serum retinol (μg/dL) in adults.

	Model 1	Model 2	Model 3
	β (95%CI) *p*-value	β (95%CI) *p*-value	β (95%CI) *p*-value
NAFLD status			
No	Reference	Reference	Reference
Yes	2.85 (1.81, 3.89) <0.0001	1.04 (0.02, 2.06) 0.0453	1.28 (0.19, 2.37) 0.0218
Liver fibrosis status			
No	Reference	Reference	Reference
Yes	−1.46 (−3.33, 0.41) 0.1269	−3.06 (−4.83, −1.29) 0.0007	−3.46 (−5.16, −1.75) <0.0001

Model 1, no adjusted covariables; Model 2, adjustments made for gender, race, and age; Model 3, in addition to the variables in Model 2, BMI, waist circumference, total cholesterol, triglyceride, ALT, AST, GGT, total bilirubin, serum albumin, serum creatinine, blood urea nitrogen, recreational physical activity, smoking status, and diabetes were added. In different stratified policies, stratified variables were not adjusted themselves.

the correlation between NAFLD and fibrosis status with serum retinol levels.

Three models were created in the study, as recommended by the guidelines for reporting observational studies (20): Model 1, no adjusted covariables; Model 2, adjustments made for sex, race, and age; Model 3, in addition to the variables in Model 2, waist circumference, BMI, total cholesterol, triglyceride, ALT, AST, GGT, total bilirubin, serum albumin, serum creatinine, blood urea nitrogen, recreational physical activity, smoking status, and diabetes status were added. Subgroup analyses were performed based on age, sex, race, and BMI.

EmpowerStats[2] and R[3] software packages were used for the analyses. *p*-values of <0.05 were defined as statistically significant.

3. Results

3.1. Population characteristics

A total of 3,537 samples were included in the study. The weighted characteristics of the samples according to NAFLD and fibrosis status are shown in Table 1. There were significant differences in sample characteristics among patients with different NAFLD statuses. Compared with the group without NAFLD, the patients with NAFLD were more likely to be older, men, non-Hispanic white, and former smokers. They also had higher BMIs, waist circumference, total cholesterol, triglyceride, ALT, AST, GGT, blood urea nitrogen, and LSM values, and lower total bilirubin and serum albumin levels. Moreover, they were less likely to undergo recreational physical activity and had a higher prevalence of diabetes ($p < 0.05$). Weighted characteristics based on liver fibrosis showed similar results. In brief, there were significant differences in most sample characteristics (Table 1), except for race, total cholesterol, total bilirubin, and blood urea nitrogen.

3.2. Relationship between NAFLD and serum retinol

We constructed three weighted linear regression models as shown in Table 2. Compared to the group without NAFLD, there was a significant positive correlation between the NAFLD group and serum retinol in Model 1 (β = 2.85, 95% CI: 1.81, 3.89). After adjusting for covariates, a significant positive correlation was still present in Model 2 (β = 1.04, 95% CI: 0.02 2.06) and Model 3 (β = 1.28, 95% CI: 0.19, 2.37). In further subgroup analyses (Table 3) stratified by age, sex, race, and body mass index (BMI), a positive correlation remained in people aged < 60 years (β = 1.40, 95% CI: 0.07, 2.72), Mexican Americans (β = 2.47, 95% CI: 0.18, 4.77), and those with a BMI < 25 (β = 6.48, 95% CI: 3.10, 9.86).

3.3. Relationship between liver fibrosis and serum retinol

In terms of the relationship between liver fibrosis status and serum retinol, compared with the group without liver fibrosis (Table 3), no significant correlation was found in Model 1 (β = −1.46, 95% CI: −3.33, 0.41) in the liver fibrosis group. Interestingly, there was a significant negative correlation after adjusting for the covariates in Model 2 (β = −3.06, 95% CI: −4.83, −1.29) and Model 3 (β = −3.46, 95% CI: −5.16, −1.75). In subgroup analyses (Table 4), the positive correlation remained in people aged < 60 years (β = −3.74, 95% CI: −5.89, −1.58), both men (β = −3.57, 95% CI: −5.79, −1.35) and women (β = −3.45, 95% CI: −6.13, −0.76), non-Hispanic white individuals (β = −3.47, 95% CI: −6.42, −0.52), non-Hispanic black individuals (β = −6.22, 95% CI: −9.87, 2.58), and those with a BMI of 25–29.9 (β = −4.77, 95% CI: −8.96, −0.59) and a BMI ≥ 30 (β = −2.87, 95% CI: −4.90, −0.85).

4. Discussion

In our study, the 2017–2018 NHANES cycle was used to examine the correlation between NAFLD and fibrosis status detected by TE with serum retinol in adults. NAFLD was positively associated with serum retinol levels, especially in those aged <60 years, Mexican Americans, and those with a BMI < 25. In contrast, liver fibrosis status

2 http://www.empowerstats.com

3 http://www.R-project.org

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TABLE 3 Relationship between NAFLD status and serum retinol (μg/dL) in adults, stratified by age, gender, race, and BMI.

NAFLD status	Model 1	Model 2	Model 3
	β (95%CI) *p*-value	β (95%CI) *p*-value	β (95%CI) *p*-value
<60 years			
No	Reference	Reference	Reference
Yes	2.16 (0.91, 3.40) 0.0007	1.53 (0.34, 2.72) 0.0121	1.40 (0.07, 2.72) 0.0397
≥60 years			
No	Reference	Reference	Reference
Yes	2.72 (0.83, 4.61) 0.0049	2.75 (0.85, 4.65) 0.0046	1.60 (−0.24, 3.44) 0.0887
Male			
No	Reference	Reference	Reference
Yes	2.26 (0.84, 3.67) 0.0018	1.35 (−0.08, 2.77) 0.0638	1.33 (−0.24, 2.90) 0.0966
Female			
No	Reference	Reference	Reference
Yes	2.21 (0.70, 3.72) 0.0041	0.63 (−0.83, 2.10) 0.3970	1.44 (−0.08, 2.97) 0.0636
Mexican American			
No	Reference	Reference	Reference
Yes	3.36 (0.98, 5.74) 0.0058	0.68 (−1.56, 2.91) 0.5537	2.47 (0.18, 4.77) 0.0353
Other Hispanic			
No	Reference	Reference	Reference
Yes	0.74 (−2.14, 3.61) 0.6161	−0.94 (−3.72, 1.84) 0.5079	0.59 (−2.29, 3.46) 0.6899
Non-Hispanic white			
No	Reference	Reference	Reference
Yes	2.66 (0.90, 4.43) 0.0031	0.67 (−1.09, 2.43) 0.4539	1.41 (−0.50, 3.32) 0.1491
Non-Hispanic Black			
No	Reference	Reference	Reference
Yes	5.21 (2.89, 7.52) <0.0001	3.05 (0.80, 5.30) 0.0081	0.76 (−1.59, 3.10) 0.5258
Other Race			
No	Reference	Reference	Reference
Yes	4.78 (2.43, 7.13) <0.0001	3.05 (0.75, 5.36) 0.0097	0.18 (−2.16, 2.52) 0.8792
BMI < 25			
No	Reference	Reference	Reference
Yes	11.33 (7.88, 14.79) <0.0001	8.46 (5.01, 11.91) <0.0001	6.48 (3.10, 9.86) 0.0002
BMI 25–29.9			
No	Reference	Reference	Reference
Yes	1.37 (−0.49, 3.24) 0.1493	−0.09 (−1.92, 1.74) 0.9244	−0.36 (−2.08, 1.36) 0.6826
BMI ≥ 30			
No	Reference	Reference	Reference
Yes	3.41 (1.69, 5.14) 0.0001	1.79 (0.12, 3.46) 0.0354	1.53 (−0.02, 3.07) 0.0528

Model 1, no adjusted covariables; Model 2, adjustments made for gender, race, and age; Model 3, in addition to the variables in Model 2, BMI, waist circumference, total cholesterol, triglyceride, ALT, AST, GGT, total bilirubin, serum albumin, serum creatinine, blood urea nitrogen, recreational physical activity, smoking status, and diabetes were added. In different stratified policies, stratified variables were not adjusted themselves.

was negatively associated with serum retinol levels, especially in people aged <60 years, non-Hispanic white/black individuals, and those with a BMI ≥ 25.

Vitamin A is necessary for glucose and lipid metabolism (21, 22) in the liver and adipose tissue (13), even in patients with NAFLD (23, 24). Retinol has an important antioxidant effect on NAFLD (25, 26). However, it remains unclear whether vitamin A levels promote or inhibit NAFLD development. Serum vitamin A levels in patients with NAFLD are controversial. A cross-sectional study reported that serum vitamin A levels were negatively correlated with NAFLD severity (11). Another study reported that serum retinol levels are inadequate in NAFLD (27). Nevertheless, another study indicated that serum retinol levels were higher in NAFLD donors than in non-NAFLD donors (12). A recent study from South Korea (13) showed that serum retinol levels

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TABLE 4 Relationship between liver fibrosis status and serum retinol (μg/dL) in adults, stratified by age, gender, race, and BMI.

Liver fibrosis status	Model 1	Model 2	Model 3
	β (95%CI) *p*-value	β (95%CI) *p*-value	β (95%CI) *p*-value
<60 years			
No	Reference	Reference	Reference
Yes	−2.96 (−5.27, −0.64) 0.0123	−4.18 (−6.36, −1.99) 0.0002	−3.74 (−5.89, −1.58) 0.0007
≥60 years			
No	Reference	Reference	Reference
Yes	−0.01 (−3.09, 3.07) 0.9957	0.26 (−2.82, 3.35) 0.8670	−2.75 (−5.50, 0.00) 0.0504
Male			
No	Reference	Reference	Reference
Yes	−2.82 (−5.16, −0.48) 0.0182	−3.57 (−5.85, −1.29) 0.0022	−3.57 (−5.79, −1.35) 0.0016
Female			
No	Reference	Reference	Reference
Yes	−1.30 (−4.26, 1.67) 0.3910	−2.24 (−5.04, 0.56) 0.1171	−3.45 (−6.13, −0.76) 0.0120
Mexican American			
No	Reference	Reference	Reference
Yes	−0.86 (−4.88, 3.17) 0.6767	−3.83 (−7.55, −0.11) 0.0440	−0.85 (−4.40, 2.70) 0.6385
Other Hispanic			
No	Reference	Reference	Reference
Yes	−0.86 (−4.88, 3.17) 0.6767	−3.83 (−7.55, −0.11) 0.0440	−0.85 (−4.40, 2.70) 0.6385
Non-Hispanic white			
No	Reference	Reference	Reference
Yes	−2.02 (−5.19, 1.16) 0.2138	−3.18 (−6.26, −0.11) 0.0428	−3.47 (−6.42, −0.52) 0.0213
Non-Hispanic Black			
No	Reference	Reference	Reference
Yes	0.97 (−3.27, 5.21) 0.6541	−2.14 (−6.18, 1.90) 0.2999	−6.22 (−9.87, −2.58) 0.0009
Other Race			
No	Reference	Reference	Reference
Yes	3.17 (−1.12, 7.46) 0.1478	1.00 (−3.11, 5.11) 0.6327	−2.38 (−6.40, 1.64) 0.2470
BMI < 25			
No	Reference	Reference	Reference
Yes	1.41 (−4.44, 7.26) 0.6362	−0.90 (−6.50, 4.69) 0.7518	−2.37 (−7.93, 3.19) 0.4030
BMI 25–29.9			
No	Reference	Reference	Reference
Yes	−1.36 (−6.15, 3.43) 0.5767	−4.09 (−8.64, 0.46) 0.0786	−4.77 (−8.96, −0.59) 0.0257
BMI ≥ 30			
No	Reference	Reference	Reference
Yes	−1.34 (−3.60, 0.92) 0.2441	−2.41 (−4.55, −0.28) 0.0271	−2.87 (−4.90, −0.85) 0.005

Model 1, no adjusted covariables; Model 2, adjustments made for gender, race, and age; Model 3, in addition to the variables in Model 2, BMI, waist circumference, total cholesterol, triglyceride, ALT, AST, GGT, total bilirubin, serum albumin, serum creatinine, blood urea nitrogen, recreational physical activity, smoking status, and diabetes were added. In different stratified policies, stratified variables were not adjusted themselves.

were positively associated with the prevalence of NAFLD. According to the results of our research, patients with NAFLD had higher serum retinol levels than those without NAFLD, especially in those aged < 60 years, Mexican Americans, and with a BMI < 25.

Liver fibrosis has a significant effect on vitamin A metabolism and storage. HSCs contain approximately 80% retinol. HSCs are activated to produce large amounts of collagen and fibroblasts under oxidative stress, eventually leading to fibrosis (7). Vitamin A reserves are also gradually lost during this conversion process (25). In most NAFLD prediction models, low serum retinol levels were significantly associated with advanced fibrosis. According to a recent study (13), serum retinol deficiency was significantly associated with advanced fibrosis. Another study showed that reduced serum retinol levels were observed in patients with advanced liver fibrosis, and liver fibrosis was

an independent risk factor correlated with decreased serum retinol levels (27). Our study showed that compared with the group without liver fibrosis, patients with liver fibrosis had lower serum retinol levels, especially those aged < 60 years, non-Hispanic white/black individuals, and individuals with a BMI ≥ 25. These findings were consistent with those of previous research (28–30).

Dysregulation of liver vitamin A homeostasis may be implicated in the onset of NAFLD (9). Disordered vitamin A homeostasis during the development and early stages of NAFLD can affect the accumulation of lipids in the liver (31, 32). This results in excessive release of retinol and elevated serum retinol levels (33). When NAFLD develops into advanced fibrosis, excessive impairment of vitamin A levels leads to a serious deficiency of vitamin A in the liver, which leads to decreased serum retinol concentrations.

This study has several limitations. First, the causal relationship between both NAFLD and liver fibrosis and serum retinol levels remains unclear; this should be confirmed by prospective studies. Second, NAFLD status and liver fibrosis were defined by the CAP and LSM values through VCTE rather than liver biopsy, which may cause identification bias. Third, the correlation between both NAFLD and liver fibrosis and serum retinol levels varied by age, sex, race, and BMI in the subgroup analysis; the mechanism remains unclear and needs further research.

5. Conclusion

Our study suggests that NAFLD status may be positively associated with serum retinol levels in adult patients and that liver fibrosis may be negatively associated with serum retinol levels. As many unknown and complicated mechanisms exist, future experimental and prospective cohort studies will be helpful.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: www.cdc.gov/nchs/nhanes/.

Ethics statement

NHANES was approved by the National Center for Health Statistics Research Ethics Review Board. The participants provided their written informed consent to participate in NHANES. Ethical approval for this study is deemed exempt because this study uses publically available data.

Author contributions

XN, JL, and KL contributed to the study's conception and design. XN and JL contributed to data collection and interpretation of data. KL contributed to data analysis and writing and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Association between fatty acids intake and bone mineral density in adults aged 20–59: NHANES 2011–2018

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Background: Previous studies have investigated the link between fatty acid intake and bone mineral density (BMD), but the results are controversial. This study aims to examine the relationship between fatty acid intake and BMD in adults aged 20–59.

Methods: The association between fatty acid consumption and BMD was analyzed using a weighted multiple linear regression model with National Health and Nutrition Examination Survey data from 2011 to 2018. The linearity relationship and saturation value of the connection between fatty acid consumption and BMD were assessed by fitting a smooth curve and a saturation effect analysis model.

Results: The study included 8,942 subjects. We found a significant positive correlation between the consumption of saturated fatty acids, monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids and BMD. In subgroup analyses that were stratified by gender and race, this association was still shown to be significant. Based on the smooth curve and saturation effect analysis, we found no saturation effect for the three fatty acids and total BMD. However, there was a turning point (20.52g/d) between MUFAs intake and BMD, and only MUFAs intake >20.52g/d showed a positive correlation between MUFAs and BMD.

Conclusion: We found that fatty acid intake is beneficial for bone density in adults. Therefore, according to our findings, it is recommended that adults consume moderate amounts of fatty acids to ensure adequate bone mass but not metabolic diseases.

KEYWORDS

nutrition, bone mineral density, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, NHANES

1. Introduction

Osteoporosis (OP) is a degenerative disease of the bones that results in weakened bones, weakened microarchitecture, increased fragility, and increased fracture risk (1). The prevalence of OP has been increasing in recent years. According to a study done by the International Society for Clinical Densitometry and the International Foundation for Osteoporosis, more than 70

million Americans will have osteoporosis or bone loss by 2030 (2). With the expansion of human life expectancy and population aging, OP will become a more severe public health problem worldwide (3, 4). Therefore, early detection and intervention of osteoporosis have attracted more and more attention.

As a controllable factor, lifestyle seems to play an important role. Numerous nutrients, especially dietary fatty acid intake, have been shown to potentially effect on bone health (5–10). Studies have shown that the type of fatty acid is critical (8, 10, 11). Saturated fatty acids (SFAs) improve bone health by enhancing osteoclast survival (12), decreasing mesenchymal stem cell differentiation (13), promoting calcium absorption or excretion (14), and suppressing inflammatory gene expression (15). Polyunsaturated fatty acids (PUFAs) affect bone metabolism by combining with PPARγ to induce bone marrow adipocyte differentiation (16), regulate inflammatory response (17), and improve bone marrow microcirculation (18). Additionally, monounsaturated fatty acids (MUFAs) may have possible impacts on prostaglandin activity, influencing bone production and bone resorption (19, 20).

However, after reviewing a lot of literature, we discovered that the effects of PUFAs on bone health have received considerable attention; nevertheless, large-scale clinical investigations are absent. At the same time, we find it interesting that no researcher has focused on the connection between the different types of fatty acids and bone mineral density (BMD). Therefore, we decided to conduct a large-scale cross-sectional study using the National Health and Nutrition Examination Survey (NHANES) Database to investigate the connection between the different types of fatty acids and BMD. This was done to help guide therapeutic efforts.

2. Methods

2.1. Data source and study population

Data collected by NHANES from 2011 to 2018 was used for this cross-sectional analysis. NHANES adopts an innovative survey mode that combines face-to-face interviews and physical examinations to comprehensively assess the health and nutritional status of residents in the United States. Questions about demographics, socioeconomics, diet, and health were included in the interviews. The medical section addresses medical, dentistry, and physiological examinations as well as laboratory testing performed by qualified medical specialists. In general, these statistics are used to assess the prevalence of diseases and the related risk factors, formulate guidelines for the implementation of practical public health policy, devise health initiatives and services, cultivate fundamental health awareness, and improve the quality of life.

Our analysis comprised 39,156 participants from NHENAS 2011 to 2018, excluding participants under 20 (16,539 individuals) and those beyond 59 (7,683 individuals). Simultaneously, missing data on fatty acids intake (1,958 individuals) and total BMD (4,034 individuals) were excluded. Following the screening mentioned above, we included a total of 8,942 individuals (Figure 1).

2.2. Ethics statement

All survey participants were informed of the poll's specifics and signed an informed consent form. The National Center for Health Statistics Ethics Review Board assessed and authorized the informed consent. Following the completion of official anonymization, all of the data is then made available to the public in order to make the most effective use of these resources. Anyone may access these statistics as long as they adhere to the NHENAS database regulations and are used exclusively for statistical analysis. All studies based on these data should adhere to applicable laws and legislation.

2.3. Covariates

The independent variable in our study was daily fatty acids consumption, which was determined through two 24-h food recall interviews. Interviews were conducted in person and over the phone, respectively. In the Mobile Examination Center (MEC), a small room was used to perform the first 24-h recall interviews. Each MEC dietary interview room had a set of uniform measuring parameters. These methods assist respondents in reporting the quantity and variety of food they consume. The information for the second food recall was gathered over the phone and was due 3 to 10 days later. After the participants had finished the in-person interviews, they were provided with measuring glasses, teaspoons, a ruler, and a food model guide to equip them with the tools necessary to record meal portions accurately during the phone interviews. All study participants were required to complete two in-person interviews, each performed by a professional dietary interviewer who spoke Spanish and English fluently. Some categorical variables, such as gender, education level, and moderate exercise, were also included in our study, as well as some continuous variables, including age, body mass index (BMI), the ratio of family income to poverty, alkaline phosphatase, blood calcium, blood phosphorus, blood uric acids, total cholesterol, triglyceride, glycohemoglobin, blood urea nitrogen, serum creatinine, urinary albumin creatinine ratio, total albumin, vitamin D intake, alcohol intake, total SFAs intake, total MUFAs intake, total PUFAs intake, and total BMD. For further details on gathering covariate data and 24-h dietary recall interviews, go to.[1]

2.4. Outcome variable

Dual-energy X-ray absorptiometry (DXA) is a clinically recognized method for measuring BMD. Its results can be used for osteoporosis fracture, fracture risk prediction, and drug efficacy evaluation (21). Total BMD, as determined by DXA whole-body scans, served as the outcome variable in our study. Because of their quickness, simplicity, and low radiation exposure are widely used to estimate body composition. In the NHANES, DXA scans of the participants have conducted on a Hologic Discovery Model A densitometer (Hologic, Inc., Bedford, Massachusetts), and data processing was carried out using Hologic APEX (version 4.0) software. Professional technicians who have received training and certificates do the operations above. The official NHANES website has a body composition manual with more information about how the DXA exam works.

1 www.cdc.gov/nchs/nhanes/

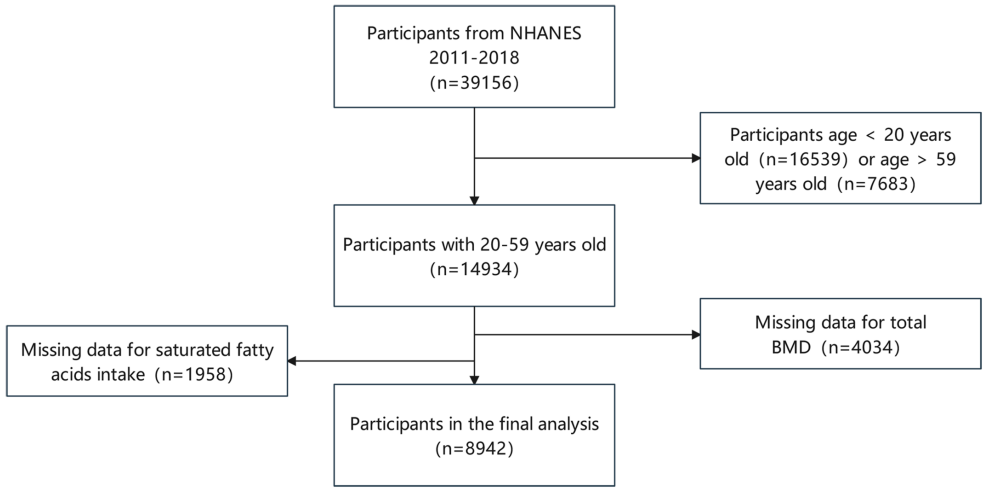

FIGURE 1
Flowchart of participant selection.

2.5. Statistical analysis

We used EmpowerStats[2] and R (3.4.4 version) software for statistical analysis. Typically, we consider a statistical result to be meaningful if the *p* value is lower than 0.05. In this study, all sample sizes are weighted. Continuous variables are reported as Mean ± SD for the comparison of baseline data, and the *p*-value was determined using a weighted linear regression model. The chi-square test was used to figure out the *p*-value, and categorical variables were given as a percentage. Weighted multiple linear regression analyses were performed to assess the effect of intake of the three types of fatty acids on BMD. We established three models of SFAs, MUFAs, and PUFAs intake and BMD, with all confounders (age, gender, race, education level, the ratio of family income to poverty, moderate activity, body mass index, alkaline phosphatase, serum calcium, serum uric acid, total cholesterol, blood urea nitrogen, serum phosphorus, triglyceride, glycohemoglobin, urinary albumin creatinine ratio, and total protein, serum creatinine, vitamin D intake, total saturated fatty acids, total monounsaturated fatty acids, and total polyunsaturated fatty acids) corrected for each model. This was done to improve the reporting of epidemiological observational studies and get the most out of the data. Concurrently, fatty acids consumption was transformed into categorical group data using the quartile approach, and *P* for trend was computed. Segregated according to age, gender, and race, subgroup analysis was performed to assess the association between fatty acids consumption and BMD in varying ages, gender, and race differences. In addition, after controlling for all confounding factors, smooth curve fitting was done, and a saturation effect analysis model was constructed to assess the correlation between the consumption of different fatty acids types and BMD. Results were expressed using turn point, effect–β (95%Cl, *p* value), and the log-likelihood ratio test (LRT test).

2 http://www.empowerstats.com

3. Results

3.1. Characteristics of participants

We stratified total bone mineral density by quartiles, Table 1 shows the study sample's baseline data, including demographic information, physical examination data, laboratory test indicators, and dietary interview information for 8,942 subjects. There are significant differences in age, gender, race, education level, the ratio of family income to poverty, moderate activity, body mass index, alkaline phosphatase, serum calcium, serum uric acid, total cholesterol, blood urea nitrogen, serum creatinine, vitamin D intake, total saturated fatty acids, total monounsaturated fatty acids, and total polyunsaturated fatty acids. However, the difference was not significant in terms of serum phosphorus, triglyceride, glycohemoglobin, urinary albumin creatinine ratio, and total protein.

3.2. Relationship between SFAs intake and BMD

Table 2 displays the weighted multiple linear regression model. The result showed that total intake of SFAs was positively linked with total BMD ($p < 0.001$). When the quartile of total SFAs intake was constructed, the lowest quartiles was used as a reference, the trend analysis was statistically significant (p for trend = 0.002), and the 4th quartile was significantly positively associated with BMD ($p < 0.01$). After controlling for all confounding variables, the association between total SFAs consumption and BMD was positive and statistically significant in subgroups stratified by age. However, in subgroups stratified by gender, total SFAs intake was statistically positively linked with BMD in male individuals but not in female subjects. In subgroups stratified by race, there was a positive correlation between total BMD and total saturated fatty acids intake among whites, blacks, and other race. These outcomes reach statistical significance. As shown in Figures 2A, B, we found no saturation effect between SFAs and BMD when we performed smooth curve fitting on the revised model.

TABLE 1 Weighted characteristics of the study sample.

Quartiles of total bone mineral density (g/cm^2)	Lowest quartiles	2nd	3rd	4th	*p*-value
Age (years)	42.52 ± 12.55	38.99 ± 11.91	38.72 ± 11.36	39.18 ± 11.29	< 0.001
Gender (%)					< 0.001
Male	29.24	41.4	54.65	70.81	
Female	70.76	58.6	45.35	29.19	
Race/ethnicity (%)					< 0.001
Mexican American	11.87	10.7	11.85	7.03	
Other Hispanic	8.59	7.55	6.82	5.61	
Non-Hispanic White	62.04	63.35	61.64	57.4	
Non-Hispanic Black	5.64	7.83	10.75	21.65	
Other race - including Multi-race	11.86	10.56	8.95	8.3	
Educational level, n (%)					< 0.001
Less than high school	13.64	12.56	12.17	9.32	
High school	22.49	21	24.05	21.04	
More than high school	63.86	66.44	63.78	69.64	
The ratio of family income to poverty (%)	2.84 ± 1.63	2.90 ± 1.65	2.90 ± 1.59	3.06 ± 1.63	< 0.001
Moderate activities (%)					< 0.001
No	31.67	31.38	29.02	25.26	
Yes	68.33	68.62	70.98	74.74	
Body mass index (kg/m^2)	27.45 ± 6.56	28.93 ± 6.98	29.14 ± 6.73	30.06 ± 6.50	< 0.001
Alkaline phosphatase (u/L)	71.33 ± 24.16	67.49 ± 22.91	65.52 ± 20.94	64.10 ± 19.62	< 0.001
Serum calcium (mmol/L)	2.35 ± 0.09	2.34 ± 0.08	2.34 ± 0.08	2.35 ± 0.08	< 0.001
Serum phosphorus (mmol/L)	1.22 ± 0.17	1.21 ± 0.18	1.21 ± 0.18	1.20 ± 0.19	0.068
Serum uric acid (umol/L)	294.10 ± 74.32	310.97 ± 75.77	320.33 ± 80.05	340.34 ± 80.47	< 0.001
Total cholesterol (mmol/L)	5.12 ± 1.02	5.00 ± 1.03	4.95 ± 1.03	4.88 ± 1.04	< 0.001
Triglyceride (mmol/L)	1.70 ± 1.20	1.70 ± 1.31	1.74 ± 1.94	1.75 ± 1.71	0.631
Glycohemoglobin (%)	5.52 ± 0.72	5.50 ± 0.84	5.51 ± 0.86	5.56 ± 1.03	0.081
Blood urea nitrogen (mmol/L)	4.43 ± 1.51	4.49 ± 1.44	4.60 ± 1.46	4.77 ± 1.58	< 0.001
Serum creatinine (umol/L)	70.10 ± 25.41	72.86 ± 20.14	77.17 ± 27.79	83.04 ± 37.56	< 0.001
Urinary albumin creatinine ratio (mg/g)	24.88 ± 138.39	18.71 ± 89.00	24.15 ± 226.05	22.54 ± 179.02	0.61
Total protein (g/L)	71.40 ± 4.25	71.51 ± 4.40	71.60 ± 4.36	71.43 ± 4.21	0.385
Vitamin D intake (mcg/d)	8.54 ± 20.59	9.60 ± 22.41	10.98 ± 25.15	12.49 ± 27.36	< 0.001
Alcohol intake (g/d)	4.18 ± 4.19	4.40 ± 4.78	4.46 ± 4.43	4.89 ± 4.60	< 0.001
Total saturated fatty acids intake (g/d)	24.52 ± 11.67	26.20 ± 13.81	27.36 ± 14.05	29.73 ± 14.31	< 0.001
Total monounsaturated fatty acids intake (g/d)	26.44 ± 12.61	28.29 ± 13.98	29.32 ± 14.62	32.44 ± 15.46	< 0.001
Total Polyunsaturated fatty acids intake (g/d)	17.93 ± 9.66	19.11 ± 10.03	19.43 ± 9.97	21.60 ± 11.30	< 0.001

Continuous variables are presented as Mean ± SD, *p*-value was calculated by a weighted linear regression model. Categorical variables are presented as %, *p*-value was calculated by chi-square test.

3.3. Relationship between MUFAs intake and BMD

As shown in Table 2, we found a significant positive connection between total MUFAs intake and BMD ($p < 0.001$) using the revised model. When quartiles of total MUFAs were quantified, the 2nd quartile was negatively connected with BMD ($p < 0.05$). In contrast, the 4th quartile was positively correlated with BMD ($p < 0.01$), and the trend analysis was statistically significant (p for trend = 0.006). There was a statistically significant correlation between total MUFAs intake and BMD across age groups in subgroups stratified by age and gender. BMD was linked to total MUFA intake in whites, blacks, and people of other race, these associations are statistically significant.

TABLE 2 Association between fatty acids intake (g/d) and total bone mineral density (g/cm^2).

Exposure	Total saturated fatty acids intake (g/d) (0.6000–190.0570g/d)	Total monounsaturated fatty acids intake (g/d) (0.6745–149.8885g/d)	Total polyunsaturated fatty acids intake (g/d) (0.2825–143.5885g/d)
	β, 95%CI, *p*-value	*β*, 95%CI, *p*-value	*β*, 95%CI, *p*-value
	0.0004 (0.0002, 0.0005)***	0.0003 (0.0002, 0.0005)***	0.0004 (0.0002, 0.0006)***
Quartiles of exposure			
Lowest quartiles	Reference	Reference	Reference
2nd	0.0036 (−0.0023, 0.0094)	−0.0064 (−0.0121, −0.0006)*	0.0034 (−0.0024, 0.0092)
3rd	0.0046 (−0.0014, 0.0105)	−0.0045 (−0.0103, 0.0013)	0.0060 (0.0002, 0.0118)*
4th	0.0101 (0.0039, 0.0163) **	0.0082 (0.0021, 0.0143)**	0.0089 (0.0029, 0.0148)**
p for trend	0.002	0.006	0.004
Stratified by age			
20–39 years old	0.0003 (0.0000, 0.0005)*	0.0003 (0.0001, 0.0005)**	0.0003 (0.0001, 0.0005)***
40–59 years old	0.0005 (0.0002, 0.0007)***	0.0003 (0.0001, 0.0005)*	0.0003 (0.0001, 0.0005)*
Stratified by gender			
Male	0.0004 (0.0002, 0.0006)***	0.0003 (0.0001, 0.0005)**	0.0004 (0.0001, 0.0006)**
Female	0.0002 (−0.0001, 0.0005)	0.0003 (0.0000, 0.0005)*	0.0004 (0.0001, 0.0007)**
Stratified by race			
Mexican American	0.0001 (−0.0003, 0.0004)	0.0000 (−0.0004, 0.0004)	0.0001 (−0.0004, 0.0006)
Other Hispanic	0.0000 (−0.0004, 0.0005)	−0.0000 (−0.0005, 0.0004)	0.0003 (−0.0003, 0.0009)
Non-Hispanic White	0.0003 (0.0000, 0.0006)*	0.0003 (0.0000, 0.0005)*	0.0003 (−0.0001, 0.0006)
Non-Hispanic Black	0.0006 (0.0003, 0.0010)**	0.0005 (0.0002, 0.0009)**	0.0005 (0.0000, 0.0010) *
Other race	0.0009 (0.0005, 0.0013)***	0.0006 (0.0003, 0.0010) ***	0.0013 (0.0008, 0.0017) ***

All confounding factors (age, gender, race, education level, the ratio of family income to poverty, moderate activity, body mass index, alkaline phosphatase, serum calcium, serum uric acid, total cholesterol, blood urea nitrogen, serum phosphorus, triglyceride, glycohemoglobin, urinary albumin creatinine ratio, total protein, serum creatinine, vitamin D intake, alcohol intake, total saturated fatty acids, total monounsaturated fatty acids, and total polyunsaturated fatty acids) were adjusted.
The model is not adjusted for the stratification variable itself in the subgroup analysis.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

We found a turning point between total MUFAs intake and BMD using a smooth curve fit inside a model that controlled all covariates Figures 2C, D. According to the saturation effect analysis model, the effect value for total MUFAs consumption was 20.52 g/d (Table 3). Taken together, the connection between MUFAs intake and total BMD showed an inverted U-shaped curve.

3.4. Relationship between PUFAs intake and BMD

In the fully adjusted model, total PUFAs intake was also found to be positively associated with BMD ($p < 0.001$). When total PUFAs intake was analyzed by quartile, fatty acids intake was positively associated with total BMD in the 3rd group ($p < 0.01$) and the 4th group ($p < 0.01$), and the trend analysis was statistically significant (p for trend = 0.004) (Table 2). In subgroups stratified by age and gender, the positive association between total PUFAs intake and total BMD remained statistically significant. In subgroups stratified by race, we observed this positive association only in blacks and other genders. These outcomes possess statistical significance. As shown in Figures 2E, F, we found no saturation effect between PUFAs and BMD when we performed smooth curve fitting on the revised model.

4. Discussion

We analyzed the association of fatty acid intake with BMD using data on adults aged 20–59 years in the NHANES from 2011 to 2018. Three classes of fatty acids (SFAs, PUFAs, and MUFAs) were favorably linked with BMD in this cross-sectional study of 8,942 people. In this study, we analyzed fatty acid intake by quartile and found that higher fatty acid intake was associated with better bone health within a certain range of fatty acid intake. Furthermore, saturation effect model analysis and smooth curve fitting showed that total MUFAs had an inverted U-shaped relationship with BMD, with a turning point of 20.52 g/d, while other fatty acids had a linear relationship with BMD once confounding factors were taken into account. When total MUFAs were higher than 20.52 g/d, there was a beneficial association between MUFAs intake and BMD ($p < 0.0001$). Study (22) has shown that MUFAs activate peroxisome proliferators receptor-β/δ, regulating the RANKL signaling pathway, and inhibiting osteoclast formation.

Currently, PUFAs have received the most attention in bone health investigations, but the results are controversial. In the ORENTRA experiment, Jørgensen et al. (23) supplied omega-3 fatty acids to kidney transplant recipients and olive oil to the control group. After 44 weeks of treatment, omega-3 fatty acids had no significant effect on BMD. The researchers believe that it may be due to the threshold effect

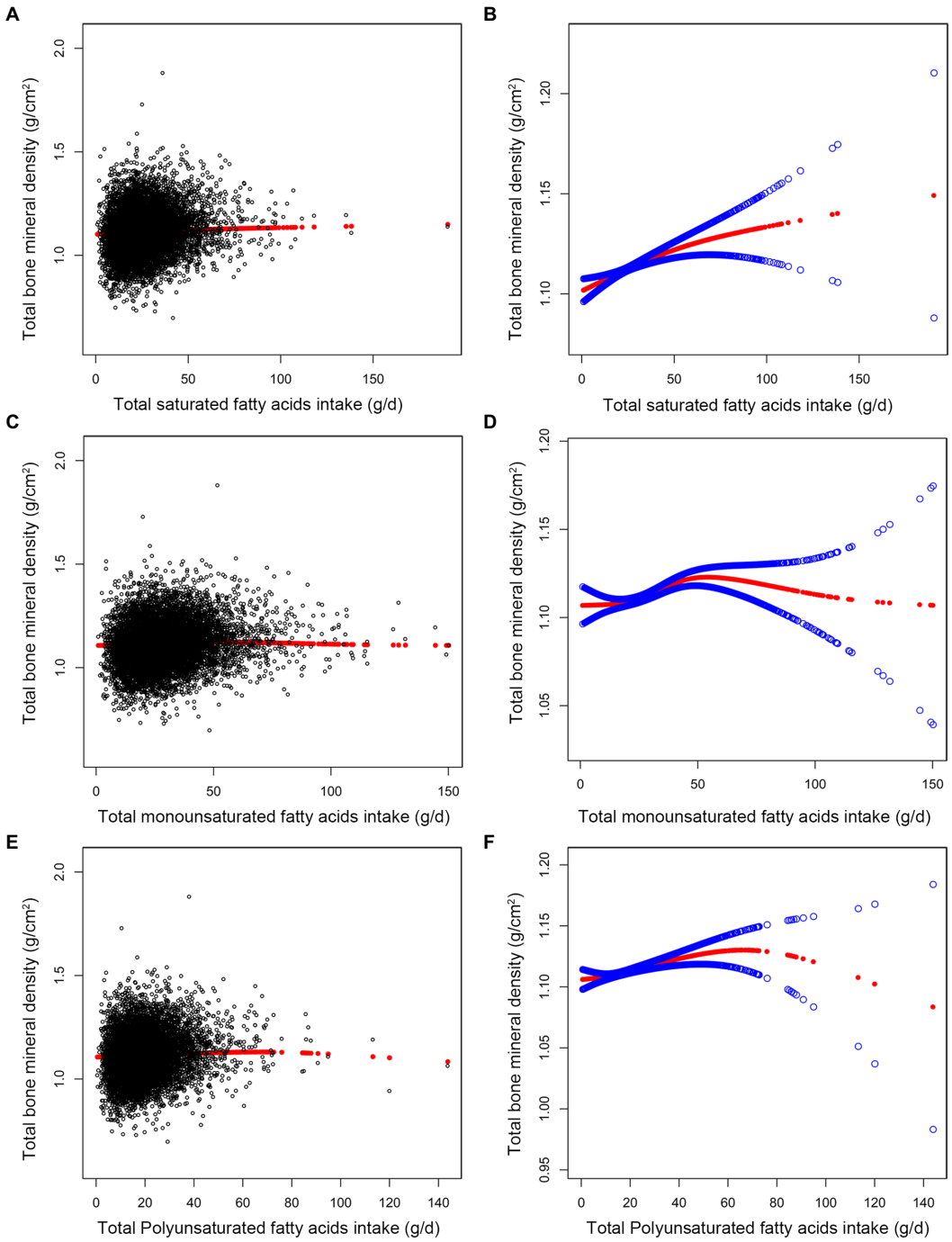

FIGURE 2
Association between fatty acids intake (g/d) and total bone mineral density (g/cm²). **(A,C,E)** Each black point represents a sample. **(B,D,F)** The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit. All confounding factors were adjusted.

of n-3 PUFAs on BMD. In a retrospective study of 275 healthy women from Japan, Kuroda et al. (24). used multiple linear regression analysis to show that omega-3 fatty acid intake contributes to hip BMD. Results from this study showed that the connection between PUFAs intake and BMD was stronger in the 3rd and 4th quartiles of the distribution, but not in the 2nd quartile. We believe that PUFAs intake is positively correlated with BMD only when PUFAs intake reaches a certain threshold, which seems to be consistent with previous research. Notably, a 5-year longitudinal study (25) found that a higher intake of PUFAs and MUFAs was linked to lower BMD in the femoral neck, even after controlling for possible confounding factors, these associations are statistically significant. This contradictory conclusion may be owing to the limited sample size of the population included in this study. Similarly, in animal study (26), BMD was significantly reduced in rats fed an atherogenic diet, and monounsaturated fatty acids ameliorated these changes but remained lower than in controls.

TABLE 3 Saturation effect analysis of fatty acids intake (g/d) and total bone mineral density (g/cm^2).

	Total bone mineral density (g/cm^2)
Turn point of total saturated fatty acids intake (g/d)	*33.67*
< 33.67, effect 1	0.000 (−0.000, 0.000) 0.2454
> 33.67, effect 2	0.001 (0.000, 0.001) 0.0002
The effect difference	0.000 (−0.000, 0.001) 0.1060
Predicted value of the equation at the folding point	1.123 (1.119, 1.127)
Log likelihood ratio test	0.105
Turn point of total monounsaturated fatty acids intake (g/d)	*20.52*
< 20.52, effect 1	−0.001 (−0.001, 0.000) 0.0546
>20.52, effect 2	0.000 (0.000, 0.001) <0.0001
The effect difference	0.001 (0.000, 0.002) 0.0036
Predicted value of the equation at the folding point	1.101 (1.098, 1.105)
Log likelihood ratio test	0.004
Turn point of total polyunsaturated fatty acids intake (g/d)	*6.23*
<6.23, effect 1	−0.003 (−0.008, 0.002) 0.2212
>6.23, effect 2	0.000 (0.000, 0.001) <0.0001
The effect difference	0.003 (−0.001, 0.008) 0.1644
Predicted value of the equation at the folding point	1.093 (1.090, 1.097)
Log likelihood ratio test	0.164

All confounding factors were adjusted.

Little research has been conducted on the correlation between saturated fatty acid consumption and BMD to date, and there has been a dearth of large-scale investigations into the topic. Because of this, we conducted this extensive retrospective study and found that consuming saturated fatty acids actually improves BMD.

In addition to the regulation of bone metabolism, fatty acids have other biological effects, such as omega-3 PUFAs to protect cardiovascular (27) and nerve (28), anti-tumor (29), possibly by inhibiting inflammation, reducing oxidative stress, regulating cell apoptosis and other mechanisms. A meta-analysis with 23 literature showed that when SFAs intake >17 g/d, there will be a clear protective effect of type 2 diabetes (30). Data from the literature indicates that diabetes is a high risk factor for osteoporosis (31). According to a number of studies, those who suffer from diabetes have lower bone mineral density than those who do not suffer from the condition (32, 33). In addition, the ratio of PUFAs (e.g., omega-6, omega-3) also affects metabolism. Studies have shown that a high intake of omega-6 fatty acids or a high omega-6/omega-3 nutrient ratio is linked to an increased risk of obesity. Obesity is also a protective factor for bone density, which partially explain why PUFAs are good for BMD (34, 35). In addition, BMD has been shown to be higher in those with adequate fat intake than in those with insufficient fat intake (36).

We performed weighted multiple linear regression analysis and smooth curve fitting analysis with data from 8,942 participants and found that fatty acid intake in adults aged 20–59 was beneficial to bone mineral density, which is also associated with osteoporosis. Prevention provides dietary guidance. However, our study also has some flaws. Because this is a cross-sectional study, it cannot show that the association between fatty acid consumption and BMD is caused by one or the other. More prospective clinical studies and basic research are needed to back up these results. According to our findings, the positive correlation between total BMD and MUFAs intake occurs only when the MUFAs intake is >20.52 g/d. We have studied a sizable amount of literature. However, to our knowledge, the saturation effect and threshold between MUFAs and BMD are not supported by any pertinent data. The exact mechanism is yet unknown, and more studies are needed to confirm it. There is no literature on the saturation effect between SFAs, PUFAs, and BMD. Therefore, in the future, we suggest carrying out a larger prospective study on SFAs, PUFAs, and BMD to further understand the causal relationship between fatty acids and BMD. Since there are only total SFAs, PUFAs, and MUFAs intakes in the NHANES database, but no specific fatty acid intakes, such as the specific intakes of n-3 and n-6 PUFAs, we suggest that future studies should focus on the association between specific fatty acids and BMD.

In conclusion, SFAs, MUFAs, and PUFAs intake were positively associated with BMD, and the associations persisted in subgroups stratified by age, gender, and race in this study. Notably, when fatty acid intake was quartiled, MUFAs in the 2nd quartile were negatively correlated with BMD and those in the 4th quartile were positively correlated. Meanwhile, this research found that MUFAs were positively correlated with BMD, but there was a threshold. Therefore, according to our findings, it is recommended that adults consume moderate amounts of fatty acids to ensure adequate bone mass but not metabolic diseases.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhanes/.

Ethics statement

All survey participants were informed of the poll's specifics and signed an informed consent form. The National Center for Health Statistics Ethics Review Board assessed and authorized the informed consent. Following the completion of official anonymization, all of the data is then made available to the public in order to make the most effective use of these resources. Anyone may access these statistics as long as they adhere to the NHENAS database regulations and are used exclusively for statistical analysis. All studies based on these data should adhere to applicable laws and legislation. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Z-BF and G-XW contributed equally to this study and made contributions to data collection, curation, statistical analysis, and

manuscript writing and revision. G-ZC and P-XZ contributed to the statistical analysis. D-LL, S-FC, and H-XZ supervised the study and contributed to the polishing and reviewing of the manuscript. S-FC provided financial assistance for this research. H-LL supervised, wrote the review, and edited this study. All authors contributed to the article and approved the submitted version.

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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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A discriminant analysis of plasma metabolomics for the assessment of metabolic responsiveness to red raspberry consumption

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Background: Many studies show that the intake of raspberries is beneficial to immune-metabolic health, but the responses of individuals are heterogeneous and not fully understood.

Methods: In a two-arm parallel-group, randomized, controlled trial, immune-metabolic outcomes and plasma metabolite levels were analyzed before and after an 8-week red raspberry consumption. Based on partial least squares discriminant analysis (PLS-DA) on plasma xenobiotic levels, adherence to the intervention was first evaluated. A second PLS-DA followed by hierarchical clustering was used to classify individuals into response subgroups. Clinical immune and metabolic outcomes, including insulin resistance (HOMA-IR) and sensitivity (Matsuda, QUICKI) indices, during the intervention were assessed and compared between response subgroups.

Results: Two subgroups of participants, type 1 responders (n=17) and type 2 responders (n=5), were identified based on plasma metabolite levels measured during the intervention. Type 1 responders showed neutral to negative effects on immune-metabolic clinical parameters after raspberry consumption, and type 2 responders showed positive effects on the same parameters. Changes in waist circumference, waist-to-hip ratio, fasting plasma apolipoprotein B, C-reactive protein and insulin levels as well as Matsuda, HOMA-IR and QUICKI were significantly different between the two response subgroups. A deleterious effect of two carotenoid metabolites was also observed in type 1 responders but these variables were significantly associated with beneficial changes in the QUICKI index and in fasting insulin levels in type 2 responders. Increased 3-ureidopropionate levels were associated with a decrease in the Matsuda index in type 2 responders, suggesting that this metabolite is associated with a decrease in insulin sensitivity for those subjects, whereas the opposite was observed for type 1 responders.

Conclusion: The beneficial effects associated with red raspberry consumption are subject to inter-individual variability. Metabolomics-based clustering appears to be an effective way to assess adherence to a nutritional intervention and to classify individuals according to their immune-metabolic responsiveness to the intervention. This approach may be replicated in future studies to provide a better

understanding of how interindividual variability impacts the effects of nutritional interventions on immune-metabolic health.

KEYWORDS

raspberry, clustering, machine learning, metabolic health, metabolomics, precision nutrition

1. Introduction

It has been shown that obesity and metabolic syndrome increase type 2 diabetes (T2D) incidence and cardiovascular disease morbidity and mortality rates (1). With both environmental and biological factors affecting the risk of an individual to develop T2D (2), the beneficial effects of plant-based diets on metabolic health have been previously highlighted (3). The consumption of fruits, and in particular berries, has been associated with beneficial health effects, especially in the prevention of metabolic disturbances (4). Berries have been consumed since the roman empire and were used to treat diseases in medieval Europe (5).

These fruits are natural source of dietary fiber and many other nutrients and phytochemicals with beneficial health properties. Berries are rich in numerous polyphenols, classified as flavonoids and non-flavonoids, which have favorable effects on obesity, hypertension, dyslipidemia and hyperglycemia, at least in part through their potential antioxidant and anti-inflammatory properties (6). In particular, the polyphenolic content and antioxidant activity of raspberries are ranked among the highest of commonly consumed fruits (7). Moreover, studies have shown that most consumed berries such as raspberries improve postprandial hyperglycemia and hyperinsulinemia in individuals with overweight or obesity, as well as with metabolic syndrome, suggesting that these fruits may have a beneficial impact on type 2 diabetes prevention and management (8, 9).

The inclusion of metabolomics-based plasma metabolic profiling has allowed the identification of nutritional markers related with intervention adherence and health response (10). The human plasma metabolome contains hundreds of circulating metabolites reflecting the physiology, genetics, environmental exposures and dietary habits of individuals (11). These metabolites include mainly xenobiotics, lipids, amino acids, vitamins and cofactors, and nucleotides. In this regard, while most of past research has demonstrated the beneficial effects of raspberry consumption on health parameters, few studies have focused on analyzing the metabolic response to raspberries through metabolomics. The main goals of the present study were to identify different types of metabolic responses to an 8-week raspberry consumption based on the plasma metabolomics signature of participants and to develop a framework for assessing adherence to a nutritional intervention's guidelines.

2. Materials and methods

2.1. Study design and participants

The study design consisted of a two-arm parallel-group, randomized, controlled trial of the effects of raspberry consumption on the metabolic parameters and plasma metabolome in subjects with metabolic disturbances. The trial, registered as NCT03620617 at clinicaltrials.gov, took place from 2018 to 2019 at the Institute of Nutrition and Functional Foods (INAF) at Université Laval. The written consent was obtained for all participants after the study was approved by the Université Laval Ethics Committee (CER-Université Laval 2017-218). Study participants were men or pre-menopausal women aged between 18 to 60 years old, with a body mass index (BMI) ranging from 25 to 40 kg/m^2 or a waist circumference greater or equal to 94 cm for men and 80 cm for women. After eligibility was confirmed and a 2-week run-in-period, subjects were randomly instructed to consume 280 g of frozen red raspberries per day ($n = 24$) or to maintain their usual diet ($n = 25$) for 8 weeks. Nutritional and clinical data of participants were collected from food frequency questionnaires (FFQ), medical questionnaires and physical examinations (12). Blood samples were taken before (week 0) and after the 8 weeks (week 8) of raspberry consumption. We have summarized the nutritional composition of raspberries in Supplementary Table 1. All data is representative of two cups of raspberries (4 portions), which participants consumed daily for 8 weeks. Further details on this clinical study are available in (12). For the present study, data from the 24 subjects of the group consuming raspberries were used. In addition to the clinical variables available in the clinical study (12), the quantitative insulin sensitivity check index (QUICKI) was computed for all participants using $1/[\log_{10}(\text{fasting insulin}) + \log_{10}(\text{fasting glucose})]$ (13). Matsuda index is used to evaluate insulin sensitivity from the data obtained by an oral glucose tolerance test (14). Homeostatic model assessment for insulin resistance (HOMA-IR) is calculated from fasting glucose and fasting insulin levels and is an index widely used to assess insulin resistance in individuals (15).

2.2. Plasma metabolome profiling

Targeted metabolomics using ultra-performance liquid chromatography–tandem mass spectrometry on the Metabolon DiscoveryHD4® platform (Morrisville, NC, United States) were performed on fasted plasma samples of the 24 participants of the raspberry group collected before (week 0) and after (week 8) the raspberry consumption (16). The dataset of metabolites consisted of a total of 1,132 biochemicals which included lipids, amino acids, xenobiotics, cofactors and vitamins, nucleotides, carbohydrates and peptides. Data were normalized by dividing the raw values in the experimental batch by the median of those samples in each instrument batch, giving each batch and thus the metabolite a median of one. After batch normalization, data were further imputed by replacing missing values for a given metabolite with its observed minimum. This was done to avoid inflating the false negative rate and weaken the statistical power of the analyses. Normalized and imputed data were then

transformed using natural log and filtered based on inter-individual variance. Metabolites presenting no variance ($n = 14$) or low variance (< 0.1; $n = 272$) were excluded from further analyses. Data were further filtered to remove unknown compounds (216 unnamed biochemicals).

2.3. Xenobiotics and adherence to the nutritional intervention

Metabolites in the Metabolon dataset classified as "xenobiotics" were used herein as metabolites reflecting the adherence of participants to the nutritional intervention. Partial least squares discriminant analysis (PLS-DA) is a supervised classification algorithm reducing the dimensionality of the data to analyze the covariance between categorical dependent variables and a very large number of independent variables. A first sparse PLS-DA (sPLS-DA) was done to confirm adherence to the nutritional intervention by discriminating trial visits, before and after raspberry consumption, using only xenobiotics ($n = 120$; Supplementary Figure 1). To identify participants with a low adherence to the protocol, we performed a second step based on the prediction of the intervention timepoints. For training and testing data, the initial dataset was split in two equal sets containing the same number of samples and equal proportion of men and women. A trained sPLS-DA model was used to predict the intervention timepoint (pre-or post-raspberry consumption) of plasma samples. This was first done while including participants whose adherence was considered as low, and then after excluding them. It served the purpose of confirming whether their exclusion from the dataset was justified by examining prediction performance statistics of the model and the model's error rate. PLS-DAs, sPLS-DAs and classification performance evaluation of the models were computed using the mixOmics R package (v6.20.0) (17).

2.4. Clustering

Metabolites for clustering analysis were filtered by removing xenobiotics ($n = 120$) and partially characterized molecules ($n = 24$). A total of 486 out of the 1,132 initial metabolites reflecting the participants' endogenous response to the intervention were used for this analysis, as shown in Supplementary Figure 1. A PLS-DA was then used in combination with a hierarchical clustering analysis (HCA) to identify clusters of participants with distinct metabolic response to raspberry consumption. The PLS-DA model was instructed to discriminate plasma samples belonging to pre-versus post-raspberry consumption timepoints. In order to identify response subgroups, the two main components resulting from the PLS-DA were used as input data for the HCA, with Euclidean distance and Ward linkage as the main parameters of the model. This was done using pvclust R package (v2.2.0) (18), which calculates approximately unbiased value of ps for all clusters by using multiscale bootstrap resampling ($n = 1{,}000$ replications). An unbiased value of p of 95% or above was considered to robustly support the identified clusters. Finally, a sPLS-DA was performed considering the newly identified clusters of participants. The sPLS-DA was done to strengthen the classification and identify the most discriminating metabolites in each subgroup. The optimal number of metabolites to use was determined during the tuning process, which was run using 10-fold cross validation and 20 repeats. A multilevel approach was used to correctly assess the structure of the data, which includes two timepoints per participant (before and after the intervention). The stability of selected metabolites within each component was computed as the proportion of folds where the loading was used to assess a given component during cross validation. The model's performance was then evaluated using the built-in tools to estimate the classification error rate.

2.5. Statistical analyses

A two-tailed unpaired t-test was first used to compare baseline characteristics between subgroups at week 0. We then explored the metabolic homogeneity of participants within each subgroup and the heterogeneity between subgroups, and assessed the physiological relevance of the metabolomics-based raspberry responsiveness classification. To do that, a linear mixed model using the nlme (v3.1.157) (19) and emmeans (v1.7.5) (20) R packages was used to compare the changes in metabolic parameters in response to raspberry consumption between subgroups. This model was used to test the effects of group, timepoint and their interaction considering the effects of age and sex. A second linear mixed model was used to test the association between plasma metabolite levels and clinical data at weeks 0 and 8. The 10 most discriminant metabolites of components 1 and 2 and the clinical variables for which the differences between subgroups were significant were used in this model. When significant interactions at $p \leq 0.05$ between metabolites and clinical variables were observed, a contrast analysis was performed to test for differences between groups.

3. Results

3.1. Adherence confirmation by xenobiotic-based PLS-DA

Xenobiotics found in the metabolome of participants were useful for identifying participants with a low adherence to the study protocol. Based on the levels of certain xenobiotics, the sPLS-DA revealed two potential non-adherent participants (Supplementary Figure 2A). The most discriminant xenobiotics were methyl glucopyranoside, 4-acetylphenyl sulfate and dihydrocaffeate sulfate (Supplementary Figure 2B). To confirm the outlier status of these two participants, we predicted the intervention timepoint. When removing these two participants, we achieved a prediction accuracy of 100%. By including these two participants, the accuracy decreased to 75%, and both subjects were systematically misclassified. For this reason, these two participants were removed from all further analyses, leaving a total of 22 study participants.

3.2. Hierarchical clustering of response subgroups to the raspberry consumption

The first two components of the PLS-DA aimed at discriminating between intervention timepoints with endogenous metabolites accounting for, respectively, 16 and 6% of the variance (Figure 1A). The HCA on the two latent variables derived from the PLS-DA revealed subgroups of matched participants with homogenous and well-discriminated metabolomic profiles at pre- and post-intervention visits, with approximately unbiased *p*-values greater than 95% (Figure 1B).

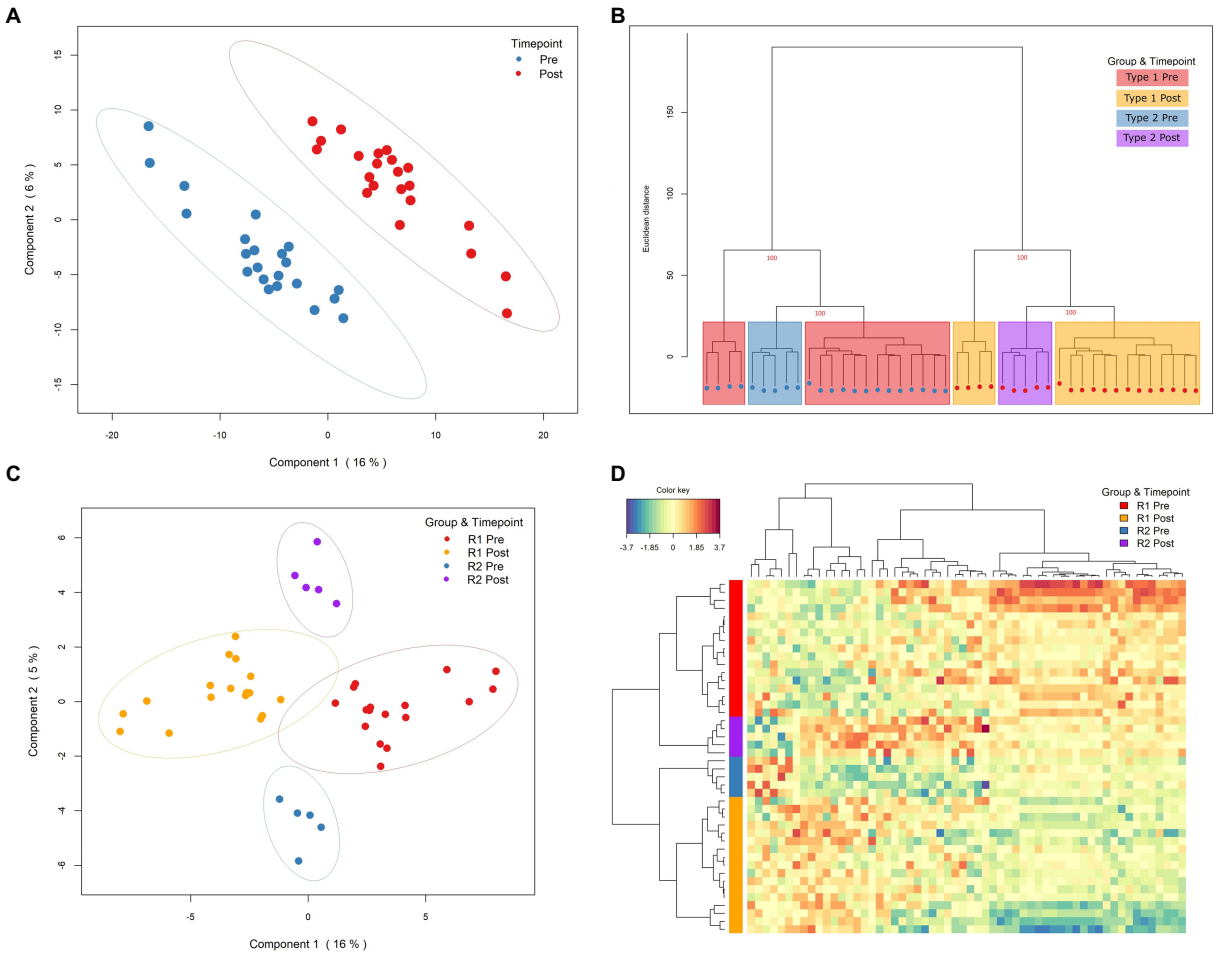

FIGURE 1
Main steps of the metabolomic-based clustering procedure. Panel **(A)** shows participants spanned by the two main components derived from partial least squares discriminant analysis (PLS-DA) grouped by timepoint (pre- and post-intervention, respectively in blue and red). Each ellipse represents the 95% confidence interval for each timepoint group. Panel **(B)** shows the four clusters of participants identified from hierarchical clustering analysis (HCA). Red and orange squares regroup type 1 responders at pre- and post-intervention timepoints. Blue and purple squares represent type 2 responders at pre-and post-intervention timepoints. Numbers in red represent the approximately unbiased *p*-values of each cluster. Panel **(C)** shows participants spanned by the two main components derived from sparse PLS-DA portraying the two distinct response subgroups identified from HCA. R1 pre-and R1 post-intervention subgroups are colored in red and orange, respectively. R2 pre- and R2 post-intervention subgroups are colored in blue and purple, respectively. **(D)** Heatmap illustrating the classification of participants based on the most discriminating metabolites derived from sparse partial least squares discriminant analysis (sPLSDA). The left dendrogram branches in four major nodes, representing the clustering of participants. The upper dendrogram branches in two major nodes, representing the first component with its 30 metabolites on the right, and the second component with its 30 metabolites on the left. The intensity of red color indicates an increase in metabolite levels between pre-and post-intervention timepoints.

Two clusters of participants were discriminated based on component 1 and were considered as type 1 responders ($n = 17$). Another two subgroups of participants were discriminated based on component 2 ($n = 5$) and were considered as type 2 responders (Figure 1B).

A multilevel sPLS-DA model was then built using these two subgroups as an input to determine which metabolites were the most discriminant and to discover the optimal number of metabolites to use in each component (Figure 1C). From this sPLS-DA, component 1 accounted for 16% of variance and was composed of 30 metabolites whereas component 2 accounted for 5% of variance and also included 30 metabolites. A heatmap illustrating these results is shown in Figure 1D. Performance evaluation of the model showed an average classification error rate of around 26% (Supplementary Figure 3A). The stability of the selected metabolites is shown in Supplementary Figure 3B. We observed a high stability for most discriminant metabolites in components 1 and 2. The top 10 metabolites in component 1 all have a stability of 0.90 or higher, while component 2 top 10 metabolites ranged from 0.98 to 0.74. This shows that metabolites in both components are highly discriminative.

3.3. Physiological relevance of clustering

We observed no significant differences between type 1 and type 2 responders for age, sex, body weight, BMI and all other clinical parameters at week 0 (Supplementary Table 2).

Changes in all clinical parameters between weeks 0 and 8 for type 1 and type 2 responders are shown in Table 1 and all the significant

TABLE 1 Changes in anthropometric and metabolic characteristics of type 1 and type 2 responders between week 0 and week 8.

Variable	Type 1 responders		Type 2 responders		*p*-Values		
	Week 0	Week 8	Week 0	Week 8	Group	Visit	Interaction
Weight (kg)	92.6 ± 4.3	93.1 ± 4.3	88.1 ± 7.1	87.5 ± 7.1	0.56	0.51	0.20
BMI (kg/m2)	31.2 ± 1.4	31.4 ± 1.4	29.2 ± 2.4	29.0 ± 2.4	0.45	0.42	0.19
Waist circumference (cm)	102.6 ± 3.3	103.7 ± 3.3	98.7 ± 5.5	96.2 ± 5.5	0.40	0.50	**0.02**
Hip circumference (cm)	113.0 ± 2.8	113.0 ± 2.8	106.8 ± 4.8	106.7 ± 4.8	0.28	0.93	0.88
Waist-Hip ratio	0.91 ± 0.02	0.92 ± 0.02	0.92 ± 0.03	0.90 ± 0.03	0.94	0.52	**0.01**
Systolic blood pressure	116.4 ± 2.4	114.4 ± 2.4	111.2 ± 4.0	110.1 ± 4.0	0.31	0.06	0.71
Diastolic blood pressure	74.9 ± 2.1	73.8 ± 2.2	68.0 ± 3.7	64.6 ± 3.7	0.06	0.14	0.41
ApoB (g/L)	0.93 ± 0.06	0.94 ± 0.06	1.06 ± 0.11	0.9 ± 0.11	0.72	0.20	**0.003**
Total-Cholesterol (mmol/L)	4.54 ± 0.24	4.54 ± 0.24	5.11 ± 0.41	4.78 ± 0.41	0.40	0.54	0.19
TG (mmol/L)	1.61 ± 0.18	1.46 ± 0.19	1.44 ± 0.32	1.19 ± 0.32	0.52	0.24	0.78
HDL-C (mmol/L)	1.18 ± 0.08	1.18 ± 0.08	1.3 ± 0.13	1.27 ± 0.13	0.49	0.80	0.85
LDL-C (mmol/L)	2.61 ± 0.22	2.7 ± 0.22	3.15 ± 0.38	2.96 ± 0.38	0.37	0.79	0.20
Cholesterol HDL-C	4.39 ± 0.33	4.28 ± 0.33	4.06 ± 0.56	3.86 ± 0.56	0.56	0.40	0.79
HbA1C	5.10 ± 0.10	5.10 ± 0.10	5.00 ± 0.10	5.10 ± 0.10	0.66	0.25	0.79
CRP (mg/L)	2.73 ± 0.86	4.2 ± 0.87	2.92 ± 1.46	1.83 ± 1.46	0.53	**0.05**	**0.02**
Fasting glucose (mmol/L)	4.86 ± 0.13	4.78 ± 0.13	4.76 ± 0.21	4.81 ± 0.21	0.88	0.32	0.30
Fasting Insulin (pmol/L)	97.1 ± 10.5	97.4 ± 10.5	87.2 ± 17.6	67.7 ± 17.6	0.35	0.22	**0.02**
Matsuda	4.84 ± 0.56	4.15 ± 0.56	3.59 ± 0.73	5.18 ± 0.73	0.91	0.63	**0.003**
HOMA-IR	2.97 ± 0.58	3.06 ± 0.58	2.96 ± 0.76	2.36 ± 0.76	0.72	0.26	**0.03**
QUICKI	0.33 ± 0.01	0.33 ± 0.01	0.33 ± 0.01	0.34 ± 0.01	0.49	0.41	**0.02**

Data are means ± standard deviation. Type 1 responders ($n = 17$) and type 2 responders ($n = 5$). A linear mixed model adjusted for age, sex, and baseline values was used. BMI, body mass index; ApoB, Apolipoprotein B; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1C, glycated hemoglobin; CRP, C-reactive protein.

visit-by-group interactions are shown in Figure 2. As compared to type 1 responders, type 2 responders showed a significant decrease in waist circumference (p for group x visit interaction, $p_i = 0.02$; Figure 2A), waist-to-hip ratio ($p_i = 0.01$; Figure 2B), plasma apolipoprotein B (ApoB; $p_i = 0.003$; Figure 2C), C-reactive protein (CRP; $p_i = 0.02$; Figure 2D), fasting insulin levels ($p_i = 0.02$; Figure 2E), and HOMA-IR ($p_i = 0.03$; Figure 2F) and a significant increase in the QUICKI index ($p_i = 0.02$; Figure 2G) and Matsuda ($p_i = 0.003$; Figure 2H).For most clinical parameters, we observed the opposite effect in type 1 responders, with fasting insulin, waist-to-hip ratio and plasma CRP levels being higher than baseline after the intervention, while QUICKI and Matsuda indices were lower. Waist circumference, HOMA-IR and ApoB levels remained stable or showed a slight increase after the intervention for type 1 responders.

3.4. Most discriminant metabolites between response subgroups

We sorted discriminant metabolites obtained through the sPLS-DA based on their loading weight, component by component. The top 10 metabolites of each component are shown in Figure 3. Type 1 responders showed higher average changes on component 1 metabolites than non-responders (Figure 3B). The top 10 metabolites of component 2 are shown in Figure 3C and Figure 3D. Briefly, type 1 responders timepoints were differentiated by metabolites of component 1, and type 2 responders timepoints were differentiated by metabolites by component 2.

Significant associations were observed between changes in clinical parameters and changes in plasma metabolite levels from both components (Figure 4). The change in fasting insulin and QUICKI index according to the change in carotene diol 1 was significantly different between type 1 and type 2 responders ($p = 0.04$ and $p = 0.02$, respectively). Concretely, we observed a positive association between the increase in carotene diol 1 and fasting insulin levels, and a negative association with the QUICKI index in type 1 responders, while the opposite was observed for type 2 responders, i.e., a decrease in fasting insulin and an increase in the QUICKI index were associated with an increase in carotene diol 1 levels (Figures 4A,B). On the other hand, we found that the change in the Matsuda index according to the change in 3-ureidopropionate was significantly different between type 1 and type 2 responders ($p = 0.05$). A negative association between the increase in the metabolite and the Matsuda index was seen for type 2 responders whereas a positive correlation was observed for type 1 responders (Figures 4C,D).

4. Discussion

The most significant finding of this study is that, using a machine learning approach, changes in the levels of plasma metabolites may be used to assess the metabolic responsiveness to raspberry

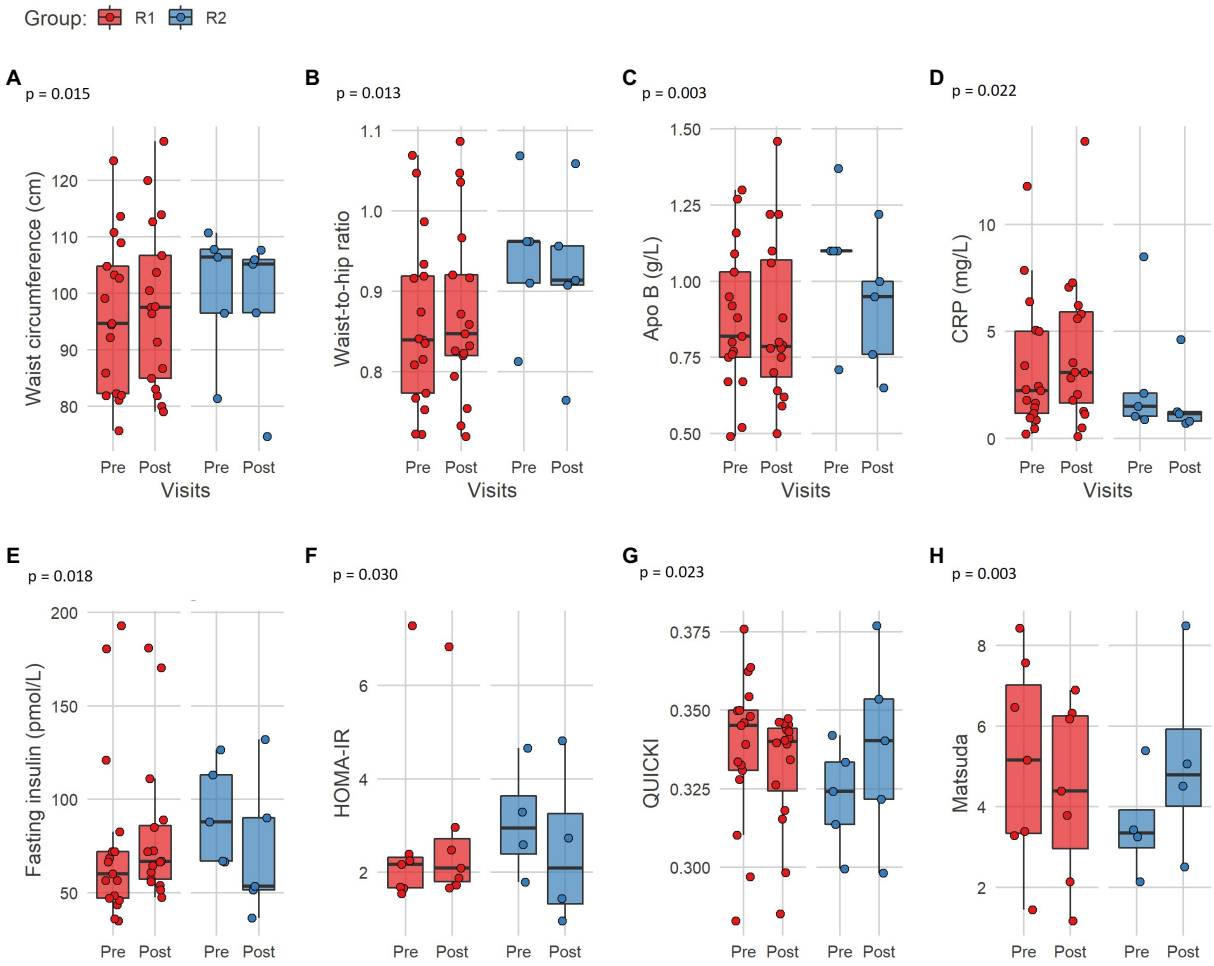

FIGURE 2
Metabolic differences between pre- and post-intervention by response subgroup. Panels **(A) – (H)** show all significant differences in metabolic parameters between pre- and post-intervention timepoints for type 1 responders (red) and type 2 responders (blue) derived from a linear mixed model. *p* Values shown above each panel represent the *p* for group × visit interaction. Differences accounted for age and sex and resulted from the interaction between the effects of group and timepoint. Each point represents a participant. The mean is represented by the horizontal line, and the standard error is represented by the vertical lines. QUICKI, Matsuda, HOMA-IR and waist-to-hip ratio have no unit.

consumption. Concretely, following the classification of study participants into two distinct response subgroups based on the levels of plasma metabolites measured before and after the 8-week raspberry consumption, it is interesting to note significant differences in metabolic health features between the two distinct response subgroups, supporting our clustering approach. In this regard, while positive metabolic responses to the raspberry consumption are already well known (21–24), the results of the present study suggest that a clustering approach of plasma metabolomic data may contribute to explain the interindividual variability observed in metabolic responsiveness to red raspberry consumption.

The use of a metabolomics-based approach for clustering was also particularly useful to assess the adherence to the nutritional intervention. The consumption of xenobiotics present in raspberries led to an increase in such metabolites in the plasma of study participants (25, 26), which were used as raspberry intake markers to identify participants who had a low adherence to the study protocol. The classification algorithm was then trained to predict if a given sample belonged to pre-or post-intervention timepoints and served the purpose of justifying the exclusion of participants suspected of low adherence. This metabolomics-based approach has been used to monitor dietary intake and adherence to a specific diet in recent studies, suggesting that metabolites could be effective biomarkers of food intake (27–31). The second part of the clustering approach was designed to address previous attempts to classify the metabolic responsiveness to an 8-week raspberry consumption, such as a transcriptomics-based approach (12). The metabolomics-based clustering through PLS-DA and sPLS-DA appeared to be relevant, as we observed a homogeneous response within subgroups of participants, as well as a heterogeneous response between subgroups.

Of all the metabolites analyzed in this study, those belonging to the carotenoid family had the most significant influence on metabolic parameters. A regular intake of raspberries has been reported to have positive effects on many metabolic parameters, including improved glucose, insulin, and lipid metabolism as well as reduced inflammation and oxidation (9, 32, 33). These berries contain many carotenoids (34), which have been linked to positive effects on metabolic health. In the present study, two carotenoids, identified as

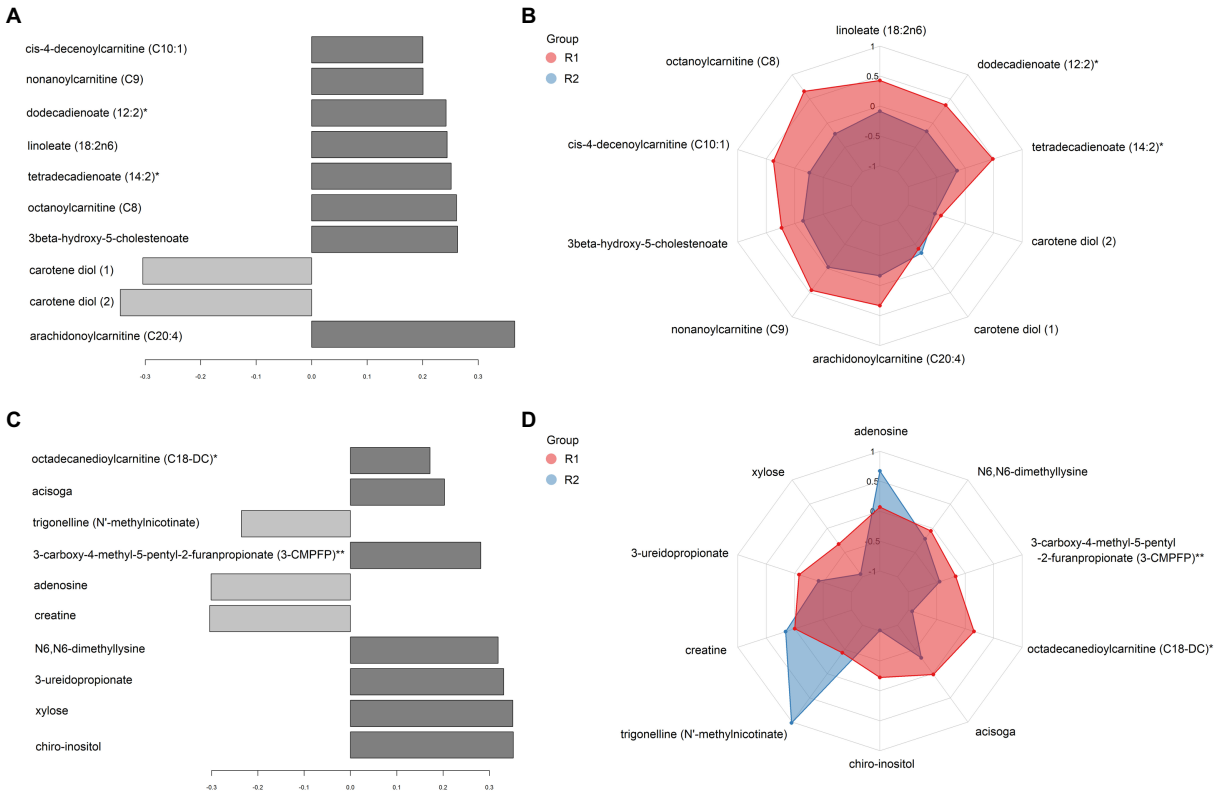

FIGURE 3
Most discriminant metabolites between type 1 and type 2 responder groups. Panels **(A)** and **(C)** show the ten most discriminant metabolites of component 1 and component 2 of the sparse partial least squares discriminant analysis (sPLS-DA) respectively, ordered from bottom to top by highest loading weight in the discrimination in their respective component. Negative loading weights are shown in light gray and positive loading weights are shown in dark gray. Panels **(B)** and **(D)** show the magnitude of change in metabolite levels for the top ten metabolites in component 1 and component 2 for each response subgroup. Changes for type 1 responders are shown in red and changes for type 2 responders are shown in blue.

carotene diol 1 and 2, were among the most discriminant metabolites in the clustering analysis, with carotene diol 1 also being significantly linked to opposite changes in fasting insulin and QUICKI index between type 1 and type 2 responders. Circulating plasma carotenoids have been associated with lower inflammation (35–37), including reduced CRP, which we observed in type 2 responders. However, the effects of carotenoids on insulin resistance and the prevention of type 2 diabetes are dichotomic, with studies showing either an inverse association or no association (37). Some studies have reported positive health outcomes on fasting plasma glucose levels and insulin resistance (38) for beta-carotene and lycopene, respectively, whereas other studies have found no correlation between lutein or lycopene and the prevention of type 2 diabetes, but association of alpha-and beta-carotene with type 2 diabetes risk reduction (39). Similarly, carotenoids have also been associated with beneficial lipid and inflammatory responses (40). Many environmental, dietary, physiologic, structural and genetic factors may influence absorption and bioavailability of carotenoids, ranging from gender to hormonal status, interactions with other nutrients or molecules and smoking status, and can affect an individual's response (41–43). Moreover, previous studies of our team suggest that the heterogeneous association observed between plasma carotenoid concentrations and lipid profiles might be mediated by genetic factors impacting on gene expression and methylation levels (40, 44, 45), which eventually may influence glucose homeostasis differently.

One of the most discriminant metabolites of component 2 in the sPLS-DA was the 3-ureidopropionate, an intermediate in the metabolism of uracil and member of the class of compounds known as ureas (46), and significantly linked to the difference in the Matsuda index between response subgroups. Another propionic derivative which was in the top 10 most discriminant metabolites of component 2 was the 3-carboxy-4-methyl-5-pentyl-2-furanpropionate (3-CMPFP). Propionate is a product of colonic fermentation of dietary fibers (47), which inhibits glucose-induced insulin secretion and glucose decarboxylation in rat pancreatic cells (48). More recent studies have confirmed that propionate improves beta-cell function in humans (49) and improved insulin sensitivity (50), which can be linked to the well documented health effects of dietary fibers on glucose homeostasis (51, 52). Discrepancies reported in the literature around the impact of raspberry consumption on metabolic health are reflected in the present study. Accordingly, most of the significant effects observed in type 2 responders were generally beneficial on metabolic health. However, the increase in 3-ureidopropionate during raspberry consumption had opposing effects. Concretely, increasing levels of 3-ureidopropionate were associated with an increase in the Matsuda index in type 1 responders, and therefore with an improvement in insulin sensitivity. In contrast, type 2 responders sustained a decrease in Matsuda index values with increasing 3-ureidopropionate levels, and a deterioration of their insulin sensitivity. However, overall insulin sensitivity of type 2 responders

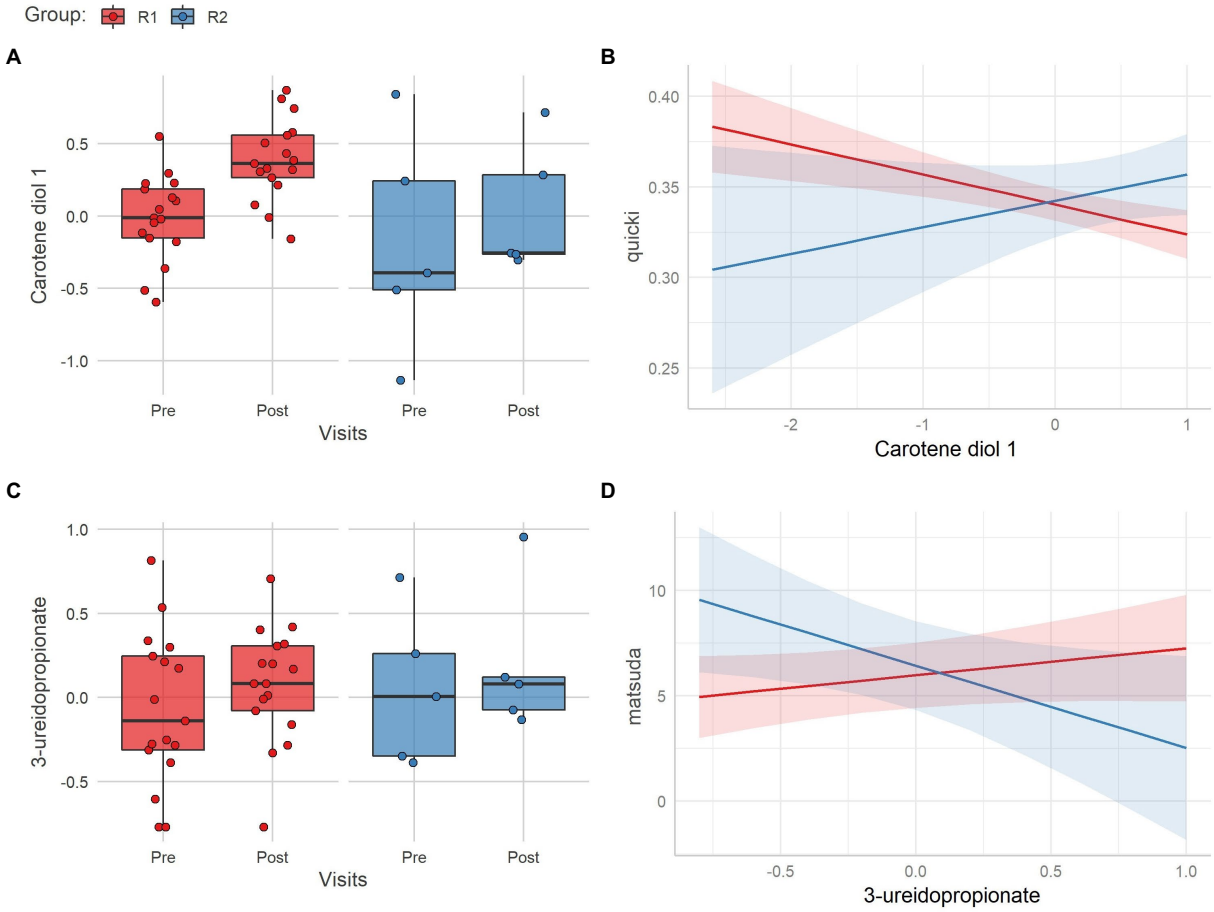

FIGURE 4
Association between changes in plasma metabolites and metabolic parameters during the intervention by response subgroup. Panels **(A)** and **(C)** show levels of carotene diol 1- and 3-ureidopropionate, respectively, for each response group, before and after the intervention. Panel **(B)** shows the changes of carotene diol 1 in relation to the changes in the QUICKI index for type 1 and type 2 responder groups. Panel **(D)** shows the changes in 3-ureidopropionate in relation to the changes in the Matsuda index for type 1 and type 2 responder groups.

after the intervention was improved. Although these results are preliminary due to the small numbers on which they are based, they reflect the interindividual variability observed in metabolic responsiveness to a nutritional intervention, possibly attributable to divergent capacity to metabolize, absorb or use these metabolites. On the other hand, chiro-inositol was the most discriminant metabolite of component 2. It has been reported to serve a purpose in the mediation of insulin action, and low concentrations have been linked to increased insulin resistance (53, 54). Xylose, the second most discriminant metabolite of component 2, has been linked to improved blood glucose levels regulation by selectively inhibiting the activity of sucrase (55, 56), and may have been a factor in the improved insulin sensitivity of type 2 responders. The different responses we observed between type 1 and type 2 responders may have different causes. Studies focusing on fruits and vegetables consumption found that many factors could influence the heterogeneity of individuals' response: health status, excess weight, chronic inflammation and hypertension can affect the absorption and metabolism of biomarkers. This may explain the varying effectiveness of the intervention in the at risk of metabolic syndrome population of this study (57). Other studies have explored the different responses in individuals to the same meal, finding many determinants of postprandial metabolism such as glycemic response and triglyceride and insulin concentrations (58). Concentrations of specific enzymes and some polymorphisms and mutations affecting key genes may also influence individual metabolic response to many nutrients and therefore the presence of their metabolites in plasma samples (59). Metabolomics alone cannot fully explain the different responses we observed between the two subgroups, and future studies may use a multi-omics approach to further understand interindividual variability and its causes.

The small sample size of the present study as well as the low number of type 2 responders can be considered as a limitation and results should be interpreted with caution. The generalization of clustering results therefore requires further studies in larger, independent study samples to confirm the present findings. The subgroup of type 2 responders with positive health outcomes consisted of only five people, which limits the impact of these results. Another limitation was the absence of polyphenols and polyphenol-derived metabolites in the Metabolon database of metabolites used in this study. The inclusion of these molecules could have revealed more meaningful interactions between metabolites and changes in clinical variables, allowing us to understand the distinct metabolic effects of red raspberry consumption. Moreover, the absence of a control group was also a limitation. The participants of the study were required to

limit their berry consumption and maintain consistent health habits during a 2-week run-in period, leading to raspberry supplementation as the primary dietary change during the intervention. However, since we do not have data from the control group, these observations should be considered exploratory in nature and further studies utilizing randomized designs will be necessary to validate our findings. Despite the small sample size, some significant interactions between metabolites and clinical features were still found, opening the door to more in-depth studies on specific metabolites. Moreover, the use of metabolomics are herein revealed as particularly promising in assessing dietary intake biomarkers in conjunction with self-reported dietary assessment methods such as food frequency questionnaires, which alone are prone to a certain error (60, 61). Such results warrant further investigation into large study samples to confirm the potential of xenobiotics as a marker of adherence to nutritional interventions.

In conclusion, this metabolomics-based clustering approach derived from an 8-week raspberry consumption allowed to develop a framework to address the impact of the interindividual variability on the metabolic responsiveness to raspberry consumption. This approach paves the way to future studies focused on further understanding the role of plasma metabolites in identifying individuals more prone to take advantage from a nutritional intervention aimed at having beneficial health effects. This framework may then be extrapolated to understand other diseases and conditions, and further enhance the development of precision nutrition.

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 Université Laval Ethics Committee (CER-Université Laval 2017-218). The patients/participants provided their written informed consent to participate in this study.

Author contributions

VB prepared the manuscript. JT-M, VB, and RS-C conducted the metabolomic and statistical analyses. MV designed and supervised the study. VG coordinated the study and PC assumed the medical supervision. MV, JT-M, RS-C, VG, GP, PC, DR, CC, and AM reviewed/revised and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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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 interpretated as a potential conflict of interest.

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Supplementary material

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Phenome-wide Mendelian randomization study evaluating the association of circulating vitamin D with complex diseases

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Background: Circulating vitamin D has been associated with multiple clinical diseases in observational studies, but the association was inconsistent due to the presence of confounders. We conducted a bidirectional Mendelian randomization (MR) study to explore the healthy atlas of vitamin D in many clinical traits and evaluate their causal association.

Methods: Based on a large-scale genome-wide association study (GWAS), the single nucleotide polymorphism (SNPs) instruments of circulating 25-hydroxyvitamin D (25OHD) from 443,734 Europeans and the corresponding effects of 10 clinical diseases and 42 clinical traits in the European population were recruited to conduct a bidirectional two-sample Mendelian randomization study. Under the network of Mendelian randomization analysis, inverse-variance weighting (IVW), weighted median, weighted mode, and Mendelian randomization (MR)–Egger regression were performed to explore the causal effects and pleiotropy. Mendelian randomization pleiotropy RESidual Sum and Outlier (MR-PRESSO) was conducted to uncover and exclude pleiotropic SNPs.

Results: The results revealed that genetically decreased vitamin D was inversely related to the estimated BMD ($\beta = -0.029$ g/cm^2, $p = 0.027$), TC ($\beta = -0.269$ mmol/L, $p = 0.006$), TG ($\beta = -0.208$ mmol/L, $p = 0.002$), and pulse pressure ($\beta = -0.241$ mmHg, $p = 0.043$), while positively associated with lymphocyte count ($\beta = 0.037\%$, $p = 0.015$). The results did not reveal any causal association of vitamin D with clinical diseases. On the contrary, genetically protected CKD was significantly associated with increased vitamin D ($\beta = 0.056$, $p = 2.361 \times 10^{-26}$).

Conclusion: The putative causal effects of circulating vitamin D on estimated bone mass, plasma triglyceride, and total cholesterol were uncovered, but not on clinical diseases. Vitamin D may be linked to clinical disease by affecting health-related metabolic markers.

KEYWORDS

circulating vitamin D, complex diseases, association, Mendelian randomization (MR) analysis, phenome wide association studies

Introduction

Vitamin D is an essential fat-soluble nutrition from cholecalciferol and steroid pro-hormone, which is predominately obtained from sunlight, dietary sources, and supplementation (1, 2). Vitamin D deficiency is common worldwide, with nearly one billion people experiencing vitamin D deficiency in 2019 (3). In this study, it is reported that 69.2% of the Asian population suffer from vitamin D deficiency, with 61% of postmenopausal women deficient in vitamin D , and the prevalence of vitamin D insufficiency among children is 56.2% (4). The deficiency in vitamin D is critical and is a contributor to the risk of metabolic diseases (5–7), cancers (8, 9), and all-cause mortality (10, 11).

Lowering 25-hydroxyvitamin D (25(OH)D) may act as a trigger for the inflammatory response to disturb the composition and function of circulating cytokines and growth factors (12), influencing endothelial cells (13), hence, increasing the risk of neurodegenerative disorders (14), cardiovascular disease (15, 16), musculoskeletal lesions (17), and mortality (11). The large-scale meta-analysis included 50 randomized controlled trials (RCTs) with a total of 74,655 participants and revealed that vitamin D supplementation statistically significantly reduced the risk of cancer death (RR: 0.85, 0.74–0.97) in adults compared with placebo (18). Moreover, vitamin D supplementation significantly reduced total cancer mortality in an updated meta-analysis of RCTs with 1,591 deaths (19). The meta-analysis comparing the highest with the lowest circulating 25(OH)D concentrations showed a 39% lower risk between levels of total 25(OH)D and colorectal cancer (CRC) risk (OR: 0.61, 0.52–0.71) with 11 case–control studies, and a 20% reduced CRC risk (HR: 0.80, 0.66–0.97) with six prospective cohort studies (20). Moreover, the effects of vitamin D supplementation on decreasing the LDL-c level (21) and releasing insulin resistance (22) were uncovered.

Inversely, the results from an RCT of vitamin D3 (cholecalciferol) at a dose of 2,000 IU per day during a median follow-up of 5.3 years found that supplementation with vitamin D3 did not result in a lower incidence of invasive cancer or cardiovascular events than placebo (23). The negative results were observed in another RCT with 5,108 older adult participants (65.9 years) during a median follow-up of 3.3 years, in which monthly high-dose vitamin D3 (200,000 IU) supplementation did not prevent CVD (24). Meanwhile, the studies did not illuminate the benefits of vitamin D3 supplementation for relapse-free survival of digestive tract cancer at 5 years (25, 26). Although numerous studies explored the association of vitamin D with many clinical diseases, inconsistent results were produced, and the potential confounders may contribute to the unobvious benefit of vitamin D.

Recently, single nucleotide polymorphism (SNP) that was identified by genome-wide association studies (GWASs) was recruited as an instrument in Mendelian randomization (MR) analyses to explore the causal effects of exposures on outcomes (6, 27–30). The MR analysis demonstrated that genetically decreased 25(OH)D was associated with the risk of multiple sclerosis (MS) and provided strong evidence for the causal role of vitamin D in MS susceptibility (8). Moreover, a significant association between 25(OH)D levels and T2DM was found in a European-descent MR with SNP instruments of vitamin D synthesis (31). Inversely, no evidence for the effects of 25(OH)D on T2DM was observed in another MR study with four instrumental SNPs of 25(OH)D concentrations (27). Meanwhile, the delicate associations between genetically predicted 25(OH)D and multiple outcomes were observed, such as in inflammatory bowel disease (IBD) (28), bone mineral density (BMD) (32), non-alcoholic fatty liver disease (NAFLD) (29), and hypertension (33). The genetic instruments of 25(OH)D recruited in a previous MR analysis were limited to four or six variants involved in vitamin D synthesis (DHCR7/NADSYN1, rs12785878 and CYP2R1, rs10741657), transportation (GC, rs3755967), and degradation (CYP24A1, rs17216707), as well as two novel vitamin D metabolism pathways, such as SEC23A (Sec23 homolog A, coat protein complex II component, rs8018720) and AMDHD1 (amidohydrolase domain containing 1, rs10745742). The overall estimate of heritability of 25(OH)D attributable to statistically significant GWAS common SNPs was 2.84% (34). The limited efficiency of SNP instruments may lead to inconsistent results.

In the current study, we conducted a systematic literature review of previous MR studies on 25(OH)D and performed a bidirectional MR analysis to examine the causal association between genetic determinants of 25(OH)D and cardiovascular diseases (CAD), fracture, rheumatoid arthritis (RA), Alzheimer's dementia (AD), inflammatory bowel disease (IBD), chronic kidney disease (CKD), and related clinical risk factors using summary data from large-scale genome-wide association studies.

Methods

Study design

The GWAS summary statistics about 10 clinical diseases (including chronic kidney disease, stroke, type-2 diabetes,

Abbreviations: BMD, bone mineral density; BMI, body mass index; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; PP, pulse pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; UAUCr, urinary albumin to creatinine ratio; UKUCr, urinary potassium to creatinine ratio; UNaUCr, urinary sodium to creatinine ratio; UNaUK, urinary sodium to potassium ratio; CAD, coronary artery disease; CD, Crohn's disease; UC, ulcerative colitis; LD, linkage disequilibrium; GWAS, genome-wide association study; MAF, minor allele frequency; MR, Mendelian randomization; IVW, inverse-variance weighting; MRPRESSO, MR-Pleiotropy RESidual Sum and Outlier method; GRASP, genome-wide repository of associations between SNPs and phenotypes; GIANT, genetic investigation of anthropometric traits; MAGIC, The Meta-Analyses of Glucose and Insulin-Related Traits Consortium; GLGC, The Global Lipids Genetics Consortium; CKDGen, The Chronic Kidney Disease Consortium; GEFOS, The GEnetic Factors for OSteoporosis consortium; ISGC, International Stroke Genetics Consortium; DIAGRAM, diabetes genetics replication and meta-analysis; CARDIoGRAMplusC4D, coronary artery disease genome-wide replication and meta-analysis plus the coronary artery disease (C4D) genetics; IBDGenetics, International Inflammatory Bowel Disease Genetics Consortium.

coronary artery disease, CAD with diabetes, Crohn's disease, ulcerative colitis, fracture, rheumatoid arthritis, and Alzheimer's dementia) and 42 clinical traits (including the anthropometric index, glycemic traits, serum lipids, cardiovascular measurements, kidney function, musculoskeletal health, bone mineral density, and blood cell and plasma cytokine) were collected from publicly available data sources and analyzed to estimate the genetic causal relationship of serum 25(OH)D with multifarious clinical outcomes through bidirectional two-sample Mendelian randomization (MR) (35). The MR procedure consists of three assumptions (36): (1) the genetic instrumental variables are strongly associated with exposures; (2) the genetic instrumental variables are not associated with any known or unmeasured confounders: influencing the association between genetic variants and outcomes; (3) the genetic variants are associated with outcomes only through exposures: variants causing significant effects on outcomes not through other pathways, no horizontal pleiotropy. The framework of the MR study is presented in Supplementary Figure 1. Ethics approval was not required for this study.

Identification of SNPs associated with 25(OH)D and clinical outcomes

Based on the large-scale GWAS, which consisted of 443,734 participants from the UK Biobank, the 138 conditionally independent SNPs for 25(OH)D, mapped to 69 distinct loci, among which 63 were previously not reported, were selected at the genome-wide significance level (6.6×10^{-9}) after adjusting for age, sex, and season of 25(OH)D measurement (37). Of these conditionally independent SNPs, 53 (38%) had a minor allele frequency (MAF) of <5%, and 85 (62%) were common (MAF $\geq$ 5%). The 53 SNPs with a MAF of <5% conferred an average absolute effect of 0.23 standard deviations on standardized log transformed 25(OH)D levels per effect allele, compared to 0.03 standard deviations of the 85 SNPs with a MAF of $\geq$5%. The average absolute effect on 25(OH)D of the 53 low frequency and rare variants was at least 7 times larger than the average effect of the 85 common SNPs. The known vitamin D loci (*CYP2R1, DHCR7, GC, CYP24A1, AMDHD1,* and *SEC23A*) were replicated in the study (Supplementary Table 1; Supplementary Figure 2). Serum 25(OH)D in this study was measured by liquid chromatography-tandem mass spectrometry.

The characteristics of selected instrumental SNPs for specific clinical diseases and clinical traits are presented in Table 1. Contributing studies received ethical approval from their respective institutional review boards. Informed consent was obtained from all participants of contributing studies. The GWAS summary statistics of 10 clinical diseases (Supplementary Tables 2, 3) and 42 clinical traits (Supplementary Table 4) were collected from publicly available resources. We conducted a comprehensive literature review to test the horizontal pleiotropy in selected SNPs and evaluate whether any of the SNPs were influenced by linkage disequilibrium (LD). To examine assumptions 2 and 3, we chose the variant with the lowest *p*-value for association with clinical outcomes.

Characteristics of selected data sources

To comprehensively explore the effective atlas of vitamin D on numerous healthy outcomes (clinical diseases and clinical traits), the specific genetic SNPs were selected from public sources (Table 1). For clinical diseases, the GWAS summary-level data were extracted from the Chronic Kidney Disease Consortium (CKDGen; 561,055 controls and 64,164 cases) for CKD; the Diabetes Genetics Replication and Meta-analysis (DIAGRAM) Consortium for T2DM (824,006 controls and 74,124 cases); the International Stroke Genetics Consortium (ISGC) for stroke (454,450 controls and 67,162 cases); the coronary artery disease genome-wide replication and meta-analysis (CARDIoGRAM) plus the Coronary Artery Disease (C4D) Genetics (CARDIoGRAMplusC4D) Consortium for CAD (123,504 controls and 60,801 cases), and CAD with diabetes (11,698 controls and 3,968 cases); the International Inflammatory Bowel Disease Genetics Consortium (IIBDGC) for IBD (48,950 for ulcerative colitis and 51,109 for Crohn's disease); the Psychiatric Genomics Consortium (PGC-ALZ), the Alzheimer's Disease Sequencing Project (ADSP), and the International Genomics of Alzheimer's Project (IGAP) for AD (383,378 controls and 71,880 cases); the GEnetic Factors for OSteoporosis consortium (GEFOS) for fracture (227,116 controls and 37,857 cases); and Genetics and Allied research in Rheumatic diseases Networking (GARNET) and Rheumatoid Arthritis Consortium International (RACI) for RA (73,758 controls and 29,880 cases).

For clinical traits, 20 GWAS summary datasets for genetic determinants were available. Genome-wide association analyses have been published for adiposity (BMI, WHR adjusted BMI, and obesity) by the Genetic Investigation of Anthropometric Traits (GIANT) Consortium; glycemic traits [hemoglobin A1c (HbA1c), fasting glucose, fasting insulin, and fasting proinsulin] by the Meta-Analyses of Glucose and Insulin-Related Traits Consortium (MAGIC), plasma lipids (HDL-C, LDL-C, TC, and TG) by the Global Lipids Genetics Consortium (GLGC), bone mineral density (estimated BMD, total body BMD, forearm, femoral neck, and lumbar spine BMD), musculoskeletal measurements (lean body mass, hand grip strength, and gait speed), blood pressure (systolic BP, diastolic BP, and pulse pressure), and heart rate variability by the GEFOS and the genome-wide repository of associations between SNPs and phenotypes (GRASP). Meanwhile, the circulating cytokines and growth factors for inflammation and urinary biomarkers for kidney function, such as the urinary albumin to creatinine ratio (UACR), the urinary potassium to creatinine ratio (UK/UCr), the urinary sodium to creatinine ratio (UNa/UCr), and the urinary sodium to potassium ratio (UNa/UK), were explored.

The definitions of clinical disease and clinical traits are explained in Supplementary material. All clinical diseases were diagnosed using ICD-10 (international classification of diseases, 10th revisions) codes. The ICD-10 codes of chronic kidney disease, stroke, type-2 diabetes, coronary artery disease,

TABLE 1 Description of selected clinical outcomes.

Disease or trait	Sample size	Population	Data sources	Years
25(OH)D (37)	401,460	European	UK BioBank	2020
Anthropometric index				
BMI (38)	526,508	Transethnic	GRASP	2018
Waist-to-hip ratio (39)	694,649	European	GIANT and UK BioBank	2019
Obesity (40)	276,007	European	GIANT	2018
Glycemic traits				
Fasting glucose (41)	133,010	European	MAGIC	2012
Fasting insulin (41)	133,010	European	MAGIC	2012
Fasting proinsulin (42)	16,378	European	MAGIC	2011
HbA1c (43)	46,368	European	MAGIC	2010
Lipids				
HDL-C (44)	188,578	Transethnic	GLGC	2013
LDL-C (44)	188,578	Transethnic	GLGC	2013
Total cholesterol (44)	188,578	Transethnic	GLGC	2013
Triglycerides (44)	188,578	Transethnic	GLGC	2013
Cardiovascular measurements				
PP (45)	1,006,863	Transethnic	GRASP	2018
SBP (5‘9)	1,006,863	Transethnic	GRASP	2018
DBP (45)	1,006,863	Transethnic	GRASP	2018
Hypertension (46)	327,288	Transethnic	GRASP	2018
Heart rate variability (47)	53,174	European	GRASP	2017
Kidney function				
eGFR (48)	765,348	Transethnic	CKDGen	2019
UAUCr (49)	327,616	European	GRASP	2019
UKUCr (49)	327,616	European	GRASP	2019
UNaUCr (49)	327,616	European	GRASP	2019
UNaUK (49)	327,616	European	GRASP	2019
Musculoskeletal health				
Lean body mass (50)	85,519	European	GEFOS	2017
Grip strength (51)	195,180	European	GRASP	2017
Gait speed (52)	34,066	European	GRASP	2017
Bone mineral density				
eBMD (53)	488,683	European	GEFOS	2017
Total body BMD (54)	666,628	Transethnic	GEFOS	2018
Forearm BMD (55)	32,965	European	GEFOS	2015
Femoral neck BMD (55)	32,965	European	GEFOS	2015
Lumbar spine BMD (55)	32,965	European	GEFOS	2015
Blood cell and plasma cytokine				
Platelet count (56)	173,480	European	GRASP	2016
Lymphocyte count (56)	173,480	European	GRASP	2016
Red blood cell count (56)	173,480	European	GRASP	2016

(Continued)

TABLE 1 (Continued)

Disease or trait	Sample size	Population	Data sources	Years
White blood cell count (56)	173,480	European	GRASP	2016
Interleukin-1-beta (57)	8,293	European	GRASP	2017
Interleukin-6 (57)	8,293	European	GRASP	2017
Interleukin-7 (57)	8,293	European	GRASP	2017
Interleukin-8 (57)	8,293	European	GRASP	2017
Interleukin-9 (57)	8,293	European	GRASP	2017
Interleukin-10 (57)	8,293	European	GRASP	2017
Beta nerve growth factor (57)	8,293	European	GRASP	2017
Tumor necrosis factor-alpha (57)	8,293	European	GRASP	2017
Vascular endothelial growth factor (57)	8,293	European	GRASP	2017
Clinical disease				
Chronic kidney disease (48)	625,219 (64,164 cases)	European	CKDGen	2019
Stroke (58)	521,612 (67,162 cases)	European	ISGC	2018
Type 2 diabetes (59)	898,130 (74,124 cases)	European	DIAGRAM	2018
Coronary artery disease (60)	184,305 (60,801 cases)	Transethnic	CARDIoGRAMplusC4D	2018
CAD with diabetes (61)	15,666 (3,968 cases)	European	GRASP	2015
Crohn's disease (62)	51,109 (22,027 cases)	European	IBDGenetics	2010
Ulcerative colitis (63)	48,950 (16,315 cases)	European	IBDGenetics	2011
Fracture (64)	264,973 (37,857 cases)	Transethnic	GEFOS	2018
Rheumatoid arthritis (65)	103,638 (29,880 cases)	Transethnic	GRASP	2014
Alzheimer's dementia (66)	455,258 (71,880 cases)	Transethnic	GRASP	2019

GRASP, genome-wide repository of associations between SNPs and phenotypes; GIANT, genetic investigation of anthropometric traits; MAGIC, The Meta-Analyses of Glucose and Insulin-Related Traits Consortium; GLGC, The Global Lipids Genetics Consortium; CKDGen, The Chronic Kidney Disease Consortium; GEFOS, The GEnetic Factors for OSteoporosis consortium; ISGC, International Stroke Genetics Consortium; DIAGRAM, diabetes genetics replication and meta-analysis; CARDIoGRAMplusC4D, coronary artery disease genome-wide replication and meta-analysis plus the coronary artery disease (C4D) genetics; IBDGenetics, International Inflammatory Bowel Disease Genetics Consortium; BMI, body mass index; eBMD, estimated bone mineral density; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; PP, pulse pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; UAUCr, urinary albumin to creatinine ratio; UKUCr, urinary potassium to creatinine ratio; UNaUCr, urinary sodium to creatinine ratio; UNaUK, urinary sodium to potassium ratio.

CAD with diabetes, Crohn's disease, ulcerative colitis, fracture, rheumatoid arthritis, and Alzheimer's dementia are presented in Supplementary Table 7.

Statistical analysis

The summary datasets consist of effect sizes and standard errors of outcomes and exposures. However, the effect/non-effect alleles must be harmonized between outcome and exposure, when the effect allele was flipped (effect/non-effect alleles were G/T for the exposure and T/G for the outcome). Meanwhile, the alleles of outcome were matched with exposure alleles and effect alleles were aligned, when the strand of SNPs was flipped (the effect/non-effect alleles were G/T for the exposure and C/A for the outcome). Finally, we eliminated the incompatible SNPs (effect/non-effect alleles were A/G for the exposure and A/T for the outcome).

All the SNPs that independently (linkage disequilibrium $r^2 < 0.01$) and strongly associated with the exposures at the genome-wide significant levels were extracted to verify the horizontal pleiotropy. The associations of curated SNPs with traits were assessed online (GWAS Catalog, https://www.ebi.ac.uk/gwas; ClinicalTrials. gov, https://clinicaltrials.gov; PhenoScanner, http://www.phenoscanner.medschl.cam.ac.uk) through Mendelian randomization analysis. Moreover, the Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) was employed to identify and remove pleiotropic SNPs by assessing outliers among the included SNPs contributing to the Mendelian randomization estimate. We repeated the analyses after excluding potentially pleiotropic SNPs. Then, inverse-variance weighting (IVW), weighted median, weighted mode, and Mendelian randomization (MR)–Egger regression were applied to explore the causal effect of genetically decreased vitamin D on a wide spectrum of phenotypes (Supplementary material). For sensitivity analysis, bidirectional Mendelian randomization was conducted to validate the causal effects of genetic determinants of clinical diseases on vitamin D. All analyses were performed using R Version 4.0.2 with the R package Mendelian Randomization, MR-PRESSO.

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TABLE 2 Association of vitamin D with clinical diseases using inverse-variance weighting Mendelian randomization study.

Clinical disease	*N* SNPs	IVW		
		Estimates (95% CI)	*p*-value	*p*_Het
Vitamin D to diseases				
Chronic kidney disease	75	0.006 (−0.075 to 0.087)	0.882	0.017
Stroke	34	0.102 (−0.224 to 0.428)	0.538	0.416
Type 2 diabetes	70	−0.023 (−0.116 to 0.07)	0.630	0.067
Coronary artery disease	29	0.065 (−0.276 to 0.405)	0.710	0.165
CAD with diabetes	76	0.032 (−0.188 to 0.253)	0.774	0.343
Crohn's disease	81	−0.070 (−0.275 to 0.135)	0.503	0.096
Ulcerative colitis	85	−0.030 (−0.212 to 0.153)	0.751	0.177
Fracture	33	0.127 (−0.049 to 0.304)	0.157	0.199
Rheumatoid arthritis	66	−0.064 (−0.186 to 0.058)	0.301	0.119
Alzheimer's dementia	95	−0.013 (−0.031 to 0.006)	0.174	0.376
Diseases to vitamin D				
Chronic kidney disease	17	0.056 (0.041 to 0.072)	2.361×10^{-26}	0.393
Stroke	13	0.001 (−0.022 to 0.025)	0.916	0.179
Type 2 diabetes	28	0.007 (−0.001 to 0.015)	0.083	0.112
Coronary artery disease	25	0.003 (−0.009 to 0.016)	0.590	0.630
CAD with diabetes	46	0.004 (−0.008 to 0.016)	0.526	0.102
Crohn's disease	60	0.001 (−0.003 to 0.004)	0.708	0.195
Ulcerative colitis	37	0.003 (−0.002 to 0.008)	0.205	0.248
Fracture	14	0.004 (−0.019 to 0.026)	0.740	0.256
Rheumatoid arthritis	26	−0.001 (−0.007 to 0.005)	0.678	0.954
Alzheimer's dementia	26	−0.004 (−0.061 to 0.052)	0.880	0.108

SNPs, single nucleotide polymorphisms; CAD, coronary artery disease; IVW, inverse-variance weighting; p_Het, heterogeneity statistics.

Results

Associations of genetic 25(OH)D with the risk of clinical diseases

The results failed to reveal any effect of 25(OH)D on many clinical diseases (All $p > 0.05$) in IVW analysis (Table 2, Figure 1). Similar results were obtained in the weighted median and weighted mode statistics ($p > 0.05$; Supplementary Table 5). There was no significant horizontal pleiotropy and heterogeneity in all selected SNPs after excluded pleiotropic variants by MR-PRESSO ($p_{\_Het} > 0.05$, intercept $p > 0.05$, global test $p > 0.05$; Table 2, Figure 1, Supplementary Table 5, Supplementary Figures 3a–j).

The inversed association between increased risk of CKD with 25(OH)D ($\beta = 0.056$, 95% CI: 0.04–0.072, $p = 2.361 \times 10^{-26}$, $p_{\_Het} = 0.393$) was found in the IVW analysis (Table 2, Figure 1). Similarly, significant associations were found in the weighted median ($\beta = 0.057$, 95% CI: 0.035–0.080, $p = 3.128 \times 10^{-25}$) and weighted mode ($\beta = 0.056$, 95% CI: 0.021–0.091, $p = 0.002$). There was no heterogeneity and horizontal pleiotropy with MR–Egger regression (Intercept $p = 0.335$) after excluded pleiotropic variants using the restrictive MR pleiotropy residual sum and outlier test (MR-PRESSO) method (Global test $p = 0.469$; Supplementary Table 5). In addition, no evidence supported the effects of clinical diseases on 25(OH)D, except CKD in reversed MR analysis (Table 2, Figure 1, Supplementary Table 5, Supplementary Figures 4a–j).

Association of genetic 25(OH)D with clinical traits

Mendelian randomization analyses were conducted to assess the association of plasma 25(OH)D with 42 clinical traits. The results revealed that genetically decreased 25(OH)D was strongly correlated with estimated BMD (g/cm^2), TC (mmol/L), TG (mmol/L), and PP (mmHg), and negatively associated with lymphocyte count (%; All $p < 0.05$, $p_{\_Het} > 0.05$) in IVW (Table 3, Figure 2). There was no evidence for the association of genetically decreased vitamin D with the anthropometric index, glycemic traits, kidney function, and musculoskeletal health (Table 3, Figure 2, Supplementary Table 6). The intercepts in MR–Egger test were tightly centered around the null, which revealed the

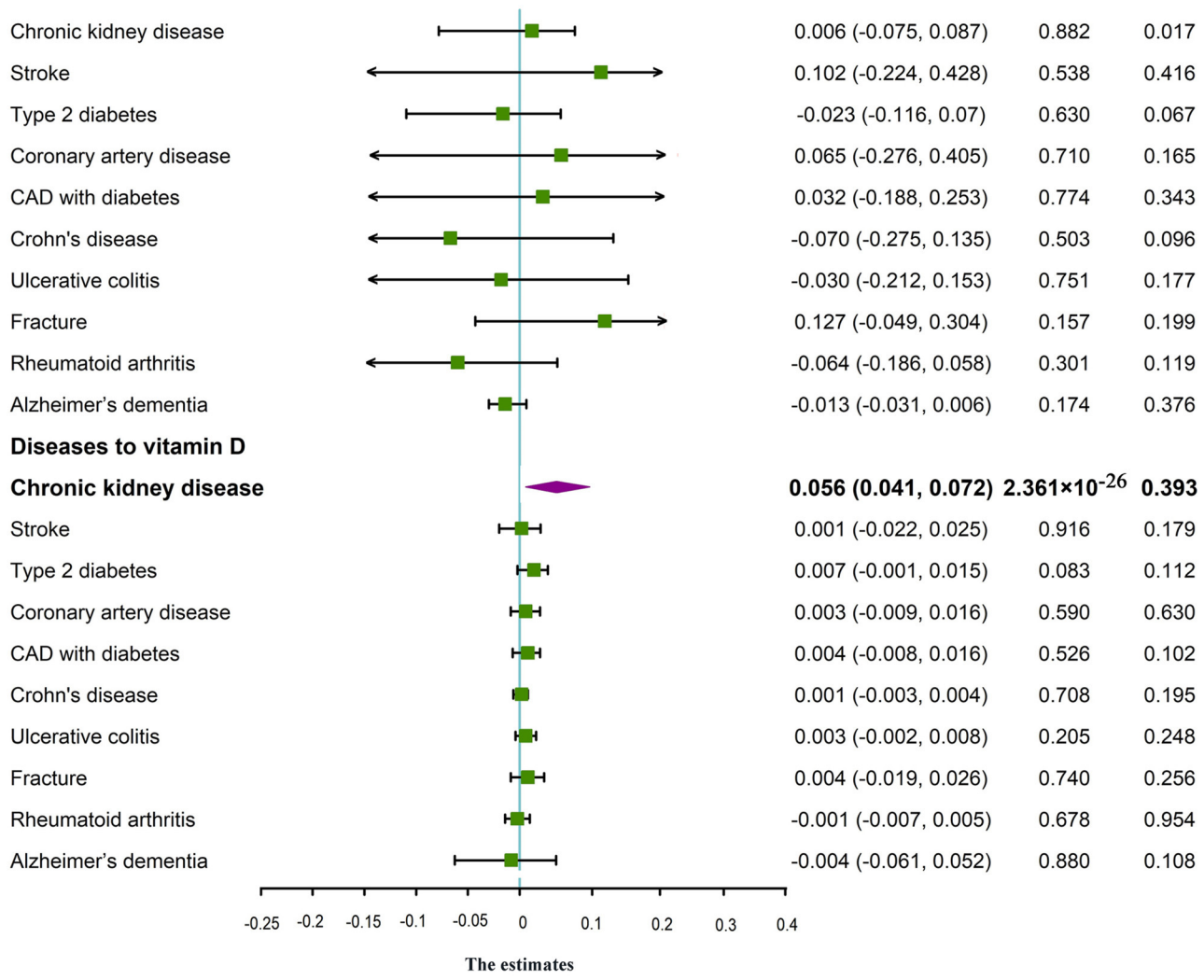

FIGURE 1
Forest plot of the association between circulating 25(OH)D and clinical diseases in bidirectional Mendelian randomization analysis. CAD, coronary artery disease; *p*_Het, heterogeneity statistics.

statistic effects of genetic instruments in Mendelian randomization analyses did not be influenced by pleiotropy.

Discussion

In the present Mendelian randomization (MR) study, our findings did not support the putative causal effects of 25(OH)D on multiple clinical diseases. Genetically decreased 25(OH)D was significantly associated with the estimated bone mineral density (eBMD), plasma cholesterol, pulse pressure, and elevated lymphocyte count. A bidirectional MR study did not reveal the significant effects of CVD, IBD, T2DM, AD, and musculoskeletal disorders on 25(OH)D concentration. However, chronic kidney disease was positively associated with decreased 25(OH)D.

Dietary and skin-derived vitamin D will play biological roles in multiple organs after hydroxylating in the liver and kidney (67). Patients with chronic kidney disease will decrease the biosynthesis of circulating 25(OH)D by reducing the production of hydroxylase (68). Vitamin D exerts a biological role by binding the vitamin D receptor (VDR) (69), which is commonly found in musculoskeletal cells and various extracellular tissues, such as parathyroid tissue, intestinal tissue, and kidneys (70). Vitamin D deficiency not only increased the risk of rickets, osteomalacia, and osteoporotic fractures (71) but also contributed to extra-skeletal disorders (72) in epidemiological studies. However, no significant associations of 25(OH)D with the risk of clinical diseases were found in our study. Meanwhile, the reversed MR analysis did not reveal any impact of multiple chronic diseases on serum 25(OH)D, except for CKD. The relationship between vitamin D and clinical disease

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TABLE 3 Association of vitamin D with clinical traits using inverse-variance weighting Mendelian randomization.

Clinical traits	*N* SNPs	IVW		
		Estimates (95% CI)	*p*-value	*p*_Het
Adiposity				
BMI	23	0.023 (−0.014 to 0.061)	0.216	0.501
Waist-to-hip ratio	23	−0.002 (−0.043 to 0.039)	0.932	0.470
Obesity	33	0.118 (−0.144 to 0.380)	0.378	0.320
Glycemic traits				
Fasting glucose	38	−0.05 (−0.111 to 0.011)	0.109	0.329
Fasting insulin	38	−0.019 (−0.077 to 0.039)	0.524	0.599
Fasting proinsulin	35	0.058 (−0.105 to 0.220)	0.486	0.055
HbA1c	36	0.029 (−0.005 to 0.063)	0.097	0.341
Lipids				
HDL-c	27	0.024 (−0.079 to 0.126)	0.650	0.474
LDL-c	23	0.017 (−0.135 to 0.168)	0.830	0.205
Total cholesterol	18	−0.269 (−0.46 to −0.077)	0.006	0.076
Triglycerides	22	−0.208 (−0.339 to −0.077)	0.002	0.649
Cardiovascular measurements				
PP	45	−0.241 (−0.474 to −0.007)	0.043	0.043
SBP	41	−0.163 (−0.503 to 0.177)	0.348	0.074
DBP	47	0.009 (−0.176 to 0.195)	0.921	0.115
Hypertension	9	−0.087 (−0.23 to 0.056)	0.232	0.544
Heart rate variability	36	−0.015 (−0.076 to 0.047)	0.640	0.816
Kidney function				
eGFRcrea	50	−0.003 (−0.007 to 0.001)	0.060	0.209
UAUCr	85	−0.01 (−0.030 to 0.009)	0.300	0.028
UKUCr	85	−0.008 (−0.029 to 0.013)	0.473	0.001
UNaUCr	13	0.033 (−0.042 to 0.109)	0.387	0.664
UNaUK	91	0.019 (0.001 to 0.039)	0.054	0.053
Musculoskeletal health				
Lean body mass	37	0.487 (−0.221 to 1.195)	0.178	0.116
Grip strength	70	0.003 (0.001 to 0.005)	0.068	0.048
Gait speed	34	0.025 (−0.01 to 0.059)	0.163	0.250
Bone mineral density				
eBMD	11	−0.029 (−0.054 to −0.003)	0.027	0.101
Total body BMD	10	−0.007 (−0.055 to 0.041)	0.771	0.121
Forearm BMD	74	0.07 (−0.057 to 0.198)	0.278	0.494
Femoral neck BMD	68	0.021 (−0.05 to 0.092)	0.555	0.089
Lumbar spine BMD	66	0.05 (−0.032 to 0.131)	0.232	0.122
Blood cell and plasma cytokine				
Platelet count	96	0.022 (−0.008 to 0.052)	0.153	0.126
Lymphocyte count	95	0.037 (0.007 to 0.066)	0.015	0.145
Red blood cell count	86	0.01 (−0.023 to 0.043)	0.541	0.076

(Continued)

TABLE 3 (Continued)

Clinical traits	*N* SNPs	IVW		
		Estimates (95% CI)	*p*-value	*p*_Het
White blood cell count	90	0.014 (−0.028 to 0.056)	0.520	0.023
Interleukin-1-beta	76	0.087 (−0.101 to 0.274)	0.365	0.020
Interleukin-6	79	0.034 (−0.104 to 0.171)	0.632	0.128
Interleukin-7	79	0.003 (−0.194 to 0.2)	0.976	0.421
Interleukin-8	79	0.023 (−0.169 to 0.214)	0.816	0.749
Interleukin-9	79	0.119 (−0.069 to 0.308)	0.215	0.532
Interleukin-10	79	0.042 (−0.089 to 0.174)	0.529	0.415
Beta nerve growth factor	79	−0.023 (−0.227 to 0.181)	0.827	0.193
Tumor necrosis factor-alpha	79	0.178 (−0.016 to 0.372)	0.072	0.661
Vascular endothelial growth factor	79	−0.041 (−0.191 to 0.109)	0.591	0.081

SNPs, single nucleotide polymorphisms; CAD, coronary artery disease; BMI, body mass index; eBMD, estimated bone mineral density; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; PP, pulse pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; UAUCr, urinary albumin to creatinine ratio; UKUCr, urinary potassium to creatinine ratio; UNaUCr, urinary sodium to creatinine ratio; UNaUK, urinary sodium to potassium ratio; IVW, inverse-variance weighting; p_Het, heterogeneity statistics.

may not be linear, and a linear analysis may yield negative results. A non-linear association of 25(OH)D deficiency with the risk of cardiovascular disease was discovered in a non-linear MR analysis (73), and the non-linear dose–response relationships between 25(OH)D concentrations and coronary heart disease, stroke, and mortality outcomes were discovered in a stratified MR analysis (74).

A weak correlation of 25(OH)D with estimated BMD of heel was found, but not with femoral neck BMD (FN-BMD) or lumbar spine BMD (LS-BMD) in our study. Meta-analysis of RCTs revealed that vitamin D supplementation was not associated with a lower risk of fractures in older adults (75). The MR study showed that the SNP instruments (rs2282679, rs117913124, rs10741657, rs12785878, and rs727479) were not associated with femoral neck BMD (FN-BMD), lumbar spine BMD (LS-BMD), or estimated BMD (eBMD), but rs6013897 near CYP24A1 was associated with FN-BMD at borderline statistical significance ($p = 0.01$). High 25(OH)D concentration was not associated with higher FN-BMD ($p = 0.37$) or LS-BMD ($p = 0.49$) in the inverse-variance weighted analysis or in sensitivity analyses but was associated with estimated BMD ($p = 0.02$) (76). The differences in the biological pathways of 25(OH)D-SNPs may be the critical factor causing the contradictory result. Mendelian randomization analyses that combine all SNP effects may mask the role of individual SNPs. Moreover, the beneficial effects of vitamin D on many diseases may be largely due to undetected confounders in an epidemiologic study.

In the present study, no significant associations of genetic 25(OH)D with the risk of CAD, stroke, and T2DM were uncovered. The putative causal effects of circulating vitamin D on plasma triglyceride and total cholesterol were uncovered. Previous observational studies revealed that circulating 25(OH)D was inversely associated with blood pressure and the risk of type-2 diabetes (77), but positively correlated with blood lipids (triglycerides, HDL-c, LDL-c, and total cholesterol) (16). Moreover, vitamin D supplementation will increase LDL-cholesterol concentrations (78). The genetic SNPs were not only related to circulating 25-hydroxyvitamin D but also plasma LDL-cholesterol and triglyceride levels. Pleiotropy-associated confounding cannot be completely ruled out (79).

The SNPs used in the previous two-stage and two-sample Mendelian randomization studies were mainly obtained from the SUNLIGHT consortium (study of underlying genetic determinants of Vitamin D and highly related traits) GWAS, which consisted of 31 cohorts from Europe, Canada, and the USA with atotal of 79,366 samples. The statistical effects of all SNPs were calculated by fixed-effect inverse-variance weighted meta-analysis. The joint test with multiple cohorts will easily induce an undetected bias due to the disparate measurements for 25(OH)D and the adjusted covariates. Meanwhile, we were unable to estimate the interactions between genetic variants and dietary intake of vitamin D as well as sunlight exposure as a source of vitamin D production in the skin. In the current study, the adequate instrumental SNPs involved in vitamin D synthesis (DHCR7/NADSYN1 and CYP2R1), transportation (GC), and degradation (CYP24A1), as well as novel vitamin D metabolism pathways, such as SEC23A (Sec23 homolog A, coat protein complex II component) and amidohydrolase domain containing 1 (AMDHD1), were recruited from the UK Biobank study with 401,460 white British participants. Moreover, the association between SNPs and 25(OH)D was estimated by linear mixed-model and the interactions of age, sex, and season with 25(OH)D were evaluated. The subjects in the GWAS analysis investigating the relationship between genetic loci and clinical features were of European and Caucasian descent, which is congruent with the population in UK Biobank. A comprehensive network of Mendelian randomization analysis, inverse-variance weighting (IVW), weighted median, weighted mode, and Mendelian randomization (MR)–Egger regression were performed to explore the causal effects and pleiotropy, as well as avoid bias. Mendelian randomization pleiotropy RESidual Sum and Outlier (MR-PRESSO) was conducted to uncover and exclude

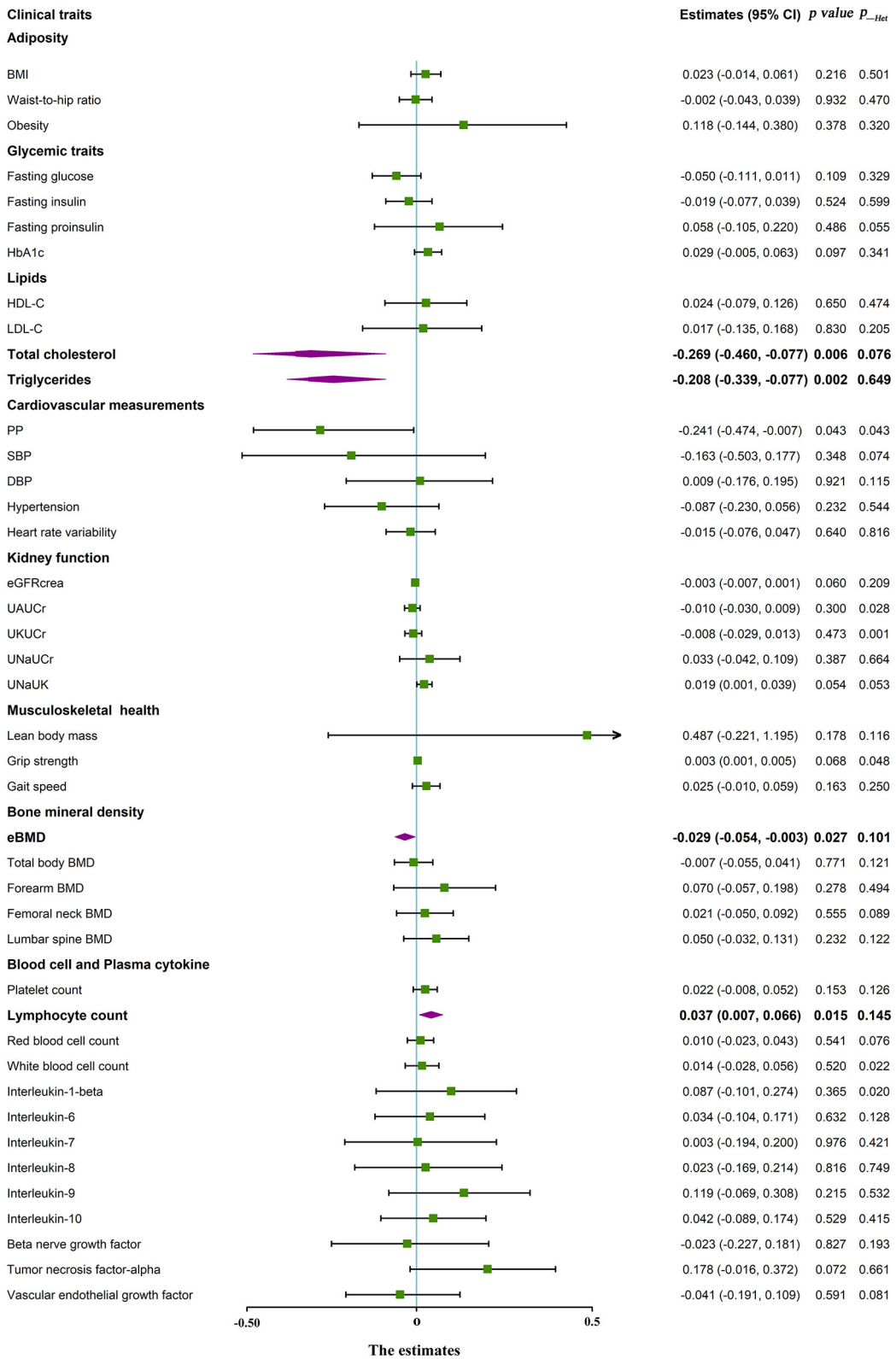

FIGURE 2
Forest plot about the association between circulating 25(OH)D and clinical traits in Mendelian randomization analysis. BMI, body mass index; eBMD, estimated bone mineral density; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; PP, pulse pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; UAUCr, urinary albumin to creatinine ratio; UKUCr, urinary potassium to creatinine ratio; UNaUCr, urinary sodium to creatinine ratio; UNaUK, urinary sodium to potassium ratio; *p*_Het, heterogeneity statistics.

pleiotropic SNPs. The methods in our study provided a credible result for the large-scale study.

Here, several strengths of our study should be mentioned. First, the panoptic atlas of 25(OH)D in many clinical diseases and healthy traits were explored in this MR analysis, which will help us to have a more comprehensive understanding of the relationship between 25(OH)D and health. In addition, the bidirectional two-sample MR study was conducted to estimate the influence of diseases on 25(OH)D concentrations. Second, the SNP biomarkers and SNP estimates were obtained in mostly European studies, thus minimizing the possibility of population stratification bias. Third, the genetic SNPs for vitamin D were derived from a recent large-scale GWAS study (n = 443,734) rather than an earlier SUNLIGHT study, which may be more representative of the genetic instruments used to explore the genetic correlation of vitamin D.

However, there are some potential limitations worth noting in the current study.

Although we performed tests to prevent pleiotropy, some genetic SNPs are not only related to circulating 25-hydroxyvitamin D but also to traits, such as plasma LDL-cholesterol and triglyceride levels, and the potential pleiotropy cannot be definitively excluded. Moreover, the non-linear association between 25(OH)D and clinical outcomes could not be assessed by our two-sample MR analysis with a summary dataset. A non-linear MR analysis using raw data is needed in future. Meanwhile, 25(OH)D was recruited as the symbol of serum vitamin D concentration. In addition, the biological roles of active metabolite 1,25-dihydroxy vitamin D in clinical outcomes were not explored. Meanwhile, we still failed to study the biological roles of 24, 25-$(OH)_2$-D_3, 1, 24, 25-$(OH)_3$-D_3, and 25, 26-$(OH)_2$-D_3 metabolites and epimer of vitamin D in health (80). In addition, our results were not likely biased by pleiotropy due to the fact that 25OHD-associated genetic variants were not associated with other lifestyles, which influenced clinical outcomes, such as drinking, physical activity, or smoking. However, we cannot exclude the possibility that such an association may have a genetic basis rather than a causal relationship.

Hence, a large-scale genome-wide scan for genetic variants of vitamin D and further investigation to understand the potential role of vitamin D in the development of clinical outcomes are required. Meanwhile, a long-term and multicentric RCT study that can avoid the interference of numerous known and unpredictable confounders on the results, such as diet, exercise, sleep, geographic latitude, and climate, is seriously needed.

Conclusion

Our study suggested that there was no evidence of the causal effect of 25(OH)D on numerous clinical diseases. Genetically decreased serum vitamin D was associated with estimated bone mineral density evaluated by ultrasound of the heel, plasma cholesterol, pulse pressure, and elevated lymphocyte count. Chronic kidney disease was inversely related to serum 25(OH)D concentration. The putative causal effects of vitamin D on multiple clinical diseases was not supported.

Author contributions

K-qL: concept and design. J-jX: data collection, statistics, and writing the manuscript. X-bZ and TY: data collection. W-tT: data interpretation. K-qL and J-jX: study supervision. All authors critically revised the manuscript and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1108477/full#supplementary-material

SUPPLEMENTARY TABLE 1
Characterics of 138 conditionally independent SNPs that reach genome-wide significance in the UKBB GWAS for 25OHD.

SUPPLEMENTARY TABLE 2
The effect estimates of the instrumental variables for 25(OH)D (exposure) and clinical diseases.

SUPPLEMENTARY TABLE 3
The effect estimates of the instrumental variables for clinical diseases (exposure) and 25(OH)D.

SUPPLEMENTARY TABLE 4
The effect estimates of the instrumental variables for 25(OH)D (exposure) and clinical traits.

SUPPLEMENTARY TABLE 5
Association of vitamin D with clinical diseases using other mendelian randomization studies in addition to inverse variance weighting.

SUPPLEMENTARY TABLE 6
Association of vitamin D with clinical traits using other mendelian randomization studies in addition to inverse variance weighting.

SUPPLEMENTARY TABLE 7
The International Classification of Diseases (ICD) codes for included clinical diseases.

SUPPLEMENTARY FIGURE 1
Assumptions of bidirectional Mendelian randomization analysis. **(a)** Main assumption of Mendelian randomization analysis. **(b)** Reversed assumption of Mendelian randomization analysis. (1) The genetic instrumental variables are strongly associated with exposures; (2) the genetic instrumental variables are not associated with any known or unmeasured confounders: influencing the association between genetic variants and outcomes; (3) the genetic variants are associated with outcomes only through exposures: variants causing significant effects on outcomes not through other pathways, no horizontal pleiotropy.

SUPPLEMENTARY FIGURE 2
Biological metabolic pathways of vitamin D.

SUPPLEMENTARY FIGURE 3
Scatter plot of the IVW MR study investigating the effect of 25(OH)D on clinical diseases. **(a)** Effect of vitamin D on chronic kidney disease. **(b)** Effect of vitamin D on stroke. **(c)** Effect of vitamin D on type-2 diabetes. **(d)** Effect of vitamin D on coronary artery disease. **(e)** Effect of vitamin D on coronary artery disease with diabetes. **(f)** Effect of vitamin D on Crohn's disease. **(g)** Effect of vitamin D on ulcerative colitis. **(h)** Effect of vitamin D on fracture. **(i)** Effect of vitamin D on rheumatoid arthritis. **(j)** Effect of vitamin D on Alzheimer's dementia. The *x*-axis represents the genetic association with 25OHD levels; the *y*-axis represents the genetic association with the risk of clinical diseases. The line represents the IVW method. 25OHD, 25-hydroxyvitamin D (vertical and horizontal blue lines around points show a 95% confidence interval for each polymorphism).

SUPPLEMENTARY FIGURE 4
Scatter plot of the IVW method in bidirectional Mendelian randomization analysis investigating the effect of clinical diseases on 25(OH)D. **(a)** Effect of chronic kidney disease on vitamin D. **(b)** Effect of stroke on vitamin D. **(c)** Effect of type-2 diabetes on vitamin D. **(d)** Effect of coronary artery disease on vitamin D. **(e)** Effect of coronary artery disease with diabetes on vitamin D. **(f)** Effect of Crohn's disease on vitamin D. **(g)** Effect of ulcerative colitis on vitamin D. **(h)** Effect of fracture on vitamin D. **(i)** Effect of rheumatoid arthritis on vitamin D. **(j)** Effect of Alzheimer's dementia on vitamin D. The *x*-axis represents the genetic association with the risk of clinical diseases; the *y*-axis represents the genetic association with 25OHD levels; The line represents the IVW method. 25OHD, 25-hydroxyvitamin D (vertical and horizontal blue lines around points show a 95% confidence interval for each polymorphism).

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Association between dietary intake of anthocyanidins and heart failure among American adults: NHANES (2007–2010 and 2017–2018)

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Background: Despite anthocyanidins have anti-inflammatory and antioxidant properties, no studies have researched association between dietary intake of anthocyanidins and heart failure.

Methods: We enrolled 15,869 participants from the National Health and Nutrition Examination Survey (NHANES) (2007–2010 and 2017–2018) in this cross-sectional study. We examined baseline data and prevalence of heart failure in different quartile groups of anthocyanin intake (Q1-4). Three models were established through logistic regression to evaluate the protective effect of Q4 (highest anthocyanidins intake) on heart failure. The protective effect of high anthocyanidins intake on heart failure was further evaluated in different subgroups.

Results: Participants with the highest anthocyanidins intake (Q4) had the lowest prevalence of heart failure (Q1:2.54%, Q2:2.33%, Q3:2.43%, Q4:1.57%, p =0.02). After adjusting for possible confounding factors, compared with the Q1 group, the highest anthocyanidins intake (Q4) was independently related to lower presence of heart failure (Q4: OR 0.469, 95%CI [0.289, 0.732], p =0.003). And this association was still stable in subgroups of female, ≥45years, smoker, non-Hispanic White or without diabetes, stroke and renal failure.

Conclusion: Dietary intake of anthocyanidins had negative association with the presence of heart failure.

KEYWORDS

anthocyanidins, heart failure, flavonoids, cardiovascular disease, NHANES

Introduction

Heart failure (HF) is the terminal manifestation of cardiovascular disease (1). In recent years, the prevalence of HF has gradually increased, and its mortality and disability rate have also increased. The number of people with HF worldwide is predicted to be close to 64.3 million (2). Despite continuous progress in the treatment of HF, due to frequent hospital stays and ongoing treatment, patients with HF have severe everyday limits and bear a heavy financial

burden (3). Moreover, 50% of people with HF with a decreased ejection fraction pass away within 5 years after being diagnosed (4). Thus, it is essential to inhibit the occurrence and development of HF.

With a focus on diet, people have realized that traditional western diet, such as red meat, high sugar food and fried food, are harmful to heart health (5), while omega-3 fatty acids (6), polyphenolic and flavonoids (7), as well as other micronutrients that are abundant in Indo-Mediterranean diets (8), may all play a protective role in maintaining the heart health. Anthocyanidins are one of the six major categories of flavonoids, and the anthocyanidins consumed in diet are mainly provided by fruits such as berries (9). Anthocyanidins have powerful anti-inflammatory and antioxidant properties, making them useful in the prevention of a variety of chronic diseases, such as eye and kidney complications and many cancer types (10–13). An increasing number of evidences show that anthocyanidins is related to circulatory disease, and have shown significant lipid-lowering effects in many studies (14, 15). Anthocyanidins also have positive effects on endothelial function and have antiatherogenic and anti-arterial stiffness properties (16). According to the meta-analysis, dietary anthocyanidins intake was linked to a lower risk of coronary heart disease and a lower mortality of cardiovascular diseases (17). Moreover, the link between anthocyanidins and cardiovascular diseases has been verified by numerous experimental research. Such as, anthocyanidins played a chemo-preventive role in atherosclerosis *via* activation of Nrf2-ARE pathway (18); through suppression of the ROS-JNK-Bcl-2 pathway, anthocyanidins reduces myocardial ischemia-induced damage (19).

However, the protective effect of anthocyanidins on HF has not been reported. Therefore, the purpose of this study was to assess the impact of dietary anthocyanidins on HF in the general American population.

Materials and methods

Study population

The National Health and Nutrition Examination Survey (NHANES) is a series of surveys designed on the basis of cross-sections to investigate the health status of all U.S. populations, which conducted by National Center for Health Statistics (NCHS). The survey included demographic information, dietary information, various physical examination indicators and health related data. All information and survey methods are available online.[1] The NCHS Research Ethics Review Board authorized the research protocols and each participant signed a written statement of informed consent. Since only three NHANES circles (2007–2008, 2009–2010 and 2017–2018) investigated the dietary intake of flavonoids, this study included an investigation of those three NHANES circles. Exclusion criteria included: age <18 years; missing HF status; missing dietary information about flavonoids (Figure 1).

1 https://www.cdc.gov/nchs/nhanes/index.htm

Assessment of dietary anthocyanidins intakes

This study mainly collects the intake of flavonoids in foods and beverages, which are usually onions, potatoes, celery, etc. According to the food code from Nutrient Database for Dietary Studies (FNDDS), the food types were refined. Different codes represent different flavonoid contents. Versions of the FNDDS that are suitable for each survey cycle were utilized: version 4.1 was used for 2007–2008, while version 5.0 was used for 2009–2010 and 2017–2018 (20). Six of the flavonoid classes (anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols and isoflavones) as well as the total daily intake of all flavonoids (the sum of the 29 individual flavonoids) were calculated from all foods and beverages.

Assessment of HF

Like previous NHANES-based articles that have been published (21), participants were asked in the health questionnaires "whether a doctor or other health professional has ever told you that you had heart failure" and those who responded "yes" were considered to have HF.

Covariates

NHANES collected demographic data on all participants. Race was divided into four categories, non-Hispanic White, non-Hispanic Black, Mexican American, and others. Smoking was divided into two categories: yes (now, former) and no (never). Diabetes, hypertension and hyperlipidemia were all diagnosed by doctors. The systolic and diastolic blood pressure, body mass index (BMI), and waist measured by experts using conventional physical examination techniques. In a typical laboratory, the level of triglycerides (TG), total cholesterol (TC), high-density cholesterol (HDL), low-density cholesterol (LDL), fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), and creatinine were all measured. We calculated the estimated glomerular filtration rate (eGFR) through the creatinine equation. Information about the specific techniques and quality of the determination of all covariate control methods were accessible from Website of NHANES.

Statistical analysis

For all statistical studies, R Programming Language (version 4.2.1) were used. Statistical significance was determined to two-tailed, $p < 0.05$. Analysis method was referred to previously NHANES-based articles (22). Participants were divided into four groups (Q1-4) according to the quartile of anthocyanidins intake. We adjusted the weights in our analysis to prevent oversampling and lower the non-response rate. Weighted means (95% confidence intervals [CIs]) and weighted percentages (95% CIs) were used to describe continuous variables and categorical variables, respectively. To evaluate differences between groups, the categorical variables used a weighted chi-square test and the continuous variables used a weighted linear regression model. Univariable and multivariable logistic regression models were

used to analyze the connections between HF and anthocyanidins consumption in all participants and different subgroups (Figure 1).

Results

Baseline characteristics of study population

This study enrolled 15,869 participants which including 513 (3.23%) HF patients (Figure 1). The average age of all participants was 47.40 (46.81, 47.99) years old, including 48.65% men and 51.35% women. The baseline characteristics are shown based on the dietary anthocyanidins intake quartiles (Q1: 0 mg; Q2: [0, 0.73] mg; Q3: [0.73, 6.29] mg and Q4: >6.29 mg) (Table 1). Compared to the other quartiles, individual who divided in Q4 group were likely to be older, female, non-Hispanic White, receive medications of statin. Regarding the traditional risk factors for cardiovascular disease, the Q4 group had increased levels of HDL cholesterol but lower levels of BMI, waist, triglycerides, and diastolic pressure. Most importantly, the prevalence of HF was lower in the Q4 group (Q1:2.54%, Q2:2.33%, Q3:2.43%, Q4:1.57%, $p = 0.02$) (Figure 2), but there is no significant difference in the prevalence of coronary heart disease, stroke and diabetes.

Association between dietary anthocyanidins intake and HF

We compared the dietary intake of flavonoids between non-HF and HF. Surprisingly, although there was no difference in the intake of total flavonoids and other five flavonoids, the intake of anthocyanidins in non-HF was higher than that in HF (13.84 [12.52, 15.17] mg vs. 8.04 [5.81, 10.28] mg; $p < 0.0001$). Moreover, the five anthocyanidins subclasses (cyanidin, delphinidin, petunidin, malvidin, peonidin) also showed the same trends (Table 2). In Supplementary Table S1, the findings of univariate logistic regression analyses for HF were shown. The dietary anthocyanidins intake were negatively correlated with HF. Conversely, risk factors for cardiovascular diseases, such as age, smoke, BMI, waist, triglycerides, fasting plasma glucose were positively with HF. Compared to the Q1 group, participants with highest dietary anthocyanidins (Q4) intake showed a lower presence of HF (OR 0.61, 95% CI [0.46–0.81]; $p < 0.001$) in the unadjusted model. Table 3 displayed the findings of multivariate logistic regression analysis for the relationship between dietary anthocyanidins intake and HF. Highest dietary anthocyanidins intake (Q4) was independently associated with lower presence of HF with adjustment for age, sex, race, smoke, BMI, waist, systolic pressure, diastolic pressure, diabetes, hypertension, hyperlipidemia, coronary heart disease, stroke, ACE inhibitor, Beta blocker, diuretics, statin, eGFR, creatinine, HbAlc, FPG, HDL, LDL, TC, TG (OR 0.467, 95% CI [0.302, 0.751]; $p = 0.003$). Additionally, we transformed intake of dietary anthocyanidins into a categorical variable (Q1-4), both the unadjusted (p for trend <0.001) and adjusted (p for trend = 0.005) models showed significant p for trends.

Subgroup analyses

Through subgroup analysis, we further investigate the correlation between dietary anthocyanidins intake and HF in different populations (Figure 3). The whole population was stratified by age, sex, race and different disease status. In the subgroups of ≥45 years (OR 0.49, 95% CI [0.35, 0.67]), female (OR 0.50, 95% CI [0.35, 0.72]), smoker (OR 0.69, 95% CI [0.48, 1.00]), non-Hispanic White (OR 0.65, 95% CI [0.45, 0.93]) or without diabetes (OR 0.45, 95% CI [0.28, 0.73]), stroke (OR 0.60, 95% CI [0.43, 0.85]), renal failure (OR 0.49, 95% CI [0.33.0.73]), this association was still stable. Furthermore, we conducted independent multivariate logistic regression analysis for each subgroup. The variables enrolled in model3 were all retained in this analysis except for the variables that were used for stratification. These trends were consistent with before.

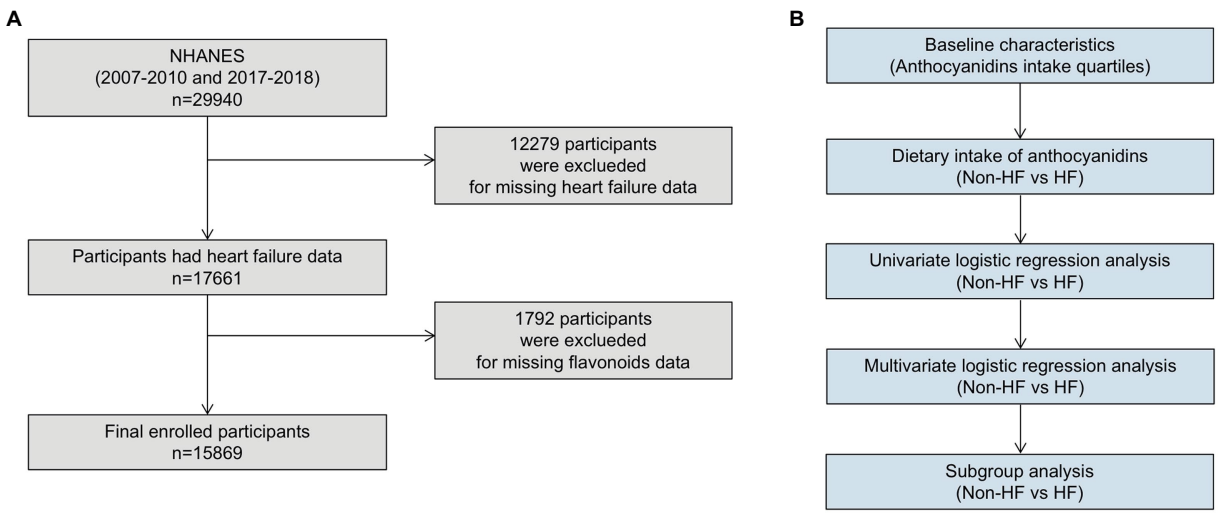

FIGURE 1
(A) Flow chart of participants selection; **(B)** flow chart of data analysis.

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TABLE 1 Baseline characteristics of participants.

Variable	Total	Q1	Q2	Q3	Q4	*p* value
Count	15,869 (100.00)	5,598 (35.28)	2,343 (14.76)	3,961 (24.96)	3,967 (25)	
Total Anthocyanidins, mg	13.72 (12.41,15.02)	0.00(0.00, 0.00)	0.24(0.22, 0.25)	2.82(2.75, 2.88)	47.59 (44.47,50.71)	<0.0001
Age	47.40 (46.81,47.99)	44.39 (43.73,45.04)	47.10 (45.98,48.22)	48.87 (48.06,49.68)	50.18 (49.24,51.11)	<0.0001
Gender						<0.0001
Male	7,720 (48.65)	2,960 (53.29)	1,086 (46.66)	1916 (49.01)	1758 (42.10)	
Female	8,149 (51.35)	2,638 (46.71)	1,257 (53.34)	2045 (50.99)	2,209 (57.90)	
Race						<0.0001
White	7,007 (44.16)	2,504 (67.08)	1,003 (66.12)	1,550 (62.93)	1950 (71.56)	
Black	3,287 (20.71)	1,390 (13.90)	465 (10.39)	783 (11.60)	649 (8.27)	
Mexican	2,592 (16.33)	751 (7.17)	435 (10.35)	838 (10.92)	568 (6.99)	
Other	2,983 (18.8)	953 (11.85)	440 (13.14)	790 (14.55)	800 (13.18)	
Smoke						<0.0001
No	8,652 (54.52)	2,678 (48.00)	1,319 (57.40)	2,256 (57.86)	2,399 (61.84)	
Yes	7,216 (45.48)	2,920 (52.00)	1,023 (42.60)	1705 (42.14)	1,568 (38.16)	
Body mass index, kg/m2	29.09 (28.86,29.32)	29.82 (29.51,30.13)	29.12 (28.65,29.59)	29.00 (28.67,29.33)	28.22 (27.90,28.53)	<0.0001
Waist, cm	99.13 (98.48,99.77)	100.93 (100.04,101.82)	99.04 (97.97,100.11)	98.94 (97.98, 99.91)	97.01 (96.27, 97.75)	<0.0001
Systolic pressure, mmHg	121.67 (121.14,122.19)	122.13 (121.39,122.87)	121.23 (120.29,122.17)	122.10 (121.11,123.10)	120.95 (120.12,121.78)	0.09
Diastolic pressure, mmHg	71.09 (70.46,71.72)	71.84 (70.98,72.70)	71.35 (70.50,72.21)	70.76 (70.05,71.46)	70.28 (69.60,70.95)	0.001
Triglycerides, mg/dl	152.81 (149.11,156.51)	154.89 (148.77,161.01)	155.22 (149.07,161.37)	161.31 (155.62,167.00)	141.77 (137.59,145.96)	<0.0001
Total cholesterol, mg/dl	194.43 (192.89,195.98)	192.81 (190.39,195.23)	195.28 (192.64,197.92)	195.22 (193.52,196.92)	195.38 (193.01,197.75)	0.19
HDL cholesterol, mg/dl	52.97 (52.42,53.52)	50.92 (50.20,51.64)	52.82 (51.85,53.79)	52.45 (51.69,53.21)	56.10 (55.26,56.94)	<0.0001
LDL cholesterol, mg/dl	114.31 (112.96,115.67)	113.88 (111.40,116.35)	115.99 (112.66,119.32)	114.83 (112.79,116.87)	113.58 (111.18,115.98)	0.72
FPG, mmol/L	5.95 (5.89,6.01)	5.94 (5.86,6.02)	5.96 (5.85,6.08)	5.97 (5.87,6.07)	5.94 (5.82,6.07)	0.94
Hemoglobin A1c, %	5.63 (5.61,5.66)	5.62 (5.60,5.65)	5.63 (5.59,5.67)	5.68 (5.63,5.73)	5.60 (5.57,5.64)	0.02
eGFR, mL/min/1.73 m^2	94.64 (93.71,95.56)	96.92 (95.87,97.97)	94.74 (93.24,96.25)	94.01 (92.90,95.12)	92.20 (90.91,93.48)	<0.0001
Creatinine, mg/dl	0.88 (0.87,0.89)	0.89 (0.88,0.91)	0.87 (0.86,0.89)	0.88 (0.87,0.89)	0.87 (0.86,0.88)	0.004
Diseases						
Heart failure	513 (3.23)	201 (2.54)	78 (2.33)	137 (2.43)	97 (1.57)	0.02
DM						0.23
DM	3,080 (19.62)	1,058 (14.10)	454 (13.93)	852 (15.96)	716 (13.49)	
IFG	814 (5.19)	311 (5.56)	114 (5.60)	184 (4.51)	205 (5.39)	
IGT	474 (3.02)	140 (2.19)	77 (2.76)	130 (2.94)	127 (3.05)	
No	11,328 (72.17)	4,042 (78.14)	1,668 (77.71)	2,756 (76.59)	2,862 (78.07)	
Hypertension	6,933 (43.69)	2,455 (38.38)	1,042 (37.38)	1765 (38.67)	1,671 (35.93)	0.33
Hyperlipidemia	11,227 (70.76)	3,917 (68.76)	1,659 (70.66)	2,858 (70.49)	2,793 (67.79)	0.27
Coronary heart disease	666 (4.21)	226 (3.19)	90 (3.17)	155 (3.36)	195 (4.20)	0.06
Stroke	702 (4.43)	252 (3.08)	132 (3.92)	174 (3.45)	144 (2.68)	0.16
Medications						
ACE inhibitors	386 (2.43)	125 (1.78)	71 (2.66)	102 (2.18)	88 (1.95)	0.31
Beta blocker	2,153 (13.58)	713 (10.29)	329 (12.17)	554 (12.39)	557 (11.40)	0.13
Diuretics	2,319 (14.62)	760 (10.40)	365 (12.90)	617 (12.59)	577 (11.51)	0.1
Statin	3,125 (19.71)	957 (13.91)	501 (17.30)	845 (19.02)	822 (18.00)	<0.0001

eGFR, glomerular filtration rate; HDL cholesterol, high-density cholesterol; LDL cholesterol, low-density cholesterol; FBG, fasting plasma glucose; DM, diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

Discussion

As far as we know, this study was the first time to demonstrate the association between dietary anthocyanidins intake and HF, dietary anthocyanidins consumption (categorical) and HF were found to be negatively correlated in the NHANES 2007–2010 and 2017–2018. When dietary anthocyanidins intake was in the Q4 group, the incidence of HF was lowered by 50% after adjusting probable confounding factors. This negative correlation is still stable in subgroups of female, ≥45 years, former smoker, non-Hispanic White or without diabetes, stroke, renal failure. Moreover, compared with anthocyanidins intake between HF and non-HF for each quartile, it was found that only anthocyanins intake of Q4 was different between HF and non-HF, the intake of anthocyanins in non-HF was higher than that in HF (Supplementary Figure S1; Supplementary Table S2).

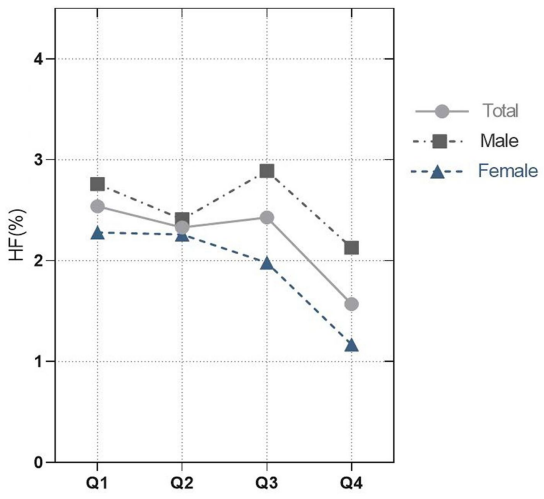

FIGURE 2
Proportion of heart failure in the quartile of anthocyanidins.

At present, cardiovascular disease has caused great social burden, so many scholars are emphasizing the importance of dietary habits in the prevention and treatment of cardiovascular diseases (23). With the increasing demand for flavonoids (24), anthocyanidins have been discovered many benefits for cardiovascular system, which is a subgroup of flavonoids (25). As some studies have shown, a high dietary intake of anthocyanidins was linked to decreased total cardiovascular disease incidence and mortality. For example, Adriouch S et al. found that participants in the highest tertiles of anthocyanidins had a 34% lower risk of major cardiovascular events than those in the lowest tertiles following multivariable adjustment (26). Besides, Lin Xu et al. confirmed that dietary intake of anthocyanidins significantly decreased the risk of death from all cardiovascular diseases in meta-analysis including 2,36,648 subjects and 9,765 cases (RR: 0.91, 95% CI [0.87, 0.96]; $p < 0.001$), it was also found that dietary anthocyanidins may have a more significant protective effect on total CVD mortality in women (27). Additionally, dietary anthocyanidins intake is also beneficial in vascular diseases. Such as, Margarethe E Goetz et al. reported that anthocyanidins intake was negatively correlated with incident of coronary heart disease after matching age, sex, race and residence (28). Moreover, anthocyanidins consumption in the diet is crucial for preventing subclinical injury of cardiovascular disease, such as hyperlipidemia, obesity, vascular endothelial function, arterial stiffness, decreased cardiac systolic function (29–32) Although some studies believed that purified anthocyanidins had more cardioprotective effects than dietary anthocyanidins (27), this conclusion is still controversial due to incompleteness of dietary data and differences in interventions (33–36). The evidence for the benefits of dietary anthocyanidins on cardiovascular system is very strong, but our research revealed for the first time a connection between dietary anthocyanidins intake and the presence of HF in the general population.

Although our clinical studies revealed an association between dietary intake of anthocyanidins and HF, the underlying mechanism had not been clarified. Thus, we summarized the

TABLE 2 Flavonoid in patients with or without heart failure.

Variable	Total	Non-HF	HF	*p* value
Total anthocyanidins, mg	13.72 (12.41, 15.02)	13.84 (12.52, 15.17)	8.04 (5.81, 10.28)	<0.0001
Total isoflavones, mg	2.01 (1.70, 2.32)	2.02 (1.71, 2.34)	1.55 (0.68, 2.42)	0.29
Total flavan-3-ols, mg	186.91 (171.93, 201.89)	186.93 (171.76, 202.10)	186.01 (127.14, 244.88)	0.98
Total flavanones, mg	12.27 (11.51, 13.02)	12.28 (11.52, 13.03)	11.90 (9.10, 14.70)	0.79
Total flavonols, mg	19.52 (18.79, 20.25)	19.58 (18.84, 20.32)	16.89 (14.13, 19.66)	0.07
Total flavones, mg	0.92 (0.86, 0.97)	0.92 (0.87, 0.97)	0.79 (0.46, 1.13)	0.46
Total sum of all flavonoids, mg	235.35 (219.62, 251.07)	235.58 (219.63, 251.52)	225.19 (163.24, 287.13)	0.75
Anthocyanidins				
Cyanidin, mg	2.55 (2.20, 2.89)	2.57 (2.22, 2.92)	1.61 (1.13, 2.09)	<0.001
Delphinidin, mg	1.76 (1.30, 2.21)	1.78 (1.32, 2.25)	0.66 (0.35, 0.97)	<0.0001
Petunidin, mg	1.13 (0.96, 1.30)	1.14 (0.97, 1.32)	0.55 (0.27, 0.82)	<0.0001
Malvidin, mg	4.75 (4.24, 5.26)	4.80 (4.28, 5.31)	2.72 (1.66, 3.78)	<0.001
Peonidin, mg	1.94 (1.65, 2.22)	1.96 (1.67, 2.24)	1.04 (0.58, 1.49)	0.001
Pelargonidin, mg	1.60 (1.37, 1.83)	1.60 (1.37, 1.83)	1.47 (0.72, 2.22)	0.75

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TABLE 3 Associations between total anthocyanidins and heart failure.

	Unadjusted model	Model 1	Model 2	Model 3
Character	95%CI, *p*	OR 95%CI, *p*	OR 95%CI, *p*	OR 95%CI, *p*
Q1	ref	ref	ref	ref
Q2	0.919 (0.644, 1.310)	0.780 (0.540, 1.128)	0.800 (0.551, 1.160)	0.583 (0.282, 1.205)
	0.633	0.182	0.232	0.112
Q3	0.957 (0.699, 1.310)	0.708 (0.513, 0.978)	0.728 (0.527, 1.004)	0.779 (0.465, 1.307)
	0.777	0.037	0.053	0.321
Q4	0.614 (0.465, 0.811)	0.426 (0.324, 0.562)	0.446 (0.336, 0.591)	0.467 (0.302, 0.751)
	<0.001	<0.001	<0.001	0.003
p for trend	<0.001	<0.001	<0.001	0.005

Unadjusted model: univariate logistic regression analyses. Model 1 was adjusted for age, sex. Model 2 was adjusted for variables included in Model 1 and race. Model 3 was adjusted for variables included in Model 1 and smoke, body mass index, waist, systolic pressure, diastolic pressure, diabetes, hypertension, hyperlipidemia, coronary heart disease, stroke, ACE inhibitor, Beta blocker, diuretics, statin, eGFR, creatinine, hemoglobin A1c, fast glucose, HDL cholesterol, LDL cholesterol, total cholesterol, triglycerides. OR, odd ratio; CI, confidence interval.

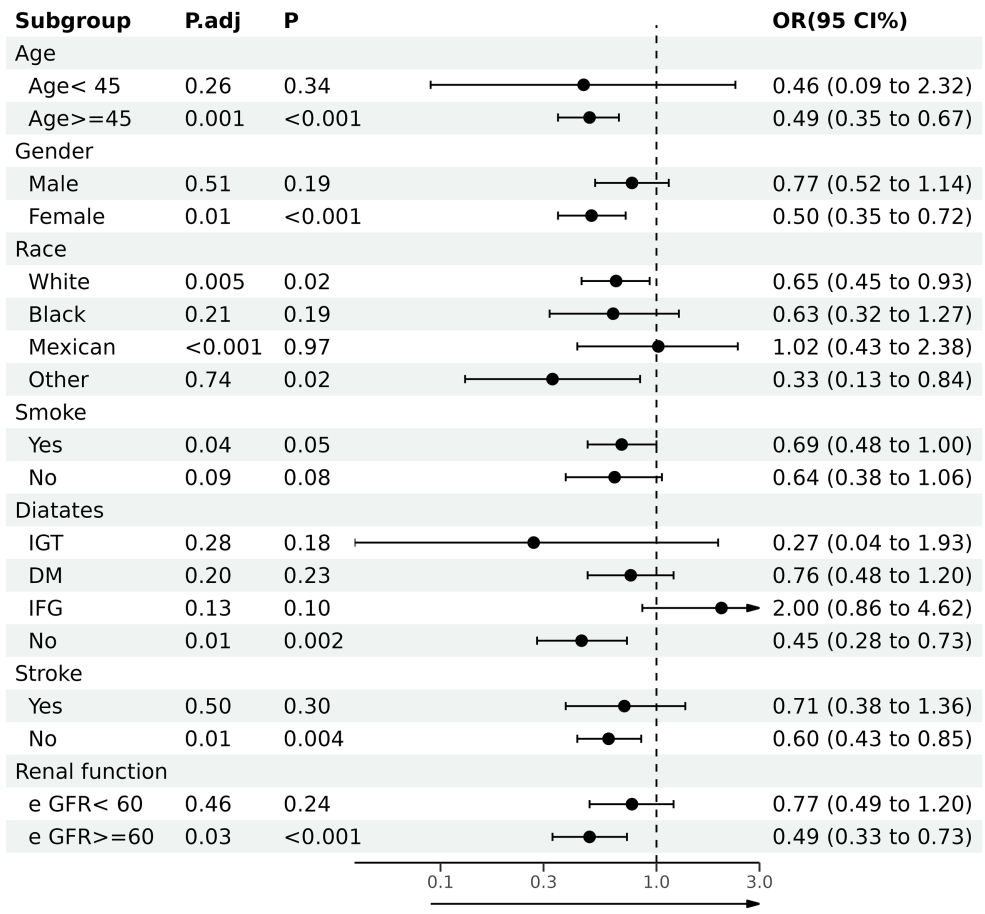

FIGURE 3
Association between total anthocyanidins (Q4) and heart failure in various stratifications. OR, odd ratio; CI, confidence interval; DM, diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; eGFR, glomerular filtration rate.

following four possible mechanisms through literature summary. First, oxidative stress disorder plays a crucial role in the in the occurrence and development of HF. When the production of reactive oxygen species (ROS) exceeds the internal defense capacity of cells, excess ROS will attack cells, leading to protein and lipid peroxidation, DNA damage, and ultimately cell death (37). Anthocyanidins, a naturally occurring plant pigment, not only serves as a colorant but also has strong antioxidant properties, ROS such superoxide anion, singlet oxygen, and peroxide free radical can be neutralized by anthocyanidins (38). Second, in the

pathophysiological process of chronic HF, a long-lasting inflammatory response causes adverse ventricular remodeling (39). The anti-inflammatory capabilities of anthocyanidins have also been proven in numerous research (40–44), anthocyanidins can inhibit NF-κB activity to reduce inflammation level. Third, some clinical studies have found that anthocyanidins can improve cardiovascular metabolic disorder and obesity, which are high risk factors for HF (29, 30). Fourth, rich-anthocyanidins foods also contain dietary fiber, vitamins and various polyphenols, which also have certain protective effects on heart health (45–48). To investigate established and speculative mechanisms, further basic and clinical research is required.

Nevertheless, there were some limitations in this study. First, as a cross-sectional study, this study was unable to confirm the causal relationship between dietary intake of anthocyanidins and HF. Second, this study only evaluated the effect of anthocyanidins in food, but whether the purified anthocyanidins had the same effect still needs further randomized controlled experiments. Third, the NHANES database does not provide brain natriuretic peptide and echocardiography data, so this study cannot further evaluate the relationship between dietary intake of anthocyanidins and the severity of HF. Moreover, uncontrollable confounding variables may also need further analysis, such as physical activity and nutritional supplements. Finally, the subjects of this study were adult Americans, excluding adolescents and children, which would affect the promotion of the research results.

Conclusion

In conclusion, dietary intake of anthocyanidins was associated with HF negatively, people can decrease the presence of HF by increasing anthocyanidins in their daily diets. To determine their clear relationship, more cellular, animal, and human investigations are necessary.

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 studies involving human participants were reviewed and approved by National Health and Nutrition Examination Survey (NHANES). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

ZT conceived and designed the study. RZ, WZ, and ZJ were responsible for the management and retrieval of data, contributed to initial data analysis, and interpretation. ZT drafted the initial manuscript. XC, RH, and XL revised the manuscript and were the guarantors of this work and had full access to all the data in the study. GM take responsibility for its integrity and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1107637/full#supplementary-material

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Association of plant-based diets with adropin, atherogenic index of plasma, and metabolic syndrome and its components: A cross-sectional study on adults

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Background: Little is known about the association of plant-based diet indices with metabolic syndrome (MetS) and its novel predictive biomarkers, including the atherogenic index of plasma (AIP) and adropin. We aimed to investigate the association of plant-based diets with adropin, atherogenic index of plasma, and MetS and its components in adults.

Methods: The present population-based cross-sectional study was conducted on a representative sample of adults aged 20–60 years in Isfahan, Iran. Dietary intake was obtained through a validated 168-item semi-quantitative food frequency questionnaire (FFQ). Peripheral blood was obtained after an overnight fast of at least 12 h from each participant. MetS was identified based on the Joint Interim Statement (JIS). AIP was calculated as a logarithmically transformed ratio of triglyceride (TG)/high-density lipoprotein cholesterol (HDL-c), and serum levels of adropin were measured by an ELISA kit.

Results: A total of 28.7% of subjects had MetS. No significant association was found between the overall plant-based diet index (PDI) and the healthful plant-based diet index (hPDI) with MetS. However, a non-linear association was observed between hPDI and MetS. Subjects in the third quartile of the unhealthful plant-based diet index (uPDI) had higher odds of MetS compared to the first quartile (OR: 2.39; 95% CI: 1.01, 5.66). The highest quartile of PDI (OR: 0.46; 95% CI: 0.21, 0.97) and the third quartile of hPDI (OR: 0.40; 95% CI: 0.18, 0.89) were associated with decreased odds of having high-risk AIP compared to the first quartile, after adjusting for potential confounders. No linear association was found between quartiles of plant-based diet indices and serum levels of adropin.

Conclusion: Plant-based diet index (PDI) and hPDI were not associated with the prevalence of MetS in adults, while moderate adherence to uPDI increased the prevalence of MetS. In addition, high adherence to PDI and moderate adherence to hPDI were associated with decreased odds of high-risk AIP. No significant association was found between plant-based diet indices and serum adropin levels. To confirm these findings, further studies with prospective designs are warranted.

KEYWORDS

plant-based diet indices, metabolic syndrome, atherogenic index of plasma, adropin, cross-sectional study

Introduction

Metabolic syndrome (MetS) is a condition defined by a cluster of metabolic disorders, including impaired fasting glucose, high blood pressure, abdominal obesity, and dyslipidemia [hypertriglyceridemia and low high-density lipoprotein cholesterol (HDL-c) levels] (1, 2). The worldwide prevalence of MetS in the adult population ranges from 20 to 25% (3), with a growing trend in both developing and developed countries (4–6). This public health problem has been considered an important risk factor for developing type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVDs), as well as all-cause mortality (7, 8). Although the exact underlying pathophysiology of MetS has not been clearly determined, abdominal obesity and insulin resistance as the results of a sedentary lifestyle and unhealthy eating patterns might play key roles in developing MetS (9, 10).

Recently, some biomarkers including adiponectin, leptin, resistin, apelin, and irisin might serve as MetS predictors (11, 12). One of the novel biomarkers that might contribute to MetS development is adropin. Adropin that contributes to nutrients and energy hemostasis is encoded by the energy homeostasis associated (Enho) gene and is expressed in the brain and the liver (13). Low serum adropin levels are associated with an increased risk of T2DM, endothelial dysfunction, obesity, and MetS (14–16). Another novel predictive biomarker of obesity and MetS is the atherogenic index of plasma (AIP) (17, 18). The sensitivity of AIP to reflect the interaction of protective lipoproteins with atherogenic ones is higher than the other atherogenic indices (17). Previous studies reported that elevated levels of AIP are associated with higher waist circumference and increased risk of chronic diseases (19, 20). In addition, AIP has been considered a strong predictor for developing MetS (17).

Environmental factors, including eating patterns, might be associated with the prevalence of MetS and related indices. Ganesh Kumar et al. reported that a low-carbohydrate high-fat diet compared to a high-carbohydrate low-fat diet increased the serum adropin levels in mice; they additionally reported that diet-induced obesity and overnight fasting conditions might suppress the serum levels of adropin (21). Recent studies reported that healthy lifestyle behaviors, including healthy eating patterns and physical activity, were associated with low levels of AIP (22, 23). In the case of MetS, previous studies suggested that adherence to the Dietary Approaches to Stop Hypertension (DASH) and Mediterranean diets decreased, and the animal-based diets increased the risk of MetS (24–26). In addition, previous studies reported that healthy plant foods including whole grains, vegetables, fruits, and nuts were associated with a lower risk of MetS. However, some less healthy plant foods such as refined grains and sugar-rich plant foods were associated with a higher risk of MetS (27, 28). This difference between plant foods and their association with the risk of disease is reflected in a graded scoring system named plant-based diet indices.

Plant-based diet indices include three indices as follows: an overall plant-based diet index (PDI) which represents the intake of all plant food with decreasing the consumption of animal food. A healthful plant-based diet index (hPDI) represents the consumption of healthy plant foods; and an unhealthful plant-based diet index (uPDI) represents the intake of less healthy plant foods (29). Previous studies demonstrated that hPDI was associated with a lower risk of chronic diseases, while adherence to uPDI was associated with a greater risk of chronic diseases (27–30).

No previous study has evaluated the association of plant-based diet indices with adropin and AIP, and a limited number of studies examined the association between plant-based diet indices and metabolic syndrome, especially in Middle Eastern countries. Therefore, we aimed to investigate the association of plant-based diets with adropin, atherogenic index of plasma, and metabolic syndrome and its components in adults.

Materials and methods

Study design and population

The present population-based cross-sectional study was conducted on a somehow representative sample of adults aged 20–60 years in Isfahan, Iran, in 2021. Isfahan was considered as big city located in the center of Iran, with six educational districts consisting of 285 primary and secondary schools. Three or four schools were chosen across each district, and 20 schools were totally defined as our final sample location, using a stratified multistage random cluster sampling method. After that, information on the study research was sent to selected schools. After the agreement of the administrators of the schools, the subjects that consented to take part in the study were recruited. To attain a relatively representative sample of the general population, all adults who were working in the selected schools, such as employees, teachers, school managers, assistants, and crews, were included in the present studies. Subjects with a history of diseases, including CVD, stroke, type 1 diabetes, and cancer, or those who used a special diet during the last 6 months, were pregnant or lactating were not included in the current study.

Since no previous study has evaluated the association between adropin and dietary indices, we used irisin (as a similar analogous factor for adropin) to calculate the sample size of the study (31, 32). Considering a power of 80%, alpha error of 0.1, and a correlation coefficient of 0.1 for the association of irisin with dietary indices, the sample size was calculated to be at least 450 subjects; by taking a low response rate into account, a total of 600 subjects were invited to the current study. Finally, a total of 527 adults were included in the current study for metabolic syndrome and AIP analysis, and

Abbreviations: MetS, metabolic syndrome; AIP, atherogenic index of plasma; FFQ, food frequency questionnaire; JIS, Joint Interim Statement; TG, triglyceride; HDL-c, high-density lipoprotein cholesterol; PDI, overall plant-based diet index; hPDI, healthful plant-based diet index; uPDI, unhealthful plant-based diet index; OR, odds ratio; 95% CI, 95% confidence interval; T2DM, type 2 diabetes mellitus; CVDs, cardiovascular diseases; Enho gene, energy homeostasis associated gene; DASH, Dietary Approaches to Stop Hypertension; BMI, body mass index; WC, waist circumference; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; LDL-c, low-density lipoprotein cholesterol; IPAQ-SF, the International Physical Activity Questionnaire Short form; HEPA, health-enhancing physical activity; ANOVA, analysis of variance; SES, socioeconomic status; MD, Mediterranean Diet; HFCS, high-fructose corn syrup.

497 subjects were included for adropin-related analysis. The Ethics Committee of the Isfahan University of Medical Sciences approved the protocol of the study (no: IR.MUI.RESEARCH.REC.1400.370), and all participants signed a written informed consent.

Dietary intake assessment

A validated 168-item semi-quantitative food frequency questionnaire (FFQ) was used to evaluate usual dietary intake during the last year in the study population (33). Based on the standard protocol, a trained dietitian instructed the participants on how to complete this self-administered dietary questionnaire. Participants were asked how often they consumed food items on the basis of 10 categories of frequency ("seldom/never," once per month, 2–3 per month, once per week, 2–3 per week, 4–6 per week, 1 per day, 2–3 per day, 4–5 per day, and 6 or more per day). In addition, they were asked to report the portion sizes of each food and beverage item. By using household measurements, the frequency of consumption was changed to daily intake, and portion sizes were converted to grams. Finally, the food intake (g/day) was transformed to Nutritionist IV software (version 7; N-squared computing, OR, United States), to compute the total energy and nutrient intake.

Plant-based diet indices

Using the dietary intake data, three types of plant-based diet indices including PDI, hPDI, and uPDI were created through the use of the method proposed by Satija et al. (29). In brief, all foods were divided into 18 groups according to similarities of nutritional and culinary characteristics. These 18 food groups belonged to broader categories of healthy plant food groups (whole grains, fruits, vegetables, nuts, legumes, vegetable oils, and tea and coffee), less healthy plant food groups (fruit juices, refined grains, potatoes, sugar-sweetened beverages, sweets, and desserts), and animal food groups (animal fats, dairy, eggs, fish, meat, and miscellaneous animal-based foods; Supplementary Table 1). On account of changing the fatty acid profile over time for margarine and hydrogenated vegetable oils, these two items were not included in the indices calculation, and instead, we made an adjustment for them in multivariable models. Because of the lack of accurate reporting due to limitations in alcohol consumption in the Iranian population, this item was not considered in the current analysis. To calculate three indices, the food groups were first adjusted for energy intake using residual methods (34, 35). A total of 18 energy-adjusted food groups were ranked into quintiles, and positive or reverse scores were assigned to them. For positive scores, subjects in the lowest quintile of food group consumption were given a score of 1, whereas those in the highest quintile were given a score of 5. For reverse scores, subjects in the lowest quintile of food group consumption received a score of 5, whereas those in the highest one received a score of 1. For the PDI, both healthy and unhealthy plant foods received positive scores. However, for the hPDI and uPDI, only healthy plant foods and unhealthy plant foods were given positive scores, respectively. In all three indices, animal food groups were assigned reverse scores (Supplementary Table 1). All plant-based diet indices theoretically ranged from 18 to 90 and higher scores were associated with greater adherence to the diet index (29).

Anthropometric and blood pressure measurement

Weight measurement was conducted using the body composition analyzer (Tanita MC-780MA, Tokyo, Japan) with 0.01 kg accuracy, while participants were minimally clothed without shoes. Height was measured using a non-stretch tape to the nearest 0.1 cm while subjects unshod. Body mass index (BMI) was computed as weight (kg)/height^2 (m^2). In addition, waist circumference (WC) was assessed to the nearest 0.1 cm at the midway level between the lower rib margin and the iliac crest at the end of exhalation in standing positions and without any pressure on the body surface.

Arterial blood pressure (BP) was measured two times, while subjects were seated comfortably after a resting period of at least 5 min. The participants were asked to be overnight fast and refrain from smoking and exercise for at least half an hour before BP measurement; if the bladder was full, it should be emptied. Systolic and diastolic BPs (SBP and DBP) were measured by a digital sphygmomanometer (OMRON, M3, HEM-7154-E, Japan), with an accuracy of 0.5 mmHg, on the left arm. BP was defined as the mean of the first and second measurements (36).

Assessment of biochemical parameters

A measure of 10 ml of peripheral blood was obtained using venipuncture, after an overnight fast of at least 12 h from each participant. The serum was separated by centrifugation at 3,500 rpm for 10 min. Biochemical parameters [including fasting blood glucose (FBG), serum levels of total cholesterol, HDL-c, low-density lipoprotein cholesterol (LDL-c), and serum triglyceride (TG)] were measured by the enzymatic colorimetric method using BioSystem Kit Company on Biosystem A15 autoanalyzer. AIP was calculated as a logarithmic transformation of the ratio of TG to HDL-c (37). ZellBio GmbH ELISA Kit (Germany) was used for the quantitative assay of human adropin on the basis of the Biotin double antibody sandwich technology. The assay range of adropin ranged from 30 to 960 pg/mL with 4 pg/mL sensitivity; the intra-assay and inter-assay CV were <10 and $<12\%$, respectively.

Definition of MetS

The Joint interim statement (JIS) was applied to define MetS. Subjects who met at least three of the following conditions were considered as MetS: (1) elevated WC (WC $\geq$ 94 cm in men and $\geq$80 cm in women), (2) elevated TG [TG $\geq$ 150 mg/dl (1.7 mmol/L) or on drug treatment for elevated triglycerides], (3) hypertension (SBP $\geq$ 130 mmHg or DBP $\geq$ 85 mmHg or on antihypertensive drug treatment in a patient with a history of hypertension), (4) hyperglycemia (FBG $\geq$ 100 mg/dl or on drug treatment for

elevated glucose), and (5) reduced HDL-c [HDL-c < 40 mg/dl (1.03 mmol/L) in men and <50 mg/dl (1.3 mmol/L) in women] (38).

Covariates assessment

Demographic and socioeconomic characteristics of the study population were evaluated by self-reported questionnaires. In addition, to evaluate the physical activity, the International Physical Activity Questionnaire Short Form (IPAQ-SF) was applied, and subjects were divided into three categories including inactive, minimally active, and health-enhancing physical activity (HEPA) (39).

Statistical analysis

To evaluate the normality of quantitative variables, the Kolmogorov–Smirnov test was applied. The quantitative variables were illustrated as mean ± SD/SE and qualitative variables as frequency (percentage). To compare the quantitative variables across quartiles of plant-based diet indices, a one-way analysis of variance (ANOVA) was applied, while for categorical variables, the chi-square test was used. In addition, the independent sample *t*-test was applied to examine the quantitative variables between subjects with and without metabolic syndrome. The general linear model was applied to evaluate the age, sex, and energy-adjusted means of nutrient intake by quartiles of the plant-based diet indices. To determine the association of plant-based diets with MetS, its component, and high-risk AIP (values greater than the median), multivariable logistic regression was applied. The odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated in crude and adjusted models. Potential confounders were selected based on the previous literature (27, 40). In Model I, adjustment was made for the main confounders (age, sex, and energy intake). In Model II, additional adjustments were conducted for education status, smoking status, marital status, socioeconomic status (SES), physical activity, and intake of margarine and hydrogenated vegetable oils. In Model III, BMI was added to previous adjustments. The first quartile of plant-based diet indices was considered the reference category in all analyses. In addition, a crude and multivariable-adjusted linear regression model was applied to predict serum adropin levels. To evaluate the non-linear association, a restricted cubic spline regression analysis was conducted. SPSS software version 20 (IBM, Chicago, IL) and STATA version 14 were used to perform analysis, and a *P*-value of < 0.05 (two-tailed) was considered statistically significant.

Results

Study population characteristics

In total, 600 subjects were invited to the current study. Among 600 invited individuals, 543 subjects provided their consent. Subjects who had left more than 70 food items unanswered ($n = 4$) reported energy intake out of the range of 800–4,200 kcal (41) ($n = 3$), and individuals with insufficient data on biochemical measurements ($n = 1$) or components of metabolic syndrome ($n = 8$) were excluded from the analysis. Finally, a total of 527 adults were included in the current cross-sectional study for metabolic syndrome and AIP analysis. In the case of adropin, 30 subjects did not have data on serum adropin levels; therefore, 497 subjects were included for adropin-related analysis (Figure 1). The overall analysis was conducted on 527 adults with a mean age and BMI of 42.65 ± 11.18 years and 26.90 ± 4.43 kg/m^2, respectively;

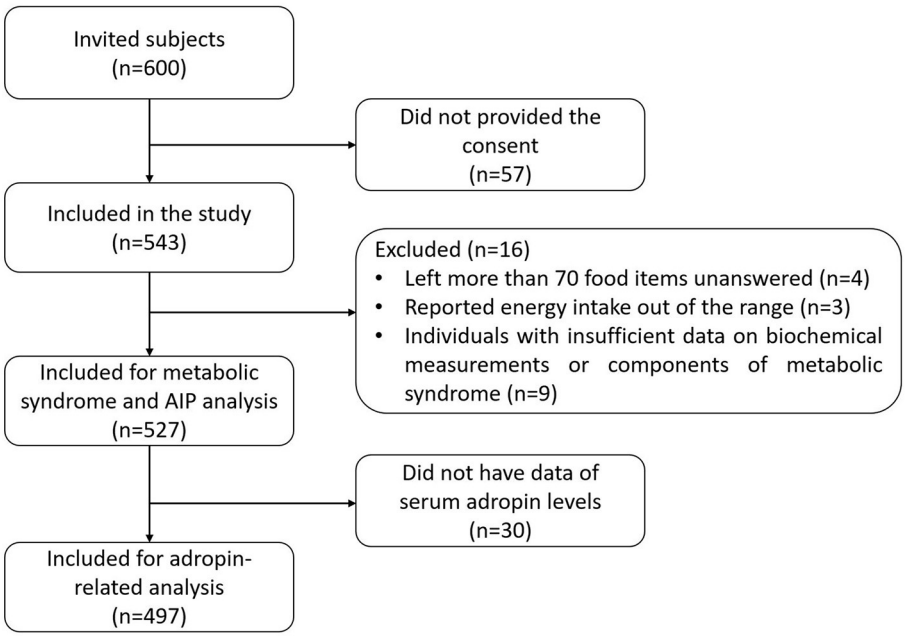

FIGURE 1
The study participant flow chart.

TABLE 1 Demographic and cardiometabolic characteristics of participants across energy-adjusted quartiles of the plant-based dietary indices[a].

Variables	PDI		*P*-value[b]	hPDI		*P*-value[b]	uPDI		*P*-value[b]
	Quartile 1	Quartile 4		Quartile 1	Quartile 4		Quartile 1	Quartile 4	
Sample size (*n*)	118	145		116	144		121	121	
Median score (range)	47 (34–49)	60 (58–71)		45 (28–48)	63 (59–81)		45 (35–48)	63 (60–76)	
Age (year)	40.48 ± 10.93	45.46 ± 11.14	<0.001	37.25 ± 10.29	47.18 ± 11.38	<0.001	44.14 ± 10.44	38.90 ± 11.18	<0.001
Body weight (kg)	77.92 ± 15.49	74.94 ± 15.18	0.12	75.95 ± 14.56	74.75 ± 15.42	0.80	74.66 ± 16.23	77.35 ± 15.20	0.51
BMI (kg/m^2)	26.98 ± 4.44	26.82 ± 4.67	0.98	26.14 ± 4.22	27.00 ± 4.82	0.18	26.98 ± 5.15	26.67 ± 4.32	0.59
WC (cm)	93.69 ± 12.33	92.89 ± 11.18	0.58	91.70 ± 11.68	92.92 ± 11.86	0.79	91.57 ± 12.66	93.15 ± 12.07	0.69
Sex			0.56			0.04			<0.001
Male	68 (57.6)	77 (53.1)		71 (61.2)	67 (46.5)		49 (40.5)	83 (68.6)	
Female	50 (42.4)	68 (46.9)		45 (38.8)	77 (53.5)		72 (59.5)	38 (31.4)	
Education			0.34			0.27			0.24
Diploma or lower	11 (9.3)	14 (9.8)		13 (11.2)	12 (8.5)		8 (6.7)	18 (14.9)	
Higher than Diploma	107 (90.7)	129 (90.2)		103 (88.8)	130 (91.5)		111 (93.3)	103 (85.1)	
Marital status			0.83			0.03			0.01
Single	18 (15.3)	25 (17.4)		23 (20.2)	28 (19.6)		19 (15.8)	31 (25.8)	
Married	98 (83.1)	116 (80.6)		91 (79.8)	112 (78.3)		98 (81.7)	88 (73.3)	
Divorced or widow	2 (1.7)	3 (2.1)		0 (0.0)	3 (2.1)		3 (2.5)	1 (0.8)	
Smoking			0.49			0.24			0.32
Non-smoker	101 (94.4)	114 (92.7)		92 (92.0)	116 (92.8)		103 (93.6)	99 (93.4)	
Ex-smoker	2 (1.9)	5 (4.1)		2 (2.0)	5 (4.0)		5 (4.5)	1 (0.9)	
Smoker	4 (3.7)	4 (3.3)		6 (6.0)	4 (3.2)		2 (1.8)	6 (5.7)	
SES			0.43			0.32			0.06
Low	26 (32.5)	31 (33.3)		29 (35.4)	23 (27.7)		17 (20.7)	33 (41.8)	
Moderate	21 (26.3)	28 (30.1)		25 (30.5)	21 (25.3)		25 (30.5)	24 (30.4)	
High	33 (41.3)	34 (36.6)		28 (34.1)	39 (47.0)		40 (48.8)	22 (27.8)	
Physical activity			0.30			0.86			0.13
Inactive	61 (51.7)	91 (63.6)		65 (56.5)	80 (55.9)		59 (49.2)	65 (54.2)	
Minimally active	48 (40.7)	41 (28.7)		43 (37.4)	54 (37.8)		51 (42.5)	40 (33.3)	
HEPA active	9 (7.6)	11 (7.7)		7 (6.1)	9 (6.3)		10 (8.3)	15 (12.5)	

(Continued)

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TABLE 1 (Continued)

Variables	PDI		*P*-value[b]	hPDI		*P*-value[b]	uPDI		*P*-value[b]
	Quartile 1	Quartile 4		Quartile 1	Quartile 4		Quartile 1	Quartile 4	
Metabolic syndrome	36 (30.5)	41 (28.3)	0.58	29 (25.0)	48 (33.3)	0.35	36 (29.8)	29 (24.0)	0.58
SBP (mmHg)	123.11 ± 16.53	121.52 ± 17.02	0.61	121.09 ± 15.91	123.41 ± 16.76	0.22	122.52 ± 16.29	120.67 ± 16.32	0.09
DBP (mmHg)	82.89 ± 9.62	83.19 ± 11.32	0.72	82.39 ± 9.10	83.12 ± 10.14	0.50	82.45 ± 8.93	82.22 ± 9.85	0.85
FBG (mg/dL)	91.75 ± 13.50	92.37 ± 18.96	0.96	90.48 ± 14.21	94.21 ± 23.74	0.41	95.08 ± 25.02	91.42 ± 18.07	0.33
TG (mg/dL)	154.32 ± 42.01	153.15 ± 42.69	0.93	154.88 ± 43.78	148.56 ± 36.44	0.37	148.99 ± 36.22	153.54 ± 38.99	0.54
TC (mg/dL)	187.01 ± 35.15	182.60 ± 30.42	0.60	180.75 ± 33.56	181.40 ± 32.99	0.30	182.28 ± 31.93	180.75 ± 30.40	0.46
LDL-C (mg/dL)	100.39 ± 31.52	95.60 ± 26.52	0.47	95.96 ± 28.60	95.91 ± 28.84	0.46	95.44 ± 28.12	95.49 ± 25.96	0.35
HDL-C (mg/dL)	55.75 ± 10.48	56.36 ± 10.16	0.38	54.27 ± 10.44	55.76 ± 10.01	0.27	57.04 ± 9.56	54.98 ± 9.46	0.21
Adropin (pg/ml)	54.35 ± 36.17	59.04 ± 43.36	0.41	57.68 ± 41.78	51.39 ± 25.13	0.08	55.51 ± 42.70	51.71 ± 24.15	0.11
AIP	0.43 ± 0.15	0.42 ± 0.15	0.71	0.44 ± 0.15	0.42 ± 0.12	0.31	0.41 ± 0.12	0.43 ± 0.13	0.25

[a]Quantitative variables: mean ± SD. Qualitative variables: frequency (percentage).
[b]Resulted from ANOVA for quantitative variables and chi-square test for categorical variables.

54.3% of included subjects were male. The prevalence of MetS and high-risk AIP were 28.7 and 49%, respectively. Demographic and cardiometabolic characteristics of the study participants, divided by quartiles of the plant-based dietary indices, are presented in Table 1. The mean age of subjects was higher in the upper quartile of PDI and hPDI compared to the first quartile, while it was lower in the top quartile of uPDI. The percentage of women in the fourth quartile of hPDI was more than the first quartile, while those in the fourth quartile of uPDI were more likely to be men. The percentage of married subjects in the highest quartile of uPDI was lower than the first one. Anthropometric measurements, education, SES, physical activity, MetS and its components, adropin levels, and AIP were not significantly different among the quartiles of the various types of plant-based dietary indices.

Multivariate adjusted dietary intakes across quartiles of the plant-based dietary indices are presented in Supplementary Table 2. The mean energy intake in the highest quartile of PDI was lower than the first one, while in hPDI and uPDI, the mean energy intake was higher in the highest quartiles. The mean intake of carbohydrates was greater, and the mean intake of proteins, fats, and cholesterol was lower in the highest quartile of the three types of plant-based diets compared to the first one. In addition, dietary fiber intake in the highest quartile of PDI and hPDI was greater than the first one. However, subjects in the highest quartile of uPDI had less intake of dietary fiber than those in the first quartile.

Association of plant-based diets with MetS and high-risk AIP

Multivariable-adjusted ORs (95% CI) for the association of plant-based diets with MetS and AIP are presented in Table 2. In the crude and adjusted models, no significant association was found between PDI and hPDI with MetS. Although no significant association was detected between uPDI and MetS in the crude model, after adjustment for potential confounders, the moderate adherence to uPDI (third quartile) was associated with an increased odds of MetS (OR: 2.39; 95% CI: 1.01, 5.66). In the case of the association of plant-based diets with high-risk AIP, the highest quartile of PDI was associated with decreased odds of high-risk AIP, after adjusting for potential confounders (OR: 0.46; 95% CI: 0.21, 0.97). The third quartile of hPDI was also associated with reduced odds of high-risk AIP compared to the lowest one, after adjusting for potential confounders (OR: 0.40; 95% CI: 0.18, 0.89). However, neither crude nor adjusted models did find any significant association between uPDI and high-risk AIP. The non-linear associations of all types of plant-based diet indices with MetS and AIP are shown in Figure 2. A significant non-linear association was observed between hPDI and MetS ($P_{\text{non-linearity}} = 0.04$).

In a sensitivity analysis, we evaluated the association of plant-based diets with MetS, after excluding subjects who reported fruit and vegetable intake >1,000 g per day (Supplementary Table 3). The results showed that higher adherence to hPDI was related to a 73% decreased odds of MetS in the fully-adjusted model ($OR_{Q4\ vs.\ Q1}$: 0.27; 95% CI: 0.09, 0.77), and moderate adherence to uPDI was linked to an increased odds of MetS ($OR_{Q3\ vs.\ Q1}$: 2.79; 95% CI: 1.14, 6.81).

TABLE 2 Multivariate adjusted odds ratio (OR) and 95% confidence interval (CI) for the association of energy-adjusted plant-based diets with metabolic syndrome and high risk AIP.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-trend
PDI					
Metabolic syndrome					
Crude model	1	1.02 (0.61, 1,71)	0.70 (0.39, 1.28)	0.90 (0.53, 1.53)	0.46
Model I[a]	1	1.08 (0.62, 1.88)	0.61 (0.32, 1.18)	0.70 (0.39, 1.26)	0.09
Model II[b]	1	1.45 (0.70, 3.04)	0.99 (0.4, 2.49)	1.10 (0.50, 2.42)	0.92
Model III[c]	1	1.35 (0.61, 2.97)	0.94 (0.35, 2.54)	0.96 (0.41, 2.49)	0.70
AIP (high risk)					
Crude model	1	1.00 (0.62, 1.61)	0.84 (0.50, 1.41)	0.68 (0.41, 1.10)	0.07
Model I[a]	1	1.04 (0.63, 1.71)	0.91 (0.52, 1.60)	0.68 (0.41, 1.15)	0.10
Model II[b]	1	1.14 (0.58, 2.24)	0.85 (0.36, 1.98)	0.52 (0.25, 1.07)	0.04
Model III[c]	1	1.09 (0.54, 2.19)	0.78 (0.33, 1.86)	0.46 (0.21, 0.97)	0.02
hPDI					
Metabolic syndrome					
Crude model	1	1.02 (0.58, 1.79)	1.32 (0.75, 2.34)	1.50 (0.87, 2.59)	0.08
Model I[a]	1	0.82 (0.45, 1.48)	0.82 (0.44, 1.51)	0.74 (0.40, 1.38)	0.38
Model II[b]	1	0.83 (0.37, 1.86)	0.82 (0.36, 1.87)	0.70 (0.30, 1.65)	0.44
Model III[c]	1	0.47 (0.19, 1.15)	0.64 (0.26, 1.58)	0.43 (0.16, 1.12)	0.18
AIP (high risk)					
Crude model	1	0.72 (0.44, 1.17)	0.89 (0.53, 1.48)	0.80 (0.49, 1.31)	0.61
Model I[a]	1	0.75 (0.45, 1.25)	0.83 (0.48, 1.42)	0.81 (0.47, 1.39)	0.57
Model II[b]	1	0.72 (0.34, 1.49)	0.48 (0.22, 1.03)	0.72 (0.32, 1.61)	0.31
Model III[c]	1	0.55 (0.25, 1.19)	0.40 (0.18, 0.89)	0.57 (0.24, 1.33)	0.17
uPDI					
Metabolic syndrome					
Crude model	1	1.09 (0.64, 1.86)	0.96 (0.57, 1.62)	0.74 (0.42, 1.32)	0.27
Model I[a]	1	1.17 (0.67, 2.04)	0.99 (0.57, 1.74)	0.88 (0.47, 1.64)	0.61
Model II[b]	1	1.13 (0.52, 2.47)	1.69 (0.78, 3.66)	1.14 (0.48, 2.71)	0.48
Model III[c]	1	1.25 (0.53, 2.95)	2.39 (1.01, 5.66)	1.44 (0.56, 3.71)	0.21
AIP (high risk)					
Crude model	1	1.16 (0.71, 1.89)	1.08 (0.67, 1.74)	1.07 (0.65, 1.77)	0.88
Model I[a]	1	1.15 (0.69, 1.92)	0.89 (0.54, 1.47)	0.80 (0.47, 1.38)	0.30
Model II[b]	1	1.65 (0.80, 3.40)	0.94 (0.46, 1.91)	0.98 (0.45, 2.15)	0.64
Model III[c]	1	1.62 (0.77, 3.44)	0.99 (0.48, 2.08)	1.02 (0.45, 2.29)	0.74

[a] Adjusted for age, sex, and energy intake.
[b] Adjusted for age, sex, energy intake, education status, smoking status, marital status, SES, physical activity, margarine, and hydrogenated oil.
[c] Adjusted for age, sex, energy intake, education status, smoking status, marital status, SES, physical activity, margarine, hydrogenated oil, and BMI.

Association of plant-based diets with components of MetS

The association of plant-based diets with components of MetS is presented in Table 3. Considering the first quartile of PDI as the reference group, higher adherence to PDI was not associated with any components of Mets in both crude and adjusted models. Subjects in the highest quartile of hPDI had lower odds of elevated fasting glucose compared to the first one in the fully-adjusted model (OR: 0.36; 95% CI: 0.13, 0.99). In addition, in Model I, those in the highest quartile of hPDI had 43% marginally significant decreased odds of hypertriglyceridemia (OR: 0.57; 95% CI: 0.32, 1.00). Participants in the third and fourth quartiles of uPDI had higher odds for hypertension after adjusting for potential confounders

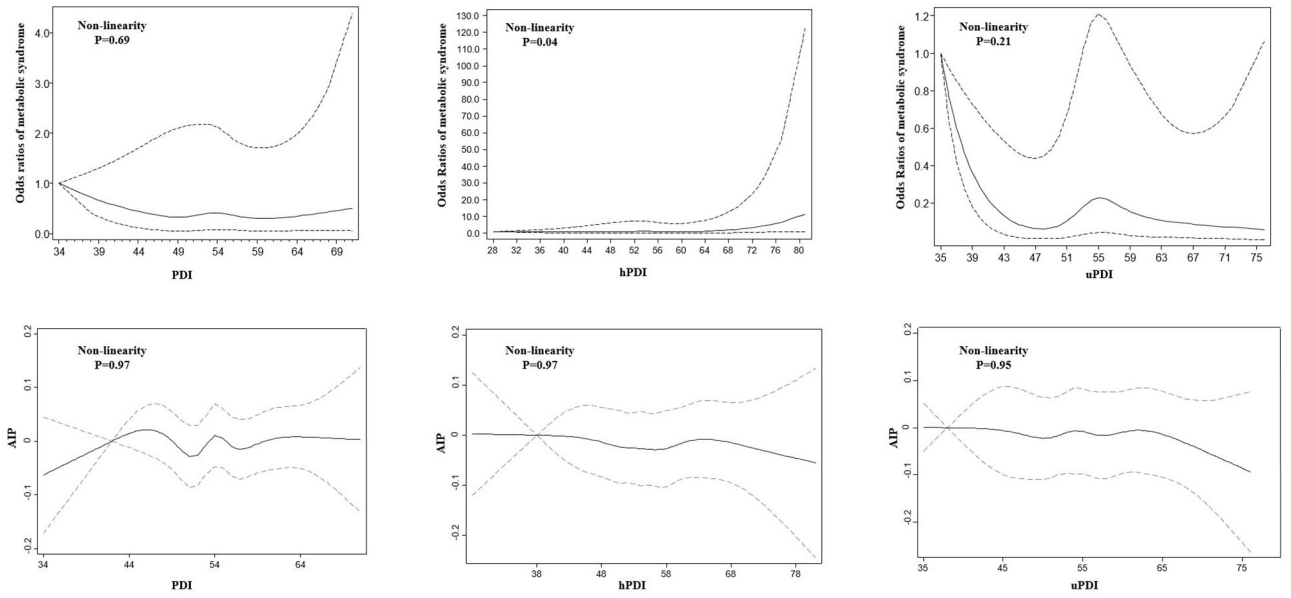

FIGURE 2
The non-linear association of all types of plant-based diet indices with MetS and AIP.

($OR_{Q3\ vs.\ Q1}$: 3.38; 95% CI: 1.51, 7.57; $OR_{Q4\ vs.\ Q1}$: 2.42; 95% CI: 1.02, 5.75).

Association of plant-based diets with serum level of adropin

Higher adherence to plant-based diet indices (per 1 quartile increment in PDI, hPDI, and uPDI) was not associated with serum level of adropin (as a continuous variable), after adjustment for potential confounders (B: 2.06, 95% CI: −0.88, 5.01; $P = 0.17$ for PDI; B: 0.57, 95% CI: −2.56, 3.70; $P = 0.72$ for hPDI; B: −0.46, 95% CI: −3.56, 2.63; $P = 0.77$ for uPDI) (Table 4).

Discussion

This population-based cross-sectional study revealed that PDI and hPDI were not associated with MetS, whereas higher adherence to PDI and moderate adherence to hPDI decreased the odds of high-risk AIP in middle-aged adults. In addition, moderate adherence to uPDI was associated with greater odds of MetS, while no significant association was found between uPDI and high-risk AIP. Among MetS components, higher adherence to hPDI was detected to be associated with lower odds of hyperglycemia and hypertriglyceridemia, while higher adherence to uPDI was found to be associated with greater odds of hypertension. No significant association was found between plant-based diet indices and serum levels of adropin.

Previous studies suggested that plant-based diets, especially healthy plant foods, might have a role in the prevention and management of MetS and high-risk AIP, and unhealthy plant foods might increase the risk of diseases (23, 42–44). Our results suggested that hPDI might be associated with decreased odds of some components of MetS, and high-risk AIP and uPDI might be associated with an increased likelihood of overall MetS.

The current study found that higher adherence to PDI was not associated with overall MetS or its components. This finding is consistent with previous population-based studies that showed greater adherence to PDI was not associated with MetS and its components in the Iranian and South Korean populations (45, 46). In addition, a study conducted on a representative sample of Canadian adults demonstrated no significant association between plant-based diet indices and the incidence of CVD and mortality (47). Our findings suggested that greater adherence to hPDI was not associated with overall MetS, while it was associated with decreased odds of having hyperglycemia and hypertriglyceridemia. In agreement with our findings, several studies suggested that hPDI was not associated with overall MetS, abdominal obesity, hypertension, and low HDL-c (45, 46). However, the majority of previous studies, especially studies on western societies, suggested that the PDI and hPDI might decline the risk of MetS and its components (28, 30, 44, 48). To interpret the lack of association between PDI and hPDI with MetS and its components, several points should be taken into account. Compared to Western countries, the consumption of animal foods including red and processed meats is less common, and the consumption of plant foods including grains, potatoes, legumes, fruits, and vegetables is more common in the Asian population (49, 50). In other words, a significant percentage of energy intake comes from carbohydrates and starchy vegetables in the Asian population. These sources of energy could limit the ability of PDI and hPDI to change the metabolic response significantly and might therefore result in a null association between PDI and hPDI with MetS. In addition, the intake of fish in the highest quartile of plant-based diets was less than the lowest one. Fish intake could decrease the risk of MetS;

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TABLE 3 Multivariate adjusted odds ratio (OR) and 95% confidence interval (CI) for the association of energy-adjusted plant-based diets with components of metabolic syndrome.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-trend
PDI					
Abdominal obesity					
Model I[a]	1	1.09 (0.62, 1.90)	1.03 (0.55, 1.94)	0.94 (0.52, 1.68)	0.74
Model II[b]	1	1.17 (0.37, 3.74)	2.74 (0.60, 12.52)	2.75 (0.77, 9.87)	0.07
High blood pressure					
Model I[a]	1	1.23 (0.73, 2.08)	0.73 (0.40, 1.32)	0.91 (0.53, 1.57)	0.37
Model II[b]	1	0.90 (0.44, 1.83)	0.45 (0.18, 1.12)	1.04 (0.49, 2.22)	0.90
High fasting glucose					
Model I[a]	1	1.26 (0.66, 2.40)	0.82 (0.39, 1.72)	0.69 (0.35, 1.38)	0.13
Model II[b]	1	1.06 (0.46, 2.41)	1.00 (0.37, 2.76)	0.54 (0.21, 1.36)	0.16
Hypertriglyceridemia					
Model I[a]	1	0.84 (0.51, 1.40)	0.87 (0.49, 1.54)	0.82 (0.48, 1.39)	0.53
Model II[b]	1	0.82 (0.42, 1.60)	0.73 (0.32, 1.69)	0.66 (0.32, 1.35)	0.25
Low HDL-C					
Model I[a]	1	1.17 (0.54, 2.52)	0.80 (0.32, 2.03)	1.42 (0.65, 3.08)	0.49
Model II[b]	1	1.71 (0.62, 4.68)	0.99 (0.25, 3.96)	1.62 (0.55, 4.80)	0.58
hPDI					
Abdominal obesity					
Model I[a]	1	1.02 (0.58, 1.79)	0.92 (0.51, 1.68)	0.79 (0.43, 1.48)	0.42
Model II[b]	1	0.26 (0.06, 1.07)	0.35 (0.09, 1.37)	0.62 (0.15, 2.65)	0.65
High blood pressure					
Model I[a]	1	0.85 (0.50, 1.45)	0.93 (0.53, 1.65)	0.65 (0.36, 1.17)	0.20
Model II[b]	1	0.76 (0.35, 1.65)	0.96 (0.43, 2.18)	0.66 (0.28, 1.56)	0.49
High fasting glucose					
Model I[a]	1	0.75 (0.38, 1.48)	0.65 (0.32, 1.31)	0.57 (0.28, 1.17)	0.13
Model II[b]	1	0.38 (0.14, 0.98)	0.54 (0.21, 1.36)	0.36 (0.13, 0.99)	0.11
Hypertriglyceridemia					
Model I[a]	1	0.85 (0.51, 1.42)	0.87 (0.51, 1.51)	0.57 (0.32, 1.00)	0.07
Model II[b]	1	0.53 (0.25, 1.13)	0.52 (0.24, 1.12)	0.46 (0.20, 1.05)	0.08
Low HDL-C					
Model I[a]	1	0.82 (0.36, 1.90)	1.23 (0.53, 2.86)	1.52 (0.68, 3.44)	0.17
Model II[b]	1	0.62 (0.20, 1.96)	1.10 (0.35, 3.45)	1.23 (0.40, 3.84)	0.46
uPDI					
Abdominal obesity					
Model I[a]	1	1.41 (0.77, 2.58)	0.96 (0.55, 1.70)	0.82 (0.45, 1.50)	0.31
Model II[b]	1	0.99 (0.29, 3.49)	1.74 (0.48, 6.32)	0.40 (0.10, 1.69)	0.46
High blood pressure					
Model I[a]	1	0.99 (0.58, 1.69)	1.69 (0.99, 2.89)	1.22 (0.69, 2.17)	0.19
Model II[b]	1	0.85 (0.39, 1.85)	3.38 (1.51, 7.57)	2.42 (1.02, 5.75)	0.004
High fasting glucose					
Model I[a]	1	0.55 (0.29, 1.06)	0.74 (0.40, 1.38)	0.77 (0.39, 1.54)	0.58

(Continued)

TABLE 3 (Continued)

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-trend
Model II[b]	1	0.51 (0.21, 1.25)	0.75 (0.31, 1.77)	0.80 (0.31, 2.07)	0.75
Hypertriglyceridemia					
Model I[a]	1	1.22 (0.72, 2.08)	1.29 (0.77, 2.16)	1.07 (0.61, 1.88)	0.75
Model II[b]	1	1.66 (0.80, 3.45)	1.43 (0.70, 2.93)	1.33 (0.60, 2.95)	0.56
Low HDL-C					
Model I[a]	1	1.70 (0.80, 3.63)	1.19 (0.54, 2.64)	0.92 (0.38, 2.23)	0.71
Model II[b]	1	2.13 (0.73, 6.21)	1.98 (0.67, 5.81)	1.40 (0.41, 4.75)	0.55

[a]Adjusted for age, sex, and energy intake.
[b]Adjusted for age, sex, energy intake, education status, smoking status, marital status, SES, physical activity, margarine, hydrogenated oil, and BMI.

therefore, it is possible that the low consumption of fish in Asian nations could interact with the effects of plant foods, especially fruits and vegetables (51, 52).

The majority of previous studies demonstrated that higher adherence to uPDI was associated with a greater risk of MetS and its components, especially in Western countries (27, 42, 43). However, several other studies, especially in the Asian population, did not observe any significant association between uPDI with MetS and its components (44, 45). In the current study, moderate adherence to uPDI (third quartile) was associated with elevated odds of MetS and in the highest quartile, and this association was not significant. In addition, moderate adherence to uPDI was associated with increased odds of hypertension, and in the highest quartile of uPDI, this association was attenuated. In the fourth quartile of uPDI, the intake of fruits, vegetables, fiber, nutrients, and antioxidants was low, while the intake of energy, carbohydrates, red and processed meats, and sodium was high. However, we expected that greater adherence to uPDI would be associated with higher odds of MetS (53–55). In this case, some points should be taken into account; high fruits and other plant-based foods, especially energy-rich plant foods, might elevate the prevalence of MetS, while in order to take advantage of the beneficial effect of fruits and vegetables on MetS, moderate consumption is suggested (56–58). In addition, heavy metals and chemical pesticide content of plant foods, especially vegetables, could be related to the prevalence of MetS (59, 60). In other words, the presence of pesticides and heavy metals in soil and plant foods was considered a concern in Iran (61–64). These issues might attenuate or change the association between different types of plant-based diet indices and MetS in our population.

A limited number of studies evaluated the association of dietary patterns, such as plant-based diet indices with AIP. Higher adherence to PDI and moderate adherence to hPDI were associated with lower odds of higher levels of AIP in the current study which was consistent with previous studies (22, 23). However, no significant association was observed between uPDI and AIP. These results might be related to the association between plant-based diet indices with triglycerides and HDL-c. Although PDI was not separately associated with triglycerides and HDL-c, it was associated with the logarithm of their ratio (AIP). Higher adherence to hPDI decreased the triglyceride levels; this reduction in triglycerides might have a role in decreased AIP. For uPDI, no significant association was observed for triglycerides, HDL-c, as well as AIP. A previous trial showed that the combination of the Mediterranean Diet (MD) and physical activity had a beneficial role in decreasing AIP levels (23). Two other studies reported that a snack rich in fiber and adherence to a healthy diet guideline were not significantly associated with AIP levels (22, 65). In addition, a meta-analysis demonstrated that total and saturated fat had no significant beneficial effect on serum triglyceride or HDL-c levels, as components of AIP (66). Another study suggested that there was a significant positive association between the quality of dietary fat and AIP (67). Furthermore, previous studies reported that low carbohydrate diets could decrease serum triglycerides and increase HDL-c levels (68, 69). However, the association of different types of plant-based diet

TABLE 4 Linear association of energy-adjusted plant-based diet indices with serum level of adropin (as a continues variable).

	B	95% CI	*P*	R^2
Per 1 quartile of PDI				
Crude model	0.71	−2.48, 3.89	0.66	-
Model I[a]	0.39	−2.91, 3.68	0.82	0.01
Model II[b]	2.09	−0.85, 5.03	0.16	0.02
Model III[c]	2.06	−0.88, 5.01	0.17	0.03
Per 1 quartile of hPDI				
Crude model	−1.14	−4.31, 2.03	0.48	0.001
Model I[a]	−0.73	−4.21, 2.76	0.68	0.01
Model II[b]	0.60	−2.53, 3.73	0.71	0.02
Model III[c]	0.57	−2.56, 3.70	0.72	0.02
Per 1 quartile of uPDI				
Crude model	−2.11	−5.39, 1.16	0.21	0.003
Model I[a]	−1.60	−5.07, 1.86	0.36	0.01
Model II[b]	−0.52	−3.62, 2.57	0.74	0.02
Model III[c]	−0.46	−3.56, 2.63	0.77	0.02

[a]Adjusted for age, sex, and energy intake.
[b]Adjusted for age, sex, energy intake, smoking status, and physical activity.
[c]Adjusted for age, sex, energy intake, smoking status, physical activity, and BMI.

indices with triglycerides and HDL-c, as the components of AIP, could predict the AIP levels. In addition, the independent effect of macronutrients on triglycerides and HDL-c, especially intake of carbohydrates and fats, should be considered in the interpretation of AIP levels.

To the best of our knowledge, there is no previous study that investigated the association of dietary patterns with adropin. The current study was the first investigation that evaluated the association between plant-based diet indices and adropin levels, although no significant association was found. Previous studies suggested that the intake of energy and macronutrients, including carbohydrates, proteins, and fats, might affect serum adropin levels (13, 70, 71). In addition, an experimental study demonstrated that a low-carbohydrate high-fat diet was associated with greater adropin levels, and a high-carbohydrate low-fat diet was associated with lower adropin levels in mice (21). Another investigation assessed the effect of dietary intake of sugars, including glucose, fructose, and high-fructose corn syrup (HFCS) on serum adropin levels. The results suggested that the intake of glucose decreased and the intake of fructose increased the adropin levels. However, HFCS intake did not change the adropin levels. In addition, the mentioned study reported that the effect of glucose and fructose intake on adropin was similar to their effect on serum triglycerides (72). However, the interaction between macronutrients and sugar intake in different types of plant-based diet indices, as well as the effect of confounders on serum levels of adropin, might be contributed to the association between plant-based diet indices and adropin levels.

Strengths and limitations

This study was conducted on a large somehow representative sample of adults. However, the finding could be extrapolated to the general adult population. As a novelty, this study is the first one that evaluated the association of plant-based diet indices with AIP and serum adropin levels. The use of validated questionnaires and adjustments for potential confounders could be considered as the other strengths of the current study. However, some limitations should be considered. First, to evaluate the dietary intake, we applied a self-reported semi-quantitative FFQ that might enhance the measurement errors and misclassification of individuals. In addition, recall bias was another limitation of such a questionnaire that should be considered. Second, unknown and residual confounders should also be taken into account. Finally, because of the nature of cross-sectional studies, the causality of the association between exposures and outcomes could not clearly be determined.

Conclusion

The current population-based cross-sectional study demonstrated that PDI and hPDI were not associated with MetS, while higher adherence to hPDI was associated with decreased odds of hyperglycemia and hypertriglyceridemia. In addition, moderate adherence to uPDI was associated with an increased prevalence of MetS and hypertension. In addition, high and moderate adherence to PDI and hPDI were associated with decreased odds of high-risk AIP, respectively. No significant association was found between plant-based diet indices and serum adropin levels. To confirm the findings of the current study and clearly determine causality, future studies with prospective design are warranted.

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 the Isfahan University of Medical Sciences (No: IR.MUI.RESEARCH.REC.1400.370). The patients/participants provided their written informed consent to participate in this study.

Author contributions

FS, PS, KL, AF, GA, and SMS contributed to conception, design, data collection, data interpretation, manuscript drafting, and agreed on all aspects of the study. All authors contributed to the article and approved the submitted version.

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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.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1077709/full#supplementary-material

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Vitamin K supplementation and vascular calcification: a systematic review and meta-analysis of randomized controlled trials

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Background: Vascular calcification (VC) is a complex process that has been linked to conditions including cardiovascular diseases and chronic kidney disease. There is an ongoing debate about whether vitamin K (VK) can effectively prevent VC. To assess the efficiency and safety of VK supplementation in the therapies of VC, we performed a systematic review and meta-analysis of recent studies.

Methods: We searched major databases, including PubMed, the Cochrane Library, Embase databases, and Web of Science up until August 2022. 14 randomized controlled trials (RCTs) describing the outcomes of treatment for VK supplementation with VC have been included out of 332 studies. The results were reported in the change of coronary artery calcification (CAC) scores, other artery and valve calcification, vascular stiffness, and dephospho-uncarboxylated matrix Gla protein (dp-ucMGP). The reports of severe adverse events were recorded and analyzed.

Results: We reviewed 14 RCTs, comprising a total of 1,533 patients. Our analysis revealed that VK supplementation has a significant effect on CAC scores, slowing down the progression of CAC [I^2 = 34%, MD= −17.37, 95% CI (−34.18, −0.56), p = 0.04]. The study found that VK supplementation had a significant impact on dp-ucMGP levels, as compared to the control group, where those receiving VK supplementation had lower values [I^2 = 71%, MD = −243.31, 95% CI (−366.08, −120.53), p = 0.0001]. Additionally, there was no significant difference in the adverse events between the groups [I^2 = 31%, RR = 0.92, 95% CI (−0.79,1.07), p = 0.29].

Conclusion: VK may have therapeutic potential for alleviating VC, especially CAC. However, more rigorously designed RCTs are required to verify the benefits and efficacy of VK therapy in VC.

KEYWORDS

vitamin K, vascular calcification, coronary artery calcification, dephospho-uncarboxylated matrix Gla protein, systematic review, meta-analysis, randomized controlled trials

Introduction

Vascular calcification (VC) is an independent predictor of morbidity and mortality in a variety of diseases, including cardiovascular disease (CVD) and chronic kidney disease (CKD) (1). The ongoing progression of VC causes impaired compliance of vascular, atherosclerotic plaque rupture, and thrombosis. Furthermore, VC occurrences in young individuals have also sharply increased in the context of diabetes mellitus (DM), CKD, and atherosclerosis (2). Based on the available evidence, it is crucial to recognize the role of VC

in the development of vascular damage. However, the creation of efficient prevention and drug therapeutic methods continues to be a serious clinical challenge due to the existing inadequate knowledge about VC.

Vitamin K (VK) is a fat-soluble vitamin with three different forms as follows: VK1 (phylloquinone), VK2 (menaquinone), and VK3 (menadione) (3). VK1 is the main source in Western countries, mainly found in green vegetables (such as spinach and broccoli) and vegetable oils (such as soybeans, olive oil, and canola). VK2, produced by gut bacteria, is less common in the diet and is found in fermented foods, such as cheese and meat. VK3 is a synthetic form that is often added to animal feed (4). VK has several crucial functions in the body. VK1 plays the most important role in the activation of blood clotting factors in the liver, while VK2 plays a role in protein synthesis in extrahepatic tissues such as blood vessel walls (5). In recent years, the awareness of the role of VK has grown significantly because of its well-known involvement in several diseases, such as osteoporosis (6), CVD (7), inflammation (8), cancer (9), Alzheimer's disease (10), and peripheral neuropathy (11). A growing body of research indicates that VK has a positive impact on cardiovascular health, providing a low-cost and safe treatment option (12, 13).

VC is a chronic inflammatory process that involves the activation of macrophages and the differentiation of vascular smooth muscle cells into osteoblasts within the intimal and medial layers of artery walls. This process is facilitated by the generation of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor α (TNF-α). This process could be primarily mediated by the NF-κB pathway (14). Previous studies have suggested that VK exhibits anti-inflammatory properties by inhibiting the NF-κB pathway, as demonstrated in both *in vitro* and *in vivo* studies. These findings suggest that VK supplementation may help inhibit VC through anti-inflammatory mechanisms (15). Moreover, VK is necessary for the activation of several proteins involved in VC. These vitamin K-dependent proteins (VKDPs) include matrix Gla proteins (MGP), growth arrest-specific 6 (Gas 6), and Gla-rich protein (GRP). MGP is an important tissue calcification inhibitor that helps to prevent both intimal and medial VC. VK may also slow the progression of VC by increasing MGP activity by facilitating its carboxylation (16). Similarly, the inhibitory activity of GRP calcification relies on the post-translational carboxylation of Glu residues. The undercarboxylated protein lacks calcification inhibitory capacity, as demonstrated by its potential to act as an inhibitor of VC (17). Gla-rich protein is a ligand for the Tyro3/Axl/Mer (TAM) family of receptor tyrosine kinases that inhibits VC by preventing endothelial cell and vascular smooth muscle cells from going through apoptosis (18). These VKDPs are thought to carry out their intended functions through γ-carboxylation with VK, and different VKDPs may have synergistic or antagonistic effects on each other (19).

Overall, VK supplementation may provide a simple and relatively safe therapeutic strategy for preventing the development of VC, particularly in individuals who are at high risk of CVD and are prone to VK deficiency. Previous studies have produced conflicting results on the effectiveness of VK supplementation in improving measures of VC, with some studies reporting positive effects (12, 13) and others reporting no improvement (20, 21). Based on this, we performed an updated systematic review and meta-analysis of randomized controlled trials (RCTs) to confirm the relationship between VK supplementation and VC diseases.

TABLE 1 Population, intervention, comparison, outcomes, and settings (PICOS) criteria for the inclusion of studies evaluating the effects and safety of VK intake on vascular properties.

Parameter	Inclusion criteria
P (population)	Adults (year ≥18)
I (intervention)	VK (VK1 or VK2) supplementation
C (COMPARISON)	Non-exposed control group
O (outcomes)	Any measurement of VC, dp-ucMGP, adverse advents
S (settings)	RCTs

Methods

Literature search

This article was reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement (22). From the period of their creation until August 2022, PubMed, Web of Science, the Cochrane Library, and Embase databases were used in our literature searches. The following search phrases were used: "vascular calcification or Calcification, Vascular or Calcifications, Vascular or Vascular Calcifications or Vascular Calcinosis or Calcinoses, Vascular or Calcinosis, Vascular or Vascular Calcinoses" and "Vitamin k1 or Phytonadione or Vitamin K1 or Phytomenadione or Phylloquinone or Phyllohydroquinone or Aquamephyton or Konakion or Vitamin k2 or Menaquinones or Vitamin K2 or Menaquinone or Vitamin K Quinone" and "Randomized Controlled Trial or random*." For more details regarding the search strategy used for each database, please refer to Supplementary Document 1. According to PICOS criteria to develop a retrieval strategy summarized in Table 1.

Study selection criteria and study types

Inclusion criteria

Articles available in English reporting RCTs involving adult participants (age ≥18 years) of any race. These articles must have concerned VK supplementation with a placebo or no-treatment control group, and the report must include an indicator associated

Abbreviations: VC, vascular calcification; DM, diabetes mellitus; CKD, chronic kidney diseases; CAC, coronary artery calcification; VKDPs, vitamin K-dependent proteins; MGP, matrix Gla protein; dp-ucMGP, dephospho-uncarboxylated matrix Gla protein; CVD, cardiovascular disease; RCTs, randomized controlled trials; CT, computed tomography; 18 F-NaF PET, 18 F-sodium fluoride positron emission tomography; MD, mean difference; CI, confidence interval; SD, standard deviation; T2DM, type 2 diabetes mellitus; KTR, kidney transplant recipient.

with VC at the baseline and study endpoint. Co-interventions were regarded as acceptable as long as both groups received them.

Exclusion criteria

Other cohort clinical trials or observational studies. Case reports, reviews, comments, letters, animal studies, and studies containing mixed pediatric and adult populations. Literature that cannot provide relevant raw data was also excluded.

Evaluation outcomes

The primary outcome was artery calcification: the change in CAC scores, artery volume, and others. We identified the following as suitable methods for evaluating VC: ① plain lateral abdominal X-ray, ② computed tomography (CT) measuring vessels and valve calcification, volume or mass calcification scores, and ③ ^{18}F-Sodium Fluoride Positron Emission Tomography (^{18}F-NaF PET) imaging. The secondary outcomes were dp-ucMGP and adverse events.

Study selection and data extraction

Two unbiased reviewers Te Li and Yun Wang used a study selection sheet to assess the articles acquired through the search strategy. Studies that did not fit the inclusion criteria were excluded after the titles and abstracts were initially screened. After a preliminary examination, the studies that had not been excluded were retrieved for full-text screening. It was decided whether the study should be considered for inclusion in our analysis based on the inclusion criteria. During the whole process, if there is a dispute, the final decision for inclusion was made by consensus by Weiping Tu.

The data were extracted by two unbiased reviewers Te Li and Yun Wang, including study characteristics (authors, publication year, country, and the number of centers), study participants (such as eligibility criteria and baseline characteristics), and study design traits. Others including study interventions, controls, period of treatment, length of follow-up, and study results were also extracted.

Bias, quality, sensitivity analysis, and subgroup analysis assessments

The intervention quality of the studies was evaluated for the risk of bias using the Cochrane Risk of Bias tool for RCTs (23). The risk was estimated using the six criteria listed below: adequate sequence generation, allocation concealment, blinding, incomplete data outcome, and selective reporting. According to these criteria, the risk was classified as low high, or unclear risk

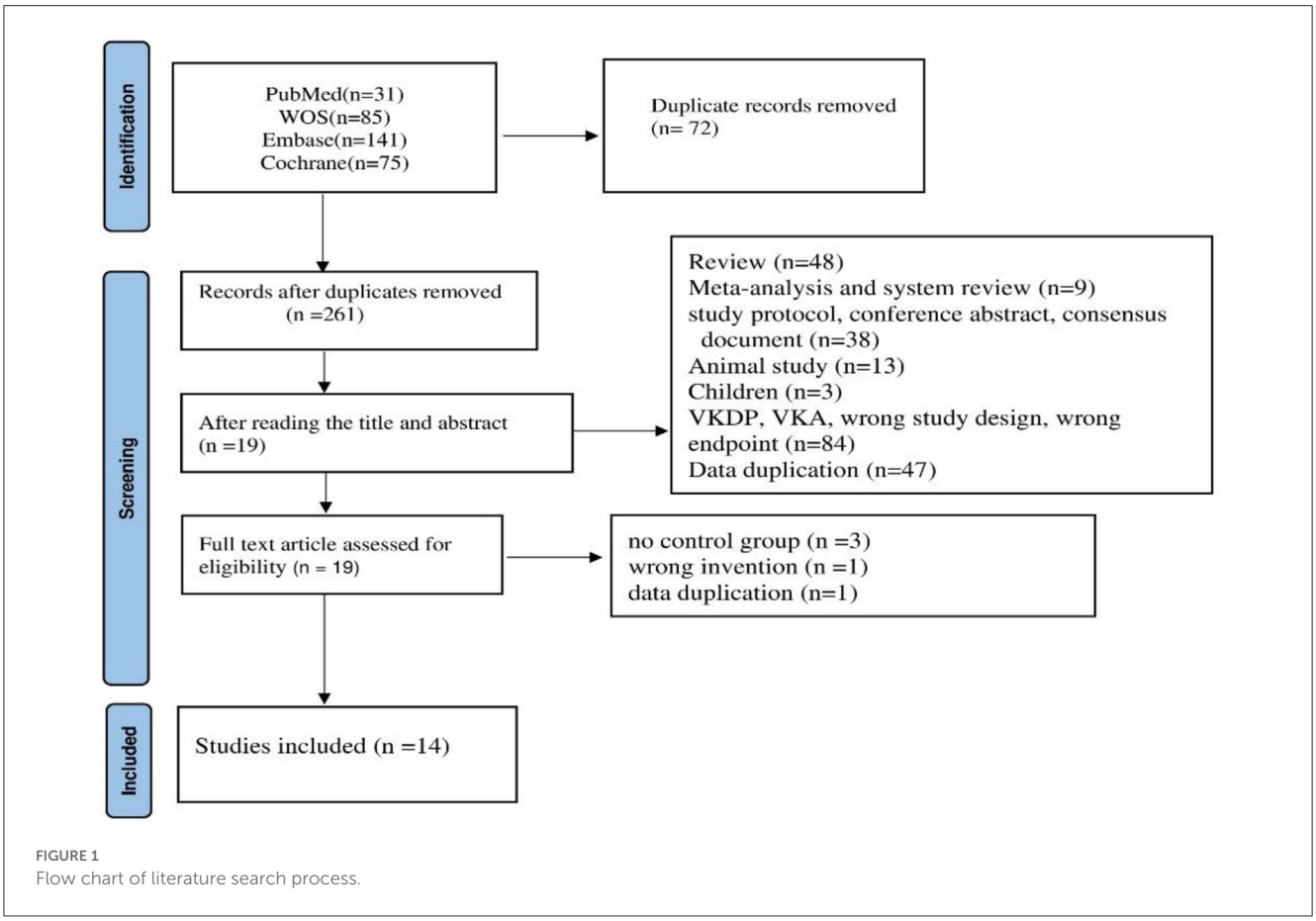

FIGURE 1
Flow chart of literature search process.

TABLE 2 Study characteristics.

References	Country	Center(s)	Population	Intervention	Control	Period of treatment (months)	Length of follow up (months)
Witham et al. (20)	USA	2	CKD	400 μg MK-7, daily	Placebo	12	12
Levy-Schousboe et al. (21)	Denmark	4	T2DM	360 μg MK-7, daily	Placebo	24	24
Lees et al. (49)	UK	1	KTR	Menadiol diphosphate 5 mg, thrice weekly	Placebo	12	12
Bellinge et al. (12)	Australia	1	T2DM	Phylloquinone 10 mg, daily	Placebo	3	3
Zwakenberg et al. (28)	The Netherlands	1	D2M and CVD	360 μg MK-7, daily	Placebo	6	6
Shea et al. (13)	USA	1	Community	Multivitamin with 500 μg phylloquinone, daily	Multivitamin, daily	36	36
Bartstra et al. (29)	The Netherlands	3	T2DM	360 μg vitamin K2, daily	Placebo	6	6
De Vriese et al. (27)	Belgium	1	Hemodialysis, nonvalvular AF	2,000 μg MK-7, thrice weekly + rivaroxaban 10 mg, daily	Rivaroxaban 10 mg, daily/VKA	18	18
Knapen et al. (25)	The Netherlands	3	Postmenopausal women	180 μg MenaQ7, daily	Placebo	36	36
Holden et al. (39)	Canada	1	Hemodialysis	10 mg of phylloquinone, thrice weekly	Placebo	12	12
Oikonomaki et al. (26)	Greece	1	Hemodialysis	200 μg MK-7, daily	No treatment	12	12
Kurnatowska et al. (30)	The Netherlands	2	CKD3–5	90 μg MK-7 + 10 μg cholecalciferol, daily	10 μg cholecalciferol	9	9
Braam et al. (50)	The Netherlands	1	Postmenopausal women	1 mg VK 1 + 10 mg zinc, 150 mg magnesium + 8 μg VD	10 mg zinc, 150 mg magnesium, and 8 μg VD/placebo	36	36
Fulton et al. (31)	Scotland	1	Older, vascular disease	100 μg MK-7, daily	Placebo	6	6

References	*N* = participation (analysis)	Intervention *N* =	Control *N* =	Age (SD) Intervention	Control	Female intervention	Control	Male intervention	Control
Witham et al. (20)	159 (124)	80 (61)	79 (63)	67.3 ± 11	65.7 ± 13.5	32	30	47	57
Levy-Schousboe et al. (21)	48	24	24	62 ± 11	66 ± 11	5	6	19	18
Lees et al. (49)	90 (83)	45 (42)	45 (41)	56.3 ± 11.1	58.9 ± 7.8	13	14	32	31
Bellinge et al. (12)	154 (149)	76 (73)	78 (76)	65.2 ± 7.1	65.2 ± 7.1	27	26	51	50
Zwakenberg et al. (28)	68 (60)	35 (33)	33 (27)	69.1 ± 8.4	69.1 ± 8.4	9	7	26	26
Shea et al. (13)	388 (295)	200 (149)	188 (146)	68 ± 6	68 ± 5	59	62	141	126
Bartstra et al. (29)	68 (60)	35 (33)	33 (27)	69 ± 8	69 ± 8	9	7	26	23

(Continued)

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TABLE 2 (Continued)

References	*N* = participation (analysis)	Intervention *N* =	Control *N* =	Age (SD) Intervention	Control	Female intervention	Control	Male intervention	Control
De Vriese et al. (27)	132	42	46/44	79.6 ± 7.3	79.9 ± 7.04/80.3 ± 9.48	14	11/9	28	35/25
Knapen et al. (25)	244 (233)	120 (111)	124 (120)	59.8 ± 3.5	59.3 ± 3.1	120	124	0	0
Holden et al. (39)	86 (69)	41 (34)	44 (35)	63 ± 11.85	61 ± 16.3	14	23	27	21
Oikonomaki et al. (26)	102 (52)	44 (22)	58 (30)	70.18 ± 66.65	66.65 ± 16.4	NR	NR	NR	NR
Kurnatowska et al. (30)	42 (40)	29 (28)	13 (12)	NR	NR	NR	NR	NR	NR
Braam et al. (50)	181 (108)	63 (38)	58 (30)/60 (40)	55.4 ± 2.8	55.9 ± 2.8/54.1 ± 3.0	38	30/40	0	0
Fulton et al. (31)	80	40	40	76 ± 4.4	77.1 ± 4.8	19	17	21	23

Age, presented as mean ± SD; NR, no report; VKA, vitamin K antagonism; AF, atrial fibrillation.

of bias. Sensitivity analyses and subgroup analyses were conducted to minimize inter-study heterogeneity. Sensitivity analyses were performed by eliminating one study at a time. Based on participants (hemodialysis vs. no-hemodialysis) and the date of publication (before 2015 vs. after 2015), subgroup analyses were undertaken.

Statistical analysis

Our article was produced utilizing the Review Manager version 5.4, Stata version 17, and SPSS version 26.0 (IBM Corp, Armonk, NY, United States) for analysis. For dichotomous or polytomous outcomes (adverse events), risk ratios (RRs) were calculated. The use of mean differences (MDs) and 95% confidence intervals (CIs) was evaluated for continuous variables. From the treatment and control groups, MD and standard deviation (SD) for VC and dp-ucMGP were extracted. If MD and SD for outcome indicators of interest were not reported, other available data were used for calculation. The MD and SD in VC were calculated from the median, interquartile range, and sample size. SD of VC or dp-ucMGP was calculated using standard methods based on the mean and 95% CIs or mean and *p*-value. Cochrane's *Q*-test and I^2 statistic were applied to examine the statistical heterogeneity (≥75%, high heterogeneity; 51%−75%, moderate heterogeneity; 26%−50%, low heterogeneity; and ≤25%, insignificant heterogeneity). It is worth noting that the I^2 estimates with 95% CIs can be used to assess heterogeneity, but it is possibly associated with fluctuation in meta-analysis with <15 trials (24). For the data analysis, we employed a random effect model if there was significant heterogeneity. If there was no significant heterogeneity, we chose a fixed effect model. Statistical significance was defined as a *p*-value of <0.05.

Results

Study selection

We conducted a comprehensive search across multiple databases and identified 332 articles. After removing duplicates, we excluded 242 studies based on title and abstract screening, leaving 19 studies for full-text review. After excluding three studies that lacked a control group, one article with duplicate data, and one study that used incorrect interventions, we included 14 RCTs in the final analysis. Figure 1 provides a summary of the included studies.

Study characteristics

Our study included 14 RCTs with 1,842 participants initially enrolled. However, due to reasons, such as automatic withdrawal, adverse reactions, loss to follow-up, and other factors, the final number of participants included in the analysis was 1,533. Among them, four studies used VK1, nine studies used VK2 (MK-7), and one study only specified the use of VK without specifying the type of supplementation. Four of the included studies focused on type 2 diabetes mellitus (T2DM), while five studies focused on CKD patients (including both hemodialysis and non-hemodialysis patients). Two studies were conducted on postmenopausal women,

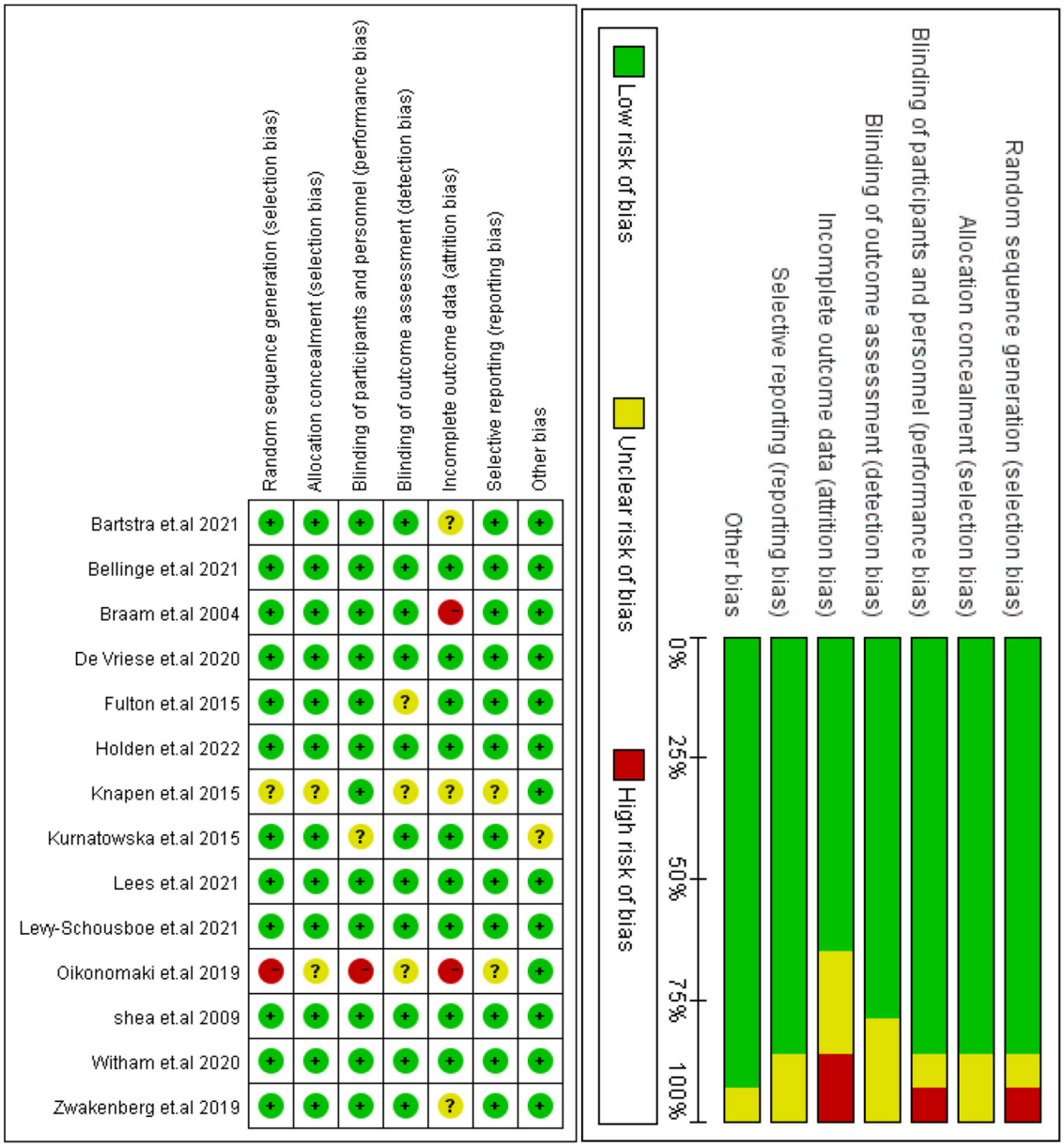

FIGURE 2
Risk of bias: review judgements about each risk of bias item for each included study.

one study focused on kidney transplant recipients (KTR), and the final two studies involved healthy individuals. Table 2 presents the main research characteristics.

Risk of bias in studies

Overall, seven studies presented a low risk of bias between all parameters. The research design methods in the Knapen et al. (25) study were poorly described. Sequence generation and allocation concealment were rated as ambiguous and highly biased in Oikonomaki et al.'s study (26). One trial used an open-label study design, which places it at high risk of bias in terms of participants and staff blinding (27). Additionally, it is unclear whether outcome data are completed for the study conducted by Zwakenberg et al. (28) with Bartstra et al. (29). Similarly, two studies made no mention of allocation concealment or blinding of outcome assessors for all outcomes (30, 31). Finally, Figure 2 presents the quality evaluation of the included RCTs.

Study outcomes

VC

① Vitamin K (VK) and VC of the change on CAC scores. A total of five studies compared VK supplementation on the

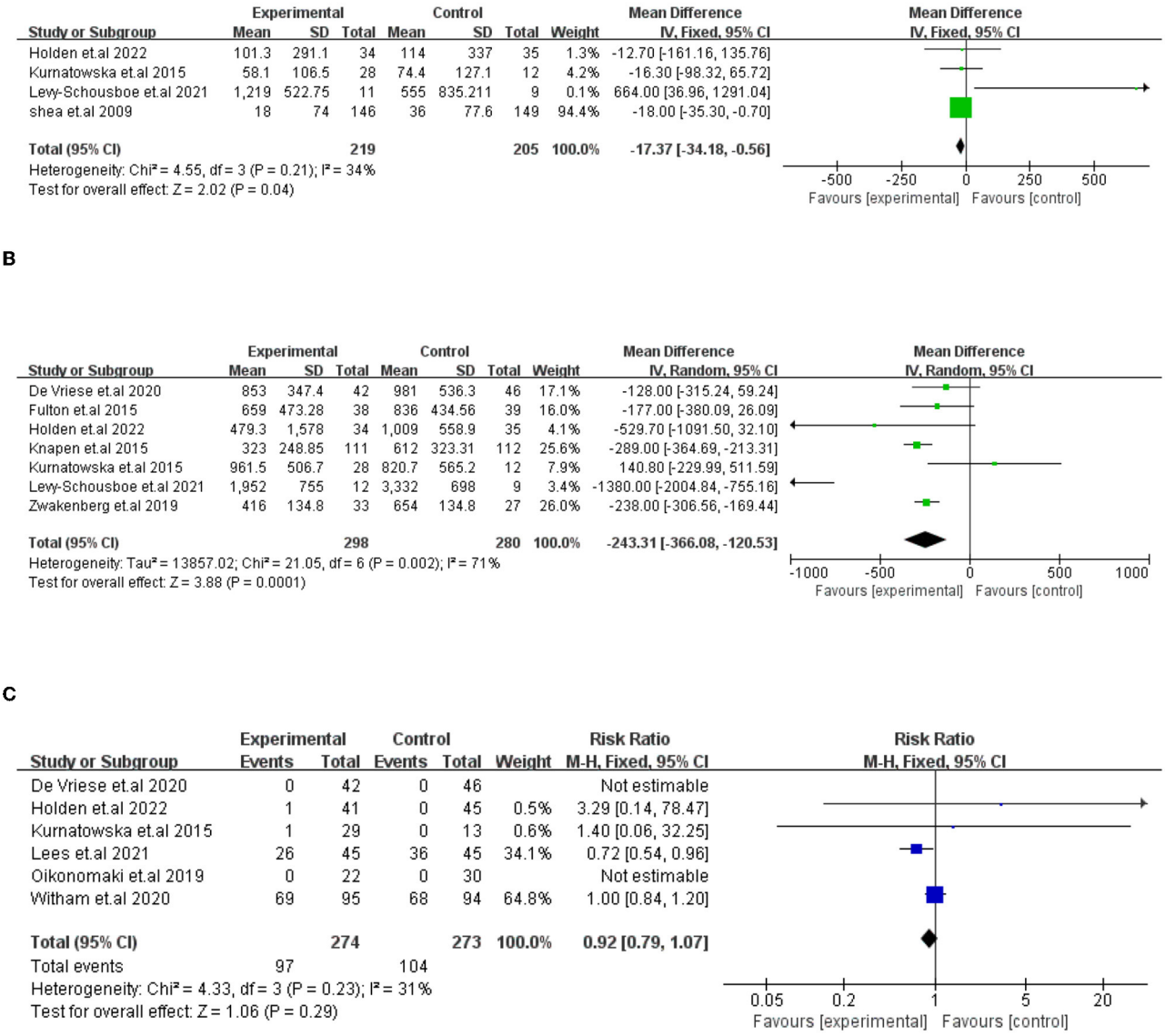

FIGURE 3
Forest plots showing the effect of VK intake on changes in CAC scores **(A)**, dp-ucMGP **(B)**, and advance events **(C)**. Data are presented as mean difference, risk radio and 95% CI.

change in CAC scores. One study (De Vriese et al.) was excluded because the results were expressed differently from other studies. In the end, four studies (424 participants) were included in the analysis. The results showed that VK supplementation has a significant effect on the decline in CAC scores, which indicated that VK supplementation slows the progression of CAC [I^2 = 34%, MD = −17.37, 95% CI (−34.18, −0.56), p = 0.04; presented in Figure 3].

② Vitamin K (VK) and VC of other measures of artery calcification. A total of eight studies describing how VK supplementation affects the calcification of other arteries or valves are presented in Table 3. In Zwakenberg et al.'s (28) study, target-to-background ratios (TBRs) tended to rise in the MK-7 group compared with placebo (0.25,95% CI [−0.02, 0.51], p = 0.06), though it would not be statistically significant. Equally, in Bellinge et al.'s study, 10 mg VK1 daily supplementation also helps to reduce the occurrence of the development of newly calcifying lesions in the aorta (OR = 0.27, 95% CI: 0.08 to 0.94, P = 0.04), coronary arteries (OR = 0.35, 95% CI:0.16 to 0.78, P = 0.01), both coronary and aortic arteries (OR = 0.28, 95% CI: 0.13 to 0.63, P = 0.002) as detected using ^{18}F-NaF PET. However, six other studies reported no significant effect of VK supplementation on vascular and valvular calcification.

TABLE 3 Measures of artery calcification.

References	Outcome	Location of measurement	Within group intervention	Control	Difference between groups	*p*-value
Levy-Schousboe et al. (21)	Change in Agatston score (AU)	CVC, MD (95% CI)	416 (−213 to 1,045)	719 (226 to 1,212)	−303 (−1,117 to 512)	0.47
	Change in calcification volume	CVC, MD (95% CI)	346 (−165 to 857)	583 (194 to 973)	−237 (?890 to 416)	0.48
		CAC, MD (95% CI)	968 (134 to 1,802)	415 (−119 to 949)	553 (−445 to 1,550)	0.28
Lees et al. (49)	Distensibility (ascending/10^{-3} mmHg)	Aortic, MD (95% CI)	−0.3 (−0.6 to 0.1)	−0.1 (−0.4 to 0.3)	−0.23(−0.75 to 0.29)	0.377
	Descending (descending/10^{-3} mmHg)	Aortic, MD (95% CI)	−0.2 (−0.6 to 0.2)	−0.5 (−0.9 to −0.1)	0.23 (−0.32 to 0.78)	0.407

References	Outcome	Location of measurement	Baseline intervention	Control	End of follow up intervention	Control	Difference between groups	*p*-value
Zwakenberg et al. (28)	Changes in TBR	Femoral arterial calcification, MD+SD	2.2 ± 0.7	2.1 ± 0.6	0.9 ± 0.55	−0.15 ± 0.44	0.25 (−0.02 to 0.51)	0.06
	Changes in calcification mass (CT)	Femoral arterial calcification, MD (IQR)	196.0 (32.5–424.0)	44.9 (9.6–409.5)	19.7 (2.2 to 51.4)	4.3(0.1 to 20.4)	0.5 (−0.23 to 1.36)	0.18
Oikonomaki et al. (26)	Agatston score (HU)	Aortic, MD + SD	7,827.88 ± 5,493.38	8,253 ± 6,298.94	10,412.53 ± 7,227.2	11,036.58 ± 9,053.34		NR
	Volume (mm 3)	Aortic, MD + SD	6,343.29 ± 4,176.29	6,529.25 ± 4,689.64	8,128.64 ± 5,534.46	8,609.25 ± 6,781.74		NR
	Mass (gr)	Aortic, MD + SD	2,394.42 ± 1,905.12	2,914.27 ± 3,786.69	3,009.51 ± 2,446. 57	3,557.06 ± 3,033.08		NR

References	Outcome	Location of measurement	Baseline intervention	Control	End of follow up Intervention	Control	*p*-value
De Vriese et al. (27)	Change of Agatston scores (%)	Total coronary artery, MD(IQR)	7,864 (4,135–14,019)	8,991 (4,165–22,185)	18.6% (7.2%−110.0%)	19.8% (7.2%−37.9%)	0.73
		Thoracic aorta, MD (IQR)	72 (8–489)	116 (22–346)	25.6% (8.2%−61.4%)	18.7% (4.6%−48.8%)	0.79
		Aortic & mitral valves, MD(IQR)	339 (40–1,028)	415 (71–2,276)	36.3% (3.1%−132.6%)	33.4% (5.8%−84.2%)	0.81
	Change of volume scores (%)	Total coronary artery, MD(IQR)	548 (108–993)	647 (195–1,199)	29.3% (9.7%−56.0%)	14.9% (2.7%−34.0%)	0.43
		Thoracic aorta, MD(IQR)	2,834 (1,478–4,541)	2,930 (1,352–6,244)	19.5% (7.3%−56.0%)	15.6% (5.1%−35.0%)	0.62
Bartstra et al. (29)	Change in arterial calcification mass score	Intracranial internal carotid artery, MD (IQR)	12 (3–25)	3 (0–35)	0 (−1; 5)	0 (−0; 2)	0.76
		Common carotid artery	3 (0–25)	2 (0–10)	0 (−0; 3)	0 (−1; 0)	0.20
		Coronary arteries	74 (13–165)	46 (1–148)	5 (−5; 12)	1 (−4; 11)	0.68

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TABLE 3 (Continued)

References	Outcome	Location of measurement	Baseline intervention	Control	End of follow up Intervention	Control	*p*-value
		Aorta	742 (322–1,337)	365 (39–1,144)	40 (−30; 125)	11 (0; 47)	0.55
		Iliac arteries	633 (242–1,148)	337 (66–764)	25 (6; 87)	5 (−4; 30)	0.07
		Leg arteries	309 (93–851)	90 (11–627)	35 (−8; 99)	7 (0; 47)	0.62
		Total arterial calcification	1,694 (812–3,584)	1,182 (235–2,445)	3 (−2; 16)	36 (1; 129)	0.38
Holden et al. (39)	Change of volume (mm^3)	CAC, MD(IQR)	647.0 [302.0, 1,415.0]	326.5 [131.0, 957.0]	106.6 [32.0, 354.8]	95.0 [2.0, 381.0]	0.96

Reference	Outcome	Location of measurement	Within group intervention	Control	Univariate OR (95% CI)	Multivariate OR (95% CI)	*p*-value
Bellinge et al. (12)	Development of ^{18}F-NaF PET positive lesions	Coronary and aortic	26%	47.4%	0.39 (0.20, 0.78)	0.28 (0.13, 0.63)	**0.002**
		Coronary arteries	20.5%	38.2%	0.42 (0.20, 0.87)	0.35 (0.16, 0.78)	**0.01**
		Aortic	5%	17.1%	0.36 (0.12, 1.06)	0.27 (0.08, 0.94)	**0.04**

The meaning of the bold values refer to $p < 0.05$.

VK and dp-ucMGP

A total of seven trials (578 participants) compared VK supplementation, which showed a substantial impact on the decline in dp-ucMGP. The difference was statistically significant between the two groups [I^2 =71%, MD= −243.31, 95% CI (−366.08, −120.53), $p = 0.0001$; presented in Figure 3].

Adverse events

The prevalence and types of adverse events that occurred in study subjects were described in six trials (547 participants). In Witham et al.'s (20) study, adverse events accounted for a large proportion. Although no adverse events or effects were connected with VK2 supplementation after dialysis in De Vriese et al.'s study, 36 life-threatening or massive hemorrhage incidents took place in the 132 patients which need us to focus on the study of De Vriese et al. (27). The adverse events were not significantly different between the groups [$I^2 = 31\%$, RR = 0.92, 95% CI [−0.79, 1.07], $p = 0.29$; presented in Figure 3].

Sensitivity analysis

We conducted a sensitivity analysis to investigate the effectiveness and safety of VK supplementation on VC and assess the stability and reliability of the meta-analysis results. After successively omitting each study, we found that the overall results had no significant impact, indicating that the conclusions drawn were stable and reliable. Figure 4 provides a summary of the results.

Test for heterogeneity and report bias

Seven studies were compared for evaluating the impact of VK supplementation on dp-ucMGP. However, the results exhibited a high degree of heterogeneity ($I^2 = 71\%$). We performed a meta-regression with the MD in dp-ucMGP as the dependent variable and the sources of heterogeneity. Meta-regression revealed that there was no significant effect on VC of VK supplementation forms or doses, year, and duration of follow-up on univariate analysis (Table 4). There were insufficient observations on the country and population. Subgroup analyses were performed based on population (hemodialysis vs. no-hemodialysis) and country (the Netherlands vs. other countries) of dp-ucMGP. Similarly, a subgroup analysis showed that country and population are not the sources of heterogeneity (Figure 5). Due to the large differences in study outcomes and the small number of included articles, we did not test for publication bias.

Discussion

We performed a systematic review and meta-analysis of the effect of VK supplementation on the progression of VC. Current research indicates that VK supplementation mitigates VC, especially CAC. Additionally, VK supplementation improves VK status, based on the carboxylation status of dp-ucMGP. Moreover, few adverse events were reported. These findings are consistent

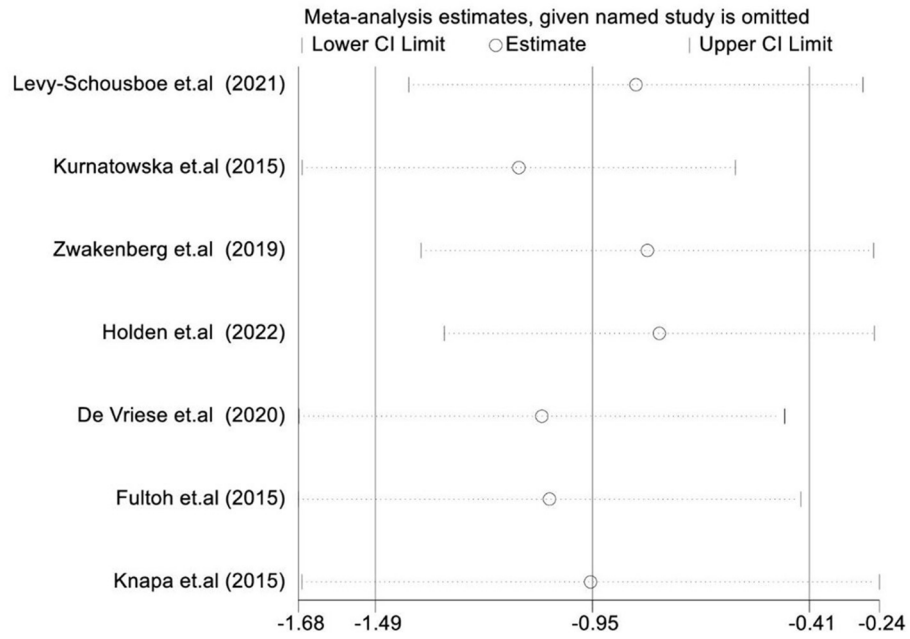

FIGURE 4
Sensitivity analyses.

TABLE 4 Meta-regression with the mean difference (%) in dp-uc MGP.

Variable	Coefficient	95% CI	*p*-value	Tau^2
Forms	−0.4123578	−1.509164 to 0.6844482	0.378	0.4862
Year	−1.901139	−4.68688 to 0.8846013	0.099	0.3276
Duration of follow-up	−1.00118	−5.289889 to 3.287529	0.421	0.9593
Dose	−0.7596538	−1.780453 to 0.2611456	0.114	0.6445

with those of a meta-analysis published in 2018 and have been confirmed by Kosciuszek et al. (32, 33). However, Vlasschaert et al. (34) stated that while VK supplementation may increase the carboxylation of dp-ucMGP, its role in preventing VC is uncertain based on the available data.

VK and calcification of vessels or valves

In this article, we testify that VK supplementation slows the development of CAC. Belling et al. found that 3 months of VK1 supplementation decreased the development of newly calcifying lesions in the aorta and the coronary arteries, as detected by using ^{18}F-NaF PET (12). Similarly, the study by Shea et al. put forward that VK supplementation delayed the course of pre-existing CAC in healthy women and elderly men (13). However, eight other trials that measured major arterial or valve calcification reported no appreciable benefits of VK intake. The role of VK in CAC seems to remain controversial.

Coronary artery calcification (CAC) means the existence of coronary artery disease that is accompanied by the progression of advanced atherosclerosis and is a well-established predictive indicator of future CVD events (35). It is easily identified by radiography as well as by CT and intravascular imaging (35, 36). At the same time, the CT scan-based Agatston score can serve as a quantitative measure of CAC (37). Their application has been expanded to examine other blood vessels. However, the Agatston score fails to capture information about the regional distribution of calcified plaque, and large variability tends to occur in baseline differences of CAC scores, which contribute to the negative results (38, 39). As a non-invasive and quantitative imaging technique, ^{18}F-NaF PET provides a biomarker of calcification activity and detects new calcium generation outside the resolution of CT (40). In Zwakenberg et al.'s (28) study, ^{18}F-NaF activity tended to rise in the MK-7 group compared with the placebo group, which is contrasting with what we found. The discrepancy in the baseline calcification mass and different calcification areas detected by ^{18}F-NaF uptake may have played a role in the disappointing trial outcome (41). In addition, a related study evaluated the uptake of ^{18}F-NaF PET in relation to gender and the number of cardiovascular risk factors (42). In the recent presentation, invasive imaging techniques, such as angiography, with or without optical coherence tomography, provide a higher spatial resolution of mineralized

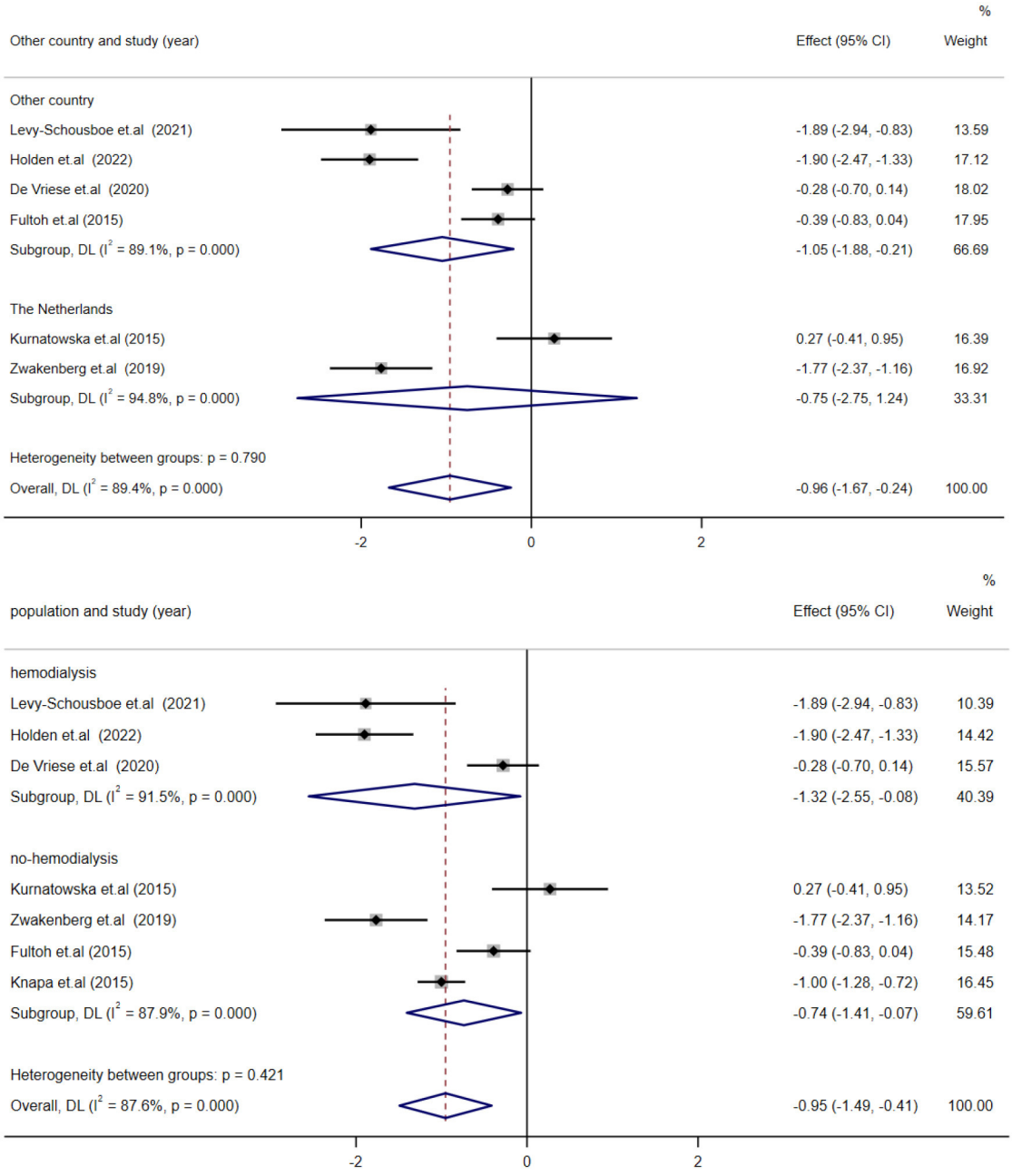

FIGURE 5
Subgroup analyses about country and population of dp-ucMGP.

sites inside and along the artery walls (43). Although these are more sensitive for the identification of VC, their applicability is restricted as an invasive procedure. Overall, limitations in image resolution and other factors may bias the evaluation of calcification scores when using traditional imaging modalities such as CT scans. However, despite these limitations, we have found conclusive evidence that VK intake may impede VC, particularly CAC. Another way to assess the status of VK is by measuring dp-ucMGP levels in the blood, which has been linked to surrogated biomarkers of VC, vascular stiffness, and cardiovascular outcomes (44).

VK and dp-ucMGP

Matrix Gla proteins (MGP) are one of the most effective naturally occurring inhibitors of VC and require VK as a cofactor to become bioactive through post-translational γ-carboxylation and phosphorylation (45). The functional status of VK related to specific tissues can be reflected indirectly by measuring un-carboxylation or carboxylation MGP *in vivo*. However, other types of MGP (except for dp-ucMGP) have a high affinity for precipitated calcium salts and hydroxyapatite, preventing them from freely entering the bloodstream (46). In addition, determining

VK content in the plasma is challenging due to the low circulating level of VK, the non-polar nature of VK, and the interference from lipids (5). As a result, measuring dp-ucMGP levels in the blood could be a useful alternative way to assess the status of VK, which is associated with a surrogate biomarker of vascular health (44).

A total of seven trials (578 participants) were compared, and there was a considerable impact on the decline in dp-ucMGP after VK supplementation. Holden et al. (39) conducted parallel RCTs in hemodialysis patients in 2022, which indicated that dp-ucMGP levels declined by 86% in the VK1 group. Levy-Schousboe et al.'s study showed that despite continuing VK supplementation within hemodialysis patients, dp-ucMGP decreased from the baseline in year 1 and increased again in year 2. It seems to demonstrate that VK supplementation significantly decreases dp-ucMGP levels but cannot prevent a subsequent increase (21). Moreover, another study of hemodialysis patients revealed that lengthy VK2 intake at therapeutic doses and VK antagonism withdrawals do not normalize systemic dp-ucMGP levels, but rather just reduce them (27). The results of the three studies are highly consistent, but they also raise questions worth considering. Specifically, the findings prompt us to inquire why VK supplementation does not restore dp-ucMGP to normal levels in hemodialysis patients.

There are two reasons to explain why dp-ucMGP levels cannot be normalized in hemodialysis patients after VK supplementation. On the one hand, multiple studies have confirmed that dp-ucMGP levels are significantly elevated in hemodialysis patients, indicating VK deficiency. The causes of VK insufficiency in hemodialysis are multifaceted and include inadequate intake, uremic suppression of the VK cycle, and possibly interfering with VK absorption by phosphate binders. On the other hand, dp-ucMGP concentrations are decided by VK status and total MGP levels which have been shown to increase with age and in CVD (47, 48). Furthermore, changes in dp-ucMGP levels were not only seen in hemodialysis patients but also in other patients. Fulton et al., Knapen et al., and Kurnatowska et al. demonstrated that dp-ucMGP levels fell significantly after VK supplementation in other patients. The result is consistent with Zwakenberg et al.'s study. Numerous studies focused on dp-ucMGP alterations in reaction to VK therapy, and it has not been mentioned whether decreasing the level of dp-ucMGP after VK supplementation could well inhibit or alter the advancement of VC. However, we have confirmed that VK supplementation reduces the absolute level of dp-ucMGP.

Conclusion and recommendation

As research progresses, numerous studies have explored ways to inhibit VC, but the complexity and variety of its pathophysiology have presented obstacles. The majority of trials are typically small, single-center studies, and are varied in terms of the type of VK administered, dose of VK, participants studied, results measured, and duration of follow-up. However, with the emergence of more RCTs in the past 2 years, these studies have demonstrated higher participant retention rates, larger cohorts, and longer follow-up periods. In addition, more studies are conducted particularly in the high-risk group of people with DM, CAC, and CKD, who would most potentially benefit from the therapeutic intervention being tested. Furthermore, new technologies, such as ^{18}F-NaF PET, have emerged to identify early and active areas of calcification with greater sensitivity than CT. In spite of this, few researchers have found evidence of VK supplementation plays a significant role in inhibiting the progression of VC. A limitation of our study is the notable variability in the results of the included research. Nonetheless, we contribute to addressing an important and unsolved issue by providing evidence that supports the potential of VK supplementation in mitigating VC, especially in the case of CAC. VK deficiency is common in populations who are at high risk of CVD and CKD and may be more easily treated with VK supplementation than with a change of lifestyle. In addition, VK supplementation does not produce serious adverse effects and may be advantageous as a long-term strategy to enhance vascular health and decrease CVD risk. In the future, we encourage further RCTs to confirm the efficacy of VK therapy. Before this can be translated into clinical practice, more studies are required.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: All data in this paper were collected from databases, including PubMed, the Cochrane Library, Embase databases, and Web of science until August 2022.

Author contributions

TL and WPT conceptualized the idea and created the protocol for this study. TL and YW developed the search strategy and performed the data collection. TL analyzed the data and drafted the manuscript. All authors have read and approved the final manuscript.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1115069/full#supplementary-material

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Reduced phosphorus is associated with older age and hypoalbuminemia. Risk factors for all-cause mortality in peritoneal dialysis patients

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Introduction/aim: Hyperphosphatemia is a mortality risk factor in dialysis patients; however, low phosphorus levels too. Diabetes and malnutrition are strongly associated with mortality and with reduced serum phosphorus. This study analyzed the pattern of serum phosphorus in patients on Peritoneal Dialysis (PD) and its association with mortality.

Methods: A Secondary analysis was performed on a multicenter cohort study in peritoneal dialysis patients from two previous studies done by our group.

Results: Six hundred fifty-four patients were included. Serum phosphorus was <3.6 mg/dL in 28.29% of patients, 3.6 to 5.2 mg/dL in 48.16%, and >5.2 mg/dL in 23.55%. In logistic regression analysis; education, age, and hypoalbuminemia were risk factors for low P levels. In multivariate Cox analysis $P < 3.6$ mg/dL, age, and low albumin were predictors for all-cause mortality. When lower P and lower albumin were combined, this group had the highest risk for all cause and cardiovascular mortality.

Conclusion: The frequency of patients with reduced serum phosphorus was higher in the Mexican population than in Europe or Asia. Low serum phosphorus levels, older age and hypoalbuminemia were risk factors for all-cause mortality. Low phosphorus combined with low albumin levels were the highest risk factor for all-cause and cardiovascular mortality.

KEYWORDS

reduced phosphorus, low serum phosphorus, all-cause mortality, cardiovascular mortality, malnutrition, peritoneal dialysis, diabetes

1. Introduction

The preservation of the biochemical variables of mineral metabolism is an important goal in the management of patients with chronic kidney disease (CKD) and is even more critical in patients with end-stage renal disease (ESRD) or on treatment with dialysis (1). Elevation in serum phosphorus (P) levels is one of the most frequent and best-known alterations and is the consequence of the incapacity of the damaged kidney to eliminate phosphate load from the diet (2, 3).

The elevated concentration of phosphorus has been related to a more rapid progression of renal disease (4), but even more critical, with all-cause mortality and cardiovascular mortality in particular (5, 6). Therefore, efforts to reduce intestinal absorption through the use of binders, increase clearance through dialysis, and limit secondary hyperparathyroidism, have been the source of abundant literature in nephrology (7).

The percentage of patients treated with dialysis who have serum phosphorus values over the standard upper limit (5.5 mg/dL) is greater than 50%, but the frequency of patients with values under the standard lower limit (3.5 mg/dL) (8), is also important, with up to 10% in Europe and some studies report that 19.7% of patients are in the lowest quintile (<4.4 mg/dL) (9–12).

There is a perception that hyperphosphatemia is less frequent in the Mexican population, given that some studies related to mineral metabolism have reported low prevalence in this country (13, 14). Among the causes that may explain these findings is the higher frequency of diabetes, malnutrition, and inflammation, conditions where phosphorus is usually reduced; these conditions are more frequent in our environment compared with other countries (15). It should also be noted that reduced preferences and less availability of food with high phosphorus content and reduced financial ability to purchase them are factors associated with patient social and cultural level in developing countries.

The low phosphorus level is relevant since phosphorus is essential for life; it is actively involved in many critical biochemical pathways, such as energy and nucleic acid metabolism, cellular signaling, and bone formation. It is a crucial component of cellular membrane phospholipids, nucleic acids, adenosine triphosphate (ATP), and phosphoproteins.

The role of hypophosphatemia on the clinical outcomes of the dialysis population is controversial. It is associated with mortality, but the finding is not consistent. However, the association remains statistically significant after adjustments for markers of malnutrition-inflammation-cachexia syndrome. The association of hypophosphatemia with other clinical conditions, such as cardiovascular dysfunction, metabolic syndrome, or glucose intolerance found in the non-CKD population, has not been analyzed in dialysis patient (16).

Recognized cardiovascular risk factors, such as diabetes, malnutrition, inflammation, low economic and cultural levels, are frequent findings in the Mexican population; these same factors are associated with low blood concentrations of phosphorus. For this reason, hyperphosphatemia and hypophosphatemia are equality relevant as a cardiovascular risk factor. This study aimed to analyze the distribution pattern of serum phosphorus in the Mexican population with peritoneal dialysis and its association with general and cardiovascular mortality.

2. Materials and methods

2.1. Design

Secondary analysis was performed on a multicenter cohort study of incident patients on peritoneal dialysis of a two previous studies done by our group, the first (study A) was a non-intervention study to analyze adherence to clinical practice guidelines in patients on dialysis, conducted between 2007 and 2009 (13), and the second (study B) was a study without intervention to find out the frequency of complications in incident patients on PD between 2015 and 2017 (17), both were from multicenter cohorts. Followed up by 18 months.

2.2. Population

Data from patients were included if they were adults (>18 years) and free of acute complications for at least 1 month before inclusion in the study; there was no restriction by gender or cause of renal disease. All patients initiated peritoneal dialysis in a planned manner.

Exclusion criteria: patients were serologically positive for hepatitis B or C or HIV, had a recent renal transplant, or were under treatment with immunosuppressant.

2.2.1. Primary outcomes

Mortality from any cause and cardiovascular mortality are defined by the following events: acute myocardial infarction, heart failure, brain-vascular disease, peripheral vascular disease, arrhythmia, and sudden death.

2.2.2. Data collection

Demographic data and history of renal disease were obtained from clinical files by nurses trained in clinical research and registered on pre-established forms. Data gathered included: age, gender, and diagnosis of diabetes. Patients received conventional treatment with dextrose solutions, and during scheduled visits, weight, height, and body mass index were recorded. Patients were followed for at least 18 months from the inclusion of the last patient and were censored at the date of death, transfer to hemodialysis or transplant, or loss to follow-up.

Laboratory Biochemical analyses were performed in a central laboratory with standard automated techniques. Measurements included: phosphorus, total calcium, total calcium corrected for albumin, C-reactive protein (CRP), glucose, urea, and creatinine. Samples were preserved at −70°C until analysis. For the ends of the analysis, only baseline data were considered. The variables related with mineral metabolism were classified as follows: calcium corrected for albumin (cCa), Q1: <8.4; Q2 to Q3: >8.4–9.5; Q4 >9.5 mg/dL; serum phosphorus in quartiles; Q1: <3.6 mg/dL, Q2 to Q3: 3.6–5.2 mg/dL, Q4: >5.2 mg/dL. Parathyroid hormone (PTH); low <150; in range >150 to <300; high >300 ng/mL).

2.3. Statistical analysis

Data are presented as mean ± standard deviation for continuous variables and percentages for discrete variables. For comparisons between groups, one-way ANOVA or Student's t was used for continuous variables and X^2 for discrete variables. The classification criteria were according to the quartiles of the concentration of phosphorus.

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TABLE 1 Demography, non-medical, and medical variables classified by phosphorus quartiles.

	Total	Q1 (< 3.6 mg/dL)	Q2 (3.6–4.2 mg/dL)	Q3 (4.3–5.1 mg/dL)	Q4 (>5.2 mg/dL)	*p*
n (%)	654 (100)	185 (28.29)	146 (22.32)	169 (25.84)	154 (23.55)	
Age (year)	48.32 ± 15.1	54.12 ± 12.5[a]	51.68 ± 13.8[b]	47.29 ± 15.2	39.30 ± 14.7[c]	0.001
Gender, *n* (%)						0.035
Male	267 (40.8)	91 (34.1)	59 (22.1)	64 (24.0)	53 (19.9)	
Female	387 (59.2)	94 (24.3)[b]	87 (22.1)	105 (24.0)	101 (26.1)	
Economic income, *n* (%)						0.190
Low	373	107 (23.4)	87 (19)	102 (2.3)	77 (16.8)	
Medium-high	84	32 (7)	19 (4)	23 (5)	10 (2.2)	
Education, *n* (%)						0.001
Low	199	80 (17.5)[a]	49 (10.7)	46 (10.1)	24 (5.2)[d]	
Intermediate	173	38 (8.3)	47 (10.3)	49 (10.7)	39 (8.5)	
High	85	21 (4.6)	10 (2.2)	30 (6.6)	24 (5.2)	
Activity, *n* (%)						0.001
Non or home	155	38 (8.3)	37 (8.1)	45 (9.8)	35 (7.7)	
Employee	274	101 (22.1)[a]	66 (14.4)	68 (14.7)	39 (8.5)[d]	
Professional	28	0	30 (0.7)	12 (2.6)	13 (2.6)	
Diabetes mellitus						0.001
Yes *n* (%)	357	125 (28.7)	93 (21.4)	99 (22.7)	59 (13.6)	
Weight (k)	65.53 ± 13.43	64.85 ± 11.87	66.16 ± 13.35	64.70 ± 13.76	66.56 ± 14.83	0.474
Height (cm)	160.21 ± 10.20	158.49 ± 8.91[b]	160.33 ± 10.95	160.34 ± 11.77	162.03 ± 8.70	0.016
BMI (k/m^2)	25.68 ± 7.23	25.79 ± 4.09	26.06 ± 9.05	25.57 ± 9.60	25.29 ± 4.87	0.818
SBP (mmHg)	131.83 ± 22.95	134.17 ± 23.14	132.15 ± 22.77	129.88 ± 21.64	130.86 ± 24.24	0.330
DBP (mmHg)	80.82 ± 14.08	79.18 ± 14.00[b]	81.40 ± 13.37	79.91 ± 14.32	83.25 ± 14.33[d]	0.045
sGlucose (mg/dL)	130.19 ± 81.51	145.85 ± 100.25[a]	143.93 ± 93.75	120.99 ± 65.61	108.46 ± 46.69	0.001
Urea (mg/dL)	102.91 ± 39.11	84.44 ± 33.81[a]	92.99 ± 30.39[b]	106.10 ± 35.33	130.99 ± 39.97[c]	0.001
Creatinine (mg/dL)	7.74 ± 3.42	6.14 ± 2.82[a]	6.47 ± 2.74	7.72 ± 2.76	10.87 ± 3.25	0.001
Cholesterol (mg/dL)	179.45 ± 42.62	175.21 ± 44.87	179.11 ± 41.61	181.54 ± 41.57	182.53 ± 41.93	0.386
HDL Cholesterol (mg/dL)	38.53 ± 20.27	39.81 ± 20.07	37.35 ± 15.37	40.35 ± 26.97	35.32 ± 13.29	0.254
Triglycerides (mg/dL)	177.53 ± 101.97	197.32 ± 142.05[e]	176.05 ± 87.99	165.00 ± 78.59	168.94 ± 72.72	0.014
sAlbumin (g/dL)	3.23 ± 0.56	3.02 ± 0.54[a]	3.20 ± 0.56[b]	3.23 ± 0.52[c]	3.50 ± 0.53[d]	0.001
CRP (mg/L)	5.23 ± 9.06	6.11 ± 10.00	5.48 ± 8.31	4.39 ± 8.6	4.9 ± 8.2	<0.315
Phosphorus (mg/dL)	4.43 ± 1.32	3.03 ± 0.52[a]	3.93 ± 0.17[b]	4.66 ± 0.26[e]	6.31 ± 0.92[c]	0.001
tCa (mg/dL)	8.71 ± 1.33	8.19 ± 1.62[a]	8.86 ± 1.14	8.84 ± 0.96	9.05 ± 1.29[d]	0.001
cCaAlb (mg/dL)	9.33 ± 1.24	8.97 ± 1.53[a]	9.50 ± 1.08	9.45 ± 0.91	9.45 ± 1.25[d]	0.001
PTH (pg/mL)	34.63 ± 66.98	27.06 ± 51.70	20.27 ± 34.04	40.31 ± 71.74	53.98 ± 96.84	0.004
RR F (mL/min)	2.85 ± 2.0	3.28 ± 2.42	3.33 ± 2.35[b]	2.76 ± 2–12[c]	1.77 ± 1.33[d]	0.001
Ultrafiltration (mL/24 h)	837.82 ± 445.73	798.24 ± 449.43	861.11 ± 430.94	913.22 ± 483.49	763.90 ± 385.45	0.061
Follow-up (months)	17.72 ± 7.79	16.63 ± 8.07	17.43 ± 6.88	18.48 ± 8.16	18.46 ± 7.73	0.078
Deaths, all-cause (n)	93	36	14	21	22	0.066
Death rate (events/100 pts/year)	9.7	3.9	1.5	2.1	2.2	
Deaths, cardiovascular (n)	54	17	7	15	16	0.383

(Continued)

TABLE 1 (Continued)

	Total	Q1 (< 3.6 mg/dL)	Q2 (3.6–4.2 mg/dL)	Q3 (4.3–5.1 mg/dL)	Q4 (>5.2 mg/dL)	*p*
Death rate, cardiovascular (events/100 pts/year)	5.6	1.9	0.7	1.5	1.6	

Data are expressed as mean ± SD, or in frequency (%). SBP, systolic blood pressure; DBP, diastolic blood pressure; CRP, C–reactive protein; cCa, albumin-corrected calcium; PTH, parathyroid hormone; RRF, residual renal function = the mean creatinine and urea clearance. One-way ANOVA test with the significance of $p < 0.005$, $p < 0.05$, test *post-hoc*: [a]Q1 vs. Q3, Q4. [b]Q2 vs. Q3, Q4. [c]Q4 vs. Q3. [d]Q1 vs. Q4. [e]Q1 vs. Q3.

Logistic regression was used to analyze the risk factors for having low phosphorus concentrations, and Cox proportional risk model was used to analyze mortality. We did the competing risk models analysis for cardiovascular mortality. Significant variables in univariate analysis were included in multivariate analysis using the step-forward method. All statistical tests were done with the program Statistical Package for Social Sciences (SPSS-PC), v.24 (SPSS, Chicago, IL, USA), and Statistics/Data Analysis (STATA), v 14.2.

3. Results

3.1. Baseline data

Table 1 shows baseline results of demography, non-medical and medical variables when patients were classified according to the quartiles of serum phosphorus. The analysis included 654 patients. The percentage of patients in quartile 1, Q1 (<3.6 mg/dL) was 28.3%. Regarding non-medical variables, patients with low phosphorus were older, mostly women, with low education levels, and had home and employee activity. They tended to have lower economic income (not significant). Regarding medical variables, the low phosphorus group had a higher percentage of diabetes, lower diastolic pressure, lower concentrations of urea, creatinine, albumin, cCa, PTH, and CRP, and higher levels of glucose, triglycerides, and residual renal function (RRF). Continuous Ambulatory Peritoneal Dialysis (CAPD) was the dialysis modality in 525 patients (80.3%), and Automated Peritoneal Dialysis (APD) in 129 patients (19.7%).

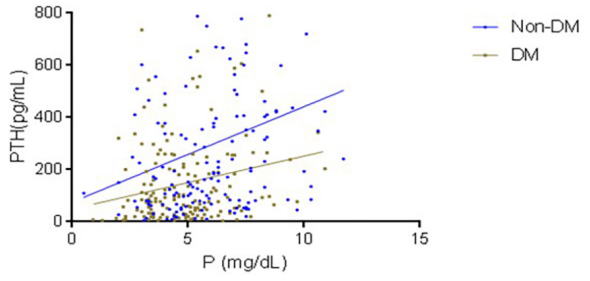

FIGURE 1
The regression line between parathyroid hormone and phosphorus levels in non-diabetic and diabetic patients. PTH was lower in diabetic than non-diabetic patients, at the same phosphorus concentrations. The regression equation for DM was: $y = 6.84(x) + 4.05$, and for non-DM: was $y = 10.1(x) + 8.34$.

3.2. Follow-up

During the follow-up, 93 patients died (14.2%), 24 patients (3.7%) were transferred to hemodialysis, 22 patients (3.4%) were transplanted and 41 patients (6.3%), were lost at the end of the follow-up (Change of address, transfer to other hospitals, loss of insurance rights, voluntary withdrawal).

The causes of Death were: Cardiovascular Death in 54 patients (58%) (which include acute myocardial infarction in 22 patients (23.6%), heart failure in 7 (7.5%), arrhythmia in 6 (6.4%), stroke in 7 patients (7.6%), sudden Death in 10 patients (10.8%), other cardiovascular causes in 2 patients, (2.1%), PD-related peritonitis in 8 patients (8.6%), infections (except peritonitis) in 11 patients (11.8%), uremia/hyperkalemia/acidosis in 13 patients (14%), unknown in 7 patients (7.6%).

Figure 1 shows the regression line between Parathyroid Hormone and Phosphorus levels in Non-diabetic (nDM) and Diabetic Patients (DM). PTH was lower in Diabetic than non-diabetic patients at the same phosphorus concentrations. The regression equation for non-DM: was $y = 10.1(x) + 8.34$, and for DM was: $y = 6.84(x) + 4.05$. DM have lower slop and intercept.

Table 2 shows logistic regression analysis to know the relative value of non-medical risk factor for low serum phosphorus, in Model 1, low education was significant. In model 2, was Model 1 plus; diabetes, serum albumin and age. Albumin and age were the factors most related to low phosphorus.

Figure 2 shows all-cause survival according to phosphorus quartiles (Cox analysis). Blue line; Q1 < 3.6 mg/dL; red line; Q4: >5.2 mg/dl, and green line, Q2-Q3: 3.6 to 5.2 mg/dL of phosphorus. The patients in Q2-Q3 had the best survival, followed by Q4 and Q1, respectively, taking the value of Q2–Q3 as a reference. That is, the phosphorus concentrations, concerning mortality risk, have a U shape. Both low and higher serum phosphorus levels were associated with elevated mortality risk.

Table 3 shows the multivariate Cox regression analysis of factors associated with all-cause mortality.

Phosphorus in quartile 1 (QI) < 3.6 mg/dL (HR: 1.89; 95% CI; 1.05–3.4, $p < 0.032$) as a risk, older age (HR: 1.05, 95% CI; 1.02–1.07, $p < 0.001$) and low albumin concentrations (HR: 0.42; 95% CI 0.28–0.64, $p < 0.001$), as a protector, were associated with elevated all-cause mortality risk, while Q2–Q3 (3.5–5.2 mg/dL), calcium and diabetes had no effect. Taking as a reference Q4. Neither level of phosphorus nor calcium had a significant effect on cardiovascular mortality.

To clarify the role of low phosphate as an independent risk factor, we classified serum Albumin (Alb), and phosphorus (P) levels, where the cut-off point was the low quintile value. We made four categories of patients; Group 1 ($n = 44$): low Alb and low P; Group 2 ($n = 85$) low Alb-high P; Group 3 ($n = 96$): high

TABLE 2 Effect of non-medical plus medical variables on low phosphorus levels.

	Variable	Significance p	RR	95% CI	
Model 1	Education level	0.000			
	Elementary	0.006	2.26	1.26	4.05
	High School	0.865	0.95	0.508	1.76
	Economic income				
	Low	0.053	0.60	0.360	1.07
	Medium, high (reference)				
Model 2	Education level	0.130			
	Elementary	0.128	1.64	0.87	3.11
	High School	0.960	1.02	0.53	1.95
	Economic income				
	Low	0.147	0.62	0.364	1.04
	Medium, high (reference)				
	Diabetes	0.125	1.51	0.89	2.55
	Albumin (g/dL)	0.001	0.49	0.32	0.76
	Age	0.011	1.03	1.00	1.04

Model 1 Logistic regression analysis with education level and economic income. Model 2; Model 1 plus diabetes, albumin and age.

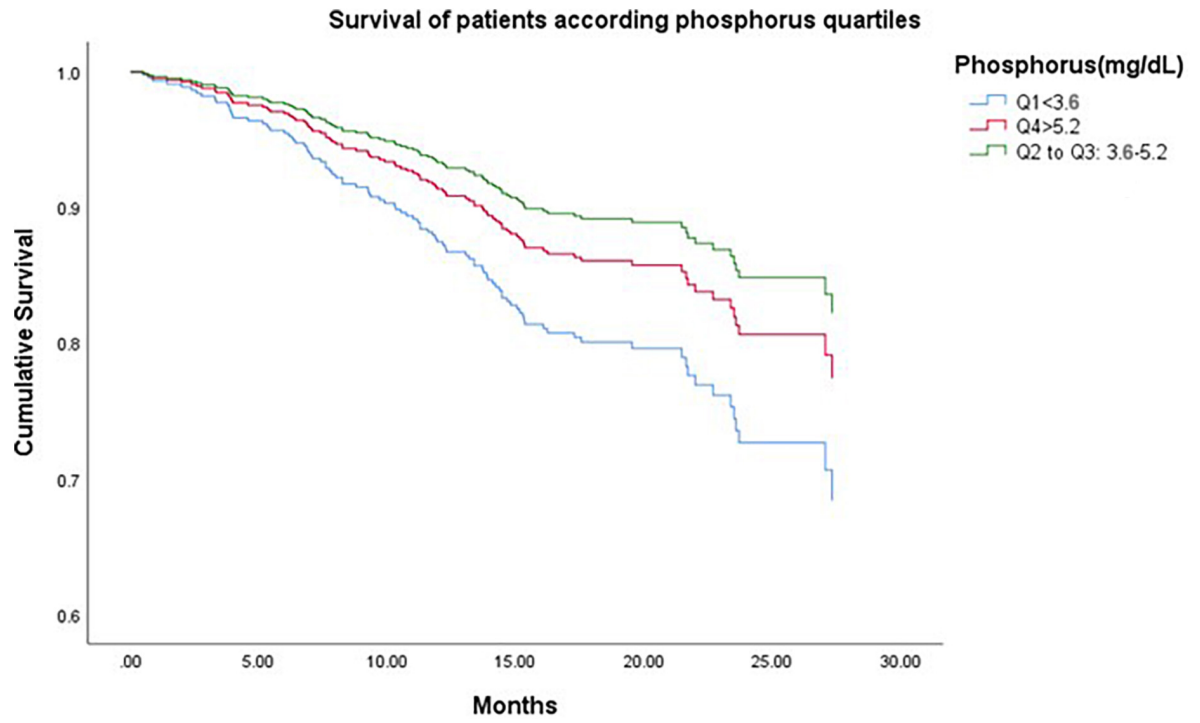

FIGURE 2
All-cause survival according to phosphorus quartiles (Cox analysis). Blue line; Q1: <3.6 mg/dL; red line; Q4 >5.2 mg/dl, and green line, Q2 to Q3 = 3.6 to 5.2 mg/dL. The patients in Q2 to Q3 had the best survival, followed by Q4 and Q1, respectively, taking the value of Q2–Q3 as a reference. Q1: HR 1.94 (95% IC 1.21–3.1, $p < 0.006$), Q4: HR 1.38 (95% IC 0.770–2.22, $p < 0.32$).

Alb-and low P and Group 4 (n = 425), high Alb and high P. Further comparisons between the subgroups were analyzed using Cox regression models. in all-cause and cardiovascular mortality.

Figure 3A shows the survival curves to know the all-cause mortality of patients classified according to the combination of Alb and P levels.

The group of patients with low Alb and low P levels had the highest risk of all-cause mortality, with a HR: 2.9 (95% CI; 1.854.7, $p < 0.001$) followed by the group with low Alb and high P, HR: 2.20 (95% CI; 1.53–3.1, $p < 0.005$), followed by the group high Alb and low P, there was not difference between this group and high Alb and high P, HR: 1.34 (95% CI; 0.92–1.9, $p < 0.128$).

TABLE 3 Factors associated with all-cause mortality on peritoneal dialysis patients.

Variable	Significance p	HR	95% CI	
P Clasification	0.002			
Q2–Q3	0.147	0.69	0.425	1.13
Q1	0.032	1.89	1.05	3.40
Ca Clasification	0.407			
Q2–Q3	0.232	1.35	0.82	2.24
Q1	0.321	1.29	0.78	2.13
Age (year)	0.001	1.05	1.02	1.07
Diabetes (no)	0.090	0.61	0.34	1.08
Albumin (g/dL)	0.001	0.42	0.28	0.64

Multivariate analysis (adjusted Cox analysis) of factors associated with all- cause mortality. Significance = $p < 0.05$, $p < 0.005$.

This means that the association between phosphorus concentration and mortality was modified by albumin level.

Figure 3B shows the survival curves to know the cardiovascular mortality of patients classified according to the combination of Alb and P levels, analyzed using Cox models.

The group of patients with low Alb and low P levels had the highest risk of cardiovascular mortality, with HR: 4.29 (95% CI; 1.69–10.4, $p < 0.002$) followed by the group with low Alb and high P; HR: 3.21 (95% CI; 1.43–7.18, $p < 0.005$), the group with the longest survival, but not significant was the group with Alb high and P low, with HR: 0.79 (95% CI; 0.23–2.69, $p < 0.70$).

These results show that low and high phosphorus levels in conjunction with low albumin values are the highest risk of death. The competing risk models analysis for cardiovascular death, shows that competing variables (change to hemodialysis, transplantation, change of address, transfer to other hospitals, loss of insurance right, voluntary withdrawal, and all-cause of mortality), not affected to cardiovascular death, it was: Q2-Q3: HR; 0.66 (95% CI 0.35–1.2, $p < 0.216$), Q4; HR: 0.69 (95% CI 0.425–1.13, $p < 0.47$), compared with Q1. For all-cause mortality, Q2-Q3: HR 0.51 (95% CI 0.32–0.82, $p < 0.05$) y Q4: HR 0.66 (95% CI 0.39–1.13, $p < 0.191$). Competing variables do not affect the mortality.

4. Discussion

The results reported in this study support the clinical perception that the proportion of patients with serum phosphorus levels lower than those recommended in clinical guideless practices is greater in Mexico than in other countries. Non-medical factors such as low education and non-professional activity are associated with low phosphorus. Medical factors such as serum markers of malnutrition and inflammation had closer associations with low phosphorus than non-medical ones. In multivariate analysis, low phosphorus levels older age, and albumin impacted all-cause mortality. But the combination of low phosphorus and low albumin levels was the strongest risk for cardiovascular and all-cause death.

Preservation of phosphorus concentrations within the accepted targets of the clinical practice guidelines has been a cause of concern in treating patients with CKD-ESRD. Nevertheless, such limits vary among the most widely distributed guidelines; even more, there are also significant variations in regional or national guidelines (9).

Concentrations of phosphorus in patients with CKD-ESRD are generally over the recommended limits; in the first reports of the Dialysis Outcomes and Practice Patients Study (DOPPS), 51.6% of the patients had >5.5 mg/dL and individual values by country were 53.6, 49.4 and 51.9% in Japan, Europe, and the USA, respectively, with a trend toward an increase in the percentage of patients within targets as reported in later studies.

In this study, the percentage of patients with hyperphosphatemia was lower, (23.5%) than in the studies mentioned above. The same DOPPS studies showed that 7.6% of the total population had phosphorus concentrations lower than the recommended limits (3.5 mg/dL). The percentage of patients with low phosphorus varied in the various countries studied, with 5.8% in Japan, 10.1% in Europe, and 6.8% in the United States (10, 11). The percentage of patients with phosphorus below 3.6 mg/dL found in this study was (28.3%) substantially higher when compared with the reports above. It confirms the clinical perception of low phosphorus in Mexican patients.

The causes of low phosphorus have not been well established although it appears to be related to medical and non-medical factors. Among the non-medical ones involved are economic and cultural factors. Population with low socio-economic level are more susceptible to have low phosphorus levels and have a faster progress of CKD (18), later access to treatment, and to be malnourished (19, 20). They more frequently consume unprocessed, rather than processed foods because in México they are cheaper. Processed foods contain much additives and preservatives, which have a high phosphorus content. Our results show that the group of patients with phosphorus <3.6 mg/dL had significant proportions of low income and education, factors that imply higher consumption of unprocessed foods, malnutrition, inflammation and low albumin levels (21, 22). Insufficiency of protein intake may also result in lower serum phosphorus concentration and a concurrent decline in serum albumin.

In patients on peritoneal dialysis, the total removal of phosphorus depends more on residual renal function than on peritoneal extraction (23). So in patients with poor renal function, such as in this study, peritoneal dialysis has little effect on phosphorus balance. The reduced food intake plays a fundamental role, and uremia *per se* is associated with spontaneous reduction of food intake (24, 25), a reduction that is even more critical in patients with the malnutrition-inflammation syndrome (26).

In this study, neither appetite nor phosphorus intake was explicitly measured, but the association with malnutrition-inflammation can be seen in the narrow correlation between serum phosphorus, serum albumin, and CRP, Table 1, which are markers of this syndrome (27).

With respect to phosphate binders, $CaCO_3$ the most frequently used. There was no significant difference in serum phosphorus levels between those who ingested $CaCO_3$ and not. We believe that it was due to the fact that the average consumption was small; 1.6 g/day/kg.

The importance of the medical factors surpassed the non-medical ones, as seen in logic regression analysis, where these latter maintained independent values as factors associated with low phosphorus levels.

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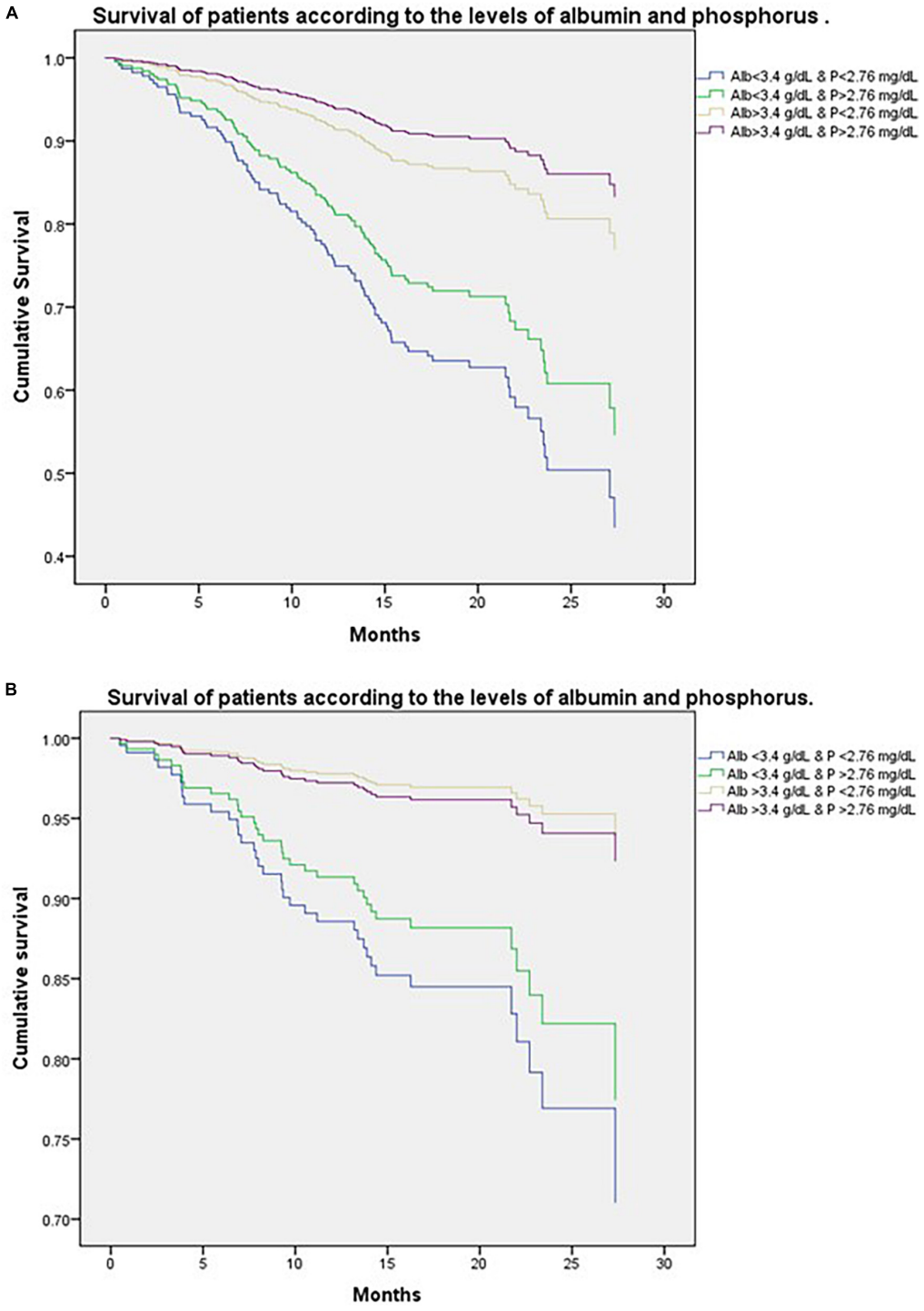

FIGURE 3
(A) Survival curves of patients classified according to the combination of albumin (Alb) and phosphorus (P) levels, analyzed using Cox models for all-cause mortality. The group of patients with low Alb and low P levels had the lowest risk of survival, followed by the group with low Alb and high P, followed by the group with Alb high and P low and Alb high and P high, respectively. **(B)** Survival of patients classified according to the combination of albumin (Alb) and phosphorus (P) levels, analyzed using Cox models for cardiovascular mortality. The group of patients with low Alb and low P levels had the lowest risk of survival, followed by the group with low Alb and high P, followed by the group with Alb high and P high, and then the group with Alb high and P low, respectively.

Among the medical factors, one should consider inadequate elimination and insufficient phosphorus ingestion. Limited studies in CKD animal models and patients with CKD suggest that there may be a break in this homeostatic response where the intestine fails to compensate for impaired renal phosphorus excretion by reducing fractional intestinal phosphorus absorption (28). Also reduced levels of 25-hidroxy vitamin D in CKD patients may impair phosphorus absorption.

The value of hyperphosphatemia as a risk factor for all-cause and cardiovascular mortality has been previously found (2, 4–6). However, the association between serum phosphorus and mortality is not linear; various studies have shown a U-shaped association with greater risk at both ends, (29) but mortality has different characteristics. While high phosphorus levels have been related to all-cause and cardiovascular death, low levels have been associated with malnutrition-inflammation and, in consequence, death by infections and all-cause mortality (30). In our study, this situation was demonstrated clearly in **Figure 2**. Mortality was higher in Q1 and Q4 than in Q2–Q3.

The present study, the univariate analysis showed the association between low phosphorus and all-cause mortality, but not with cardiovascular mortality. In multivariate analysis, conditions such as age, and hypoalbuminemia were independent risk factors for mortality with greater significance than phosphorus reduction, **Table 3**. With the competition analysis we were able to corroborate that the competition variables, such as losses (change to hemodialysis, transplantation, change of address, transfer to other hospitals, loss of insurance rights, voluntary withdrawal) of patients from the study and all-cause mortality, did not affect cardiovascular death.

We wanted to know the mortality risk of phosphorus in combination with albumin, and we found that the association between phosphorus concentration and mortality was modified by albumin level. It is important to know that low phosphorus and low albumin were risk factors for all-cause and cardiovascular mortality, but low phosphorus with high albumin blunted these effects of the low phosphorus on all-cause and cardiovascular mortality, such in others studies (31).

Low phosphorus concentrations added to malnutrition significantly increase the risk of death in vulnerable populations, such as geriatric patients (32).

Our study has strengths and weaknesses. The strength lies in a sample size considered large from a multi-center cohort with and selection criteria allowed us to analyze a representative sample of a common population of PD patients in México. The main weakness is that this is a secondary study without intervention on the supply or elimination of phosphorus in urine or dialysis. However, there are currently no controlled clinical studies that show that lowering P leads to better survival.

The data in this study do not contradict the predictive value or the importance of hyperphosphatemia in all-cause or cardiovascular mortality. They simply warm of the clinical importance of low phosphorus levels and their relation with the diabetic state, malnutrition, inflammation, and the effect of socio-economic factors as impulses for this dangerous relation for the life of patients.

In conclusion, the present study showed that the frequency of patients with serum phosphorus less than 3.6 mg/dL was higher in the Mexican population than in Europe or Asia; it also shows that low education level, older age, women, and less productive activities were risk factors for low serum phosphorus. Reduced serum phosphorus levels, older age and hypoalbuminemia were risk factors for all-cause mortality. But low phosphorus combined with low albumin values were the highest risk for all-cause mortality and cardiovascular mortality in peritoneal dialysis patients.

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 original protocol was approved by the Comité Nacional de Investigación Científica, and the Comité de Bioética from Instituto Mexicano del Seguro Social (IMSS). Registration number, R-2016-785-058. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MÁ and RP: conceptualization, formal analysis, and writing review and editing. MP: methodology. MÁ and MP: investigation. MC-B: data curation. MÁ and MC-B: writing and original draft preparation. RP: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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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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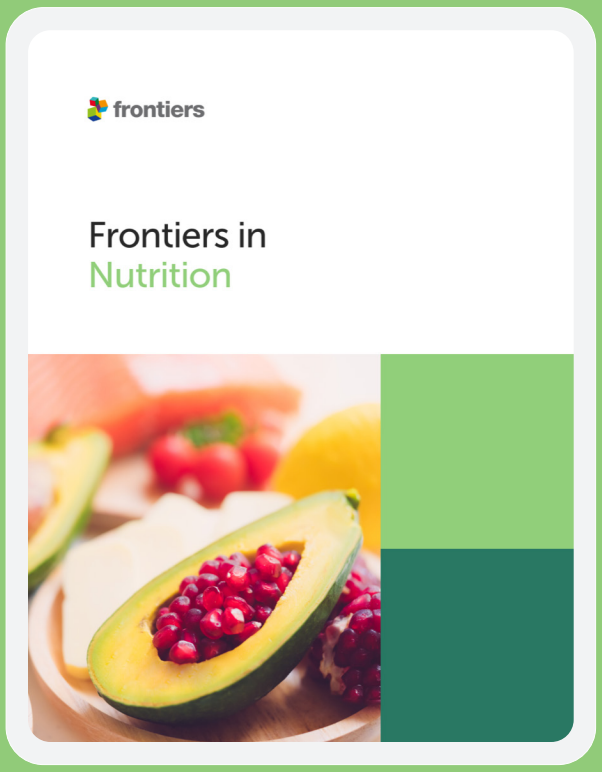

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