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Micro- and macronutrient malnutrition in cardiovascular disease

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Micro- and macronutrient malnutrition in cardiovascular disease

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Editorial: Micro- and macronutrient malnutrition in cardiovascular disease

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Editorial on the Research Topic
Micro- and macronutrient malnutrition in cardiovascular disease

According to the European Society of Clinical Nutrition and Metabolism (ESPEN), malnutrition can be defined as "a state resulting from lack of intake or uptake of nutrition that leads to altered body composition (decreased fat free mass) and body cell mass leading to diminished physical and mental function and impaired clinical outcome from disease". Malnutrition can result from starvation, disease or advanced ageing (e.g., >80 years), alone or in combination (1). The World Health Organization (WHO) classifies malnutrition as one of the biggest threats to public health. Identifying patients at risk or with malnutrition can be undertaken by numerous screening tools, for example, the Malnutrition Universal Screening Tool (MUST) which utilises BMI, acute weight loss and loss of appetite. Blood biomarker panels have also evolved comprising of clinically adoptable measures (e.g., albumin, t-lymphocytes, ferritin, cholesterol).

In patients with acute coronary syndrome and heart failure, there is a high prevalence of malnutrition, assessed post-hoc using blood biomarker panels (2, 3). In these studies, there was no direct comparisons with other screening tools (MUST and other physical clinical assessments, e.g., sarcopenic obesity. Understanding the limits of agreements between screening tools and blood biomarker panels are urgently required, particularly where a large number of patients are normal or overweight and are typically not characterised as at risk of malnutrition *via* MUST classification. For example, Roubin et al. (2) reported the prevalence of moderate or severe malnutrition in patients with acute coronary syndrome based on three scores (CONUT, NRI, PNI). The prevalence was calculated to be 11.3%, 39.5% and 9.0% respectively. The higher observed prevalence of malnutrition was obtained with the NRI tool, which includes excess weight in its calculation (i.e., actual—ideal weight). This does raise an important question; does obesity really matter when considering nutritional risk?

Nutrient deficiencies associated with obesity may be partly due to overconsumption of foods that have a high caloric value but have a low nutrient density. Cross-sectional data from the UK National Diet and Nutrition Survey (2008–2014) in 9,364 individuals indicated that ultra-processed foods accounted for 57% of total energy intake. In that study a 10% increase in the consumption of ultra-processed foods linked with a 18% rise in the prevalence of obesity in both sexes (4). A recent systematic review/meta-analysis discussing trends of ultra-processed food consumption has underlined the importance of

undernourishment in chronic noncommunicable diseases (5). Traditionally, global health has focused on two distinct issues in nutrition; overnutrition, which includes being overweight or obese; or undernutrition, which includes underweight, frailty or having nutrient deficiencies. However, both conditions can be seen in the same individual, a "double burden of malnutrition" (DBM) by increased consumption of ultra-processed food, which is considered addictive based on established scientific criteria (6), is nutrient density poor and is displacing traditional dietary habits and practices.

It is important to recognise nutritional inadequacies/deficiencies in patients regardless of BMI classification. In the US NHANES study, the population prevalence not achieving estimated average requirement (EAR) in vitamins A, C, D, E, calcium and magnesium were significantly higher moving up the BMI classifications, but still worryingly high even in the normal weight group (7). Methodologically, there are precision issues with the subjective recall of dietary intake. Additionally, physiological concentrations are mediated *via* the absorption, distribution and metabolism of the micronutrient.

There has been a proposal to harmonise nutrient intake reference values and apply on a global scale to assess intakes across populations (8). The approach incorporates the framework and terminology recommended by reports from the United Nations University, the National Academies of Sciences, Engineering, and Medicine (NASEM), the Institute of Medicine (IOM), and the European Food Safety Authority (EFSA). A recent study, Beal et al. (9) developed global food composition database and calculated recommended nutrient intakes for five populations groups with varying nutritional requirements. In addition, ratings of micronutrients across different food sources were calculated. In brief, the top sources of micronutrients were organ meat, dark leafy vegetables, crustaceans, bivalves (clams, mussels, oysters, and scallops), goat, beef, lamb, fish and milk. Interestingly, foods promoted as nutrient dense, including many fruit and vegetables and whole grains, are not particularly dense in bioavailable micronutrients. These foods provide nutritional benefits beyond specific nutrients, such as fibre, which is important for the gut microbiota.

Integrating nutrients, through better food choices i.e., less ultra-processed food consumption, and foods that have a higher nutrient density is vital for everyone, not just those with CVD. High diet quality messages is an essential component of dietary recommendations, national food policies and health promotion. Specifically, being overfed and/or undernourished is a phenotype, that in our opinion, requires more scientific and clinical discussion and empirical testing in well-designed clinical outcome studies. This is particularly given recent (and worryingly) worldwide high obesity statistics, consumption of nutrient poor processed food and the displacement of traditional eating practices. Nutritional screening in obesity, through diet quality screeners is absolutely key (10) and understanding the barriers, motivators and enablers to improve nutritional status is a priority.

Author contributions

JH wrote the article. All authors were involved in the conception of the article and provided critical revisions. 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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SYSTEMATIC REVIEW
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The Effect of Magnesium on Reperfusion Arrhythmias in STEMI Patients, Treated With PPCI. A Systematic Review With a Meta-Analysis and Trial Sequential Analysis

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Aims: The restoration of coronary circulation plays a crucial role in treating ST-segment elevation myocardial infarction (STEMI), however successful reperfusion with primary percutaneous coronary intervention (PPCI) may induce life-threatening arrhythmias. The relation between myocardial electrical instability, as a background factor in reperfusion arrhythmia, and magnesium administered periprocedurally is still questionable. Several randomized clinical trials have been conducted predominantly in the thrombolysis era. Due to the contradictory results of these studies, there is little evidence of the potential preventive effect of magnesium on reperfusion arrhythmias. The aim of our study is to review and meta-analytically analyze data from all studies published so far in the PPCI era, comparing STEMI patients who have undergone primary PCI and received either magnesium or a placebo before the reperfusion procedure.

Methods and Results: Our meta-analysis follows the points in the PRISMA protocol and, meets all of their criteria. We conducted a search in five scientific databases using the following keyword combination: (myocardial infarction OR myocardial injury OR acute coronary syndrome OR acs OR stemi) AND magnesium. The 7,295 collected publications were filtered with the Endnote program by title, abstract and full-text based on predefined criteria. A statistical analysis was performed on three randomized-controlled trials using three common parameters, involving 336 patients Trial sequential analysis (TSA) was applied to assess the risk of random error associated with sparse data and multiple testing which can affect cumulative meta-analysis. The incidence of ventricular tachycardias (VTs) was not significantly increased in the non-magnesium control group. (OR: 1.36; CI: 0.619; −2.986, $P = 0.263$). For the ejection fraction (EF), a non-significant decrease was observed in the magnesium group by weighted mean difference calculation. (WMD: 7.262, 95% CI: −0.238; 0.053; $P = 0.057$). There was significant decrease in the infarct zone wall motion index (IZWMSI) in the magnesium

treatment group. (WMD: 0.384, 95% CI: −0.042; 0.811, $P = 0.015$). Based on the TSA assessments, the results of all parameters are not significant, objectively demonstrating the lack of reasonable data pertaining to our question.

Conclusions: The preventive effect of magnesium on reperfusion arrhythmia associated with primary PCI can still be considered contradictory based on previous studies. In our study, we found, that magnesium is ineffective with a very weak evidence, due to the small number of patients and the biases of the included studies, and a well-designed clinical trial is needed in this area, based on the TSA.

Keywords: PCI, percutaneous coronary intervention, magnesium, STEMI, reperfusion arrhythmia

INTRODUCTION

The restoration of blood flow plays a crucial part-in the treatment of patients with ST-segment elevation myocardial infarction (STEMI), however reperfusion can act as a double-edged-sword. Reperfusion injury could manifest in different modes of cell death (e.g., necrosis and apoptosis), microvascular dysfunctions (e.g., impaired vasomotion, microvascular obstruction, and intramyocardial hemorrhage), and malignant ventricular arrhythmias [e.g., ventricular tachycardia (VT), ventricular fibrillation (VF)]. These clinical complications play an important role in postinfarction mortality (1).

Reperfusion induced VT or VF is defined as arrhythmia occurring during the restoration of coronary circulation. It is a relatively frequent condition among STEMI patients, who have undergone primary percutaneous coronary intervention (PPCI), 4–5% of them being affected (2). However, in the OACIS study, a much higher incidence was reported, with 23% of patients who received PCI suffering from reperfusion-related ventricular arrhythmia within 12 h of the onset of symptoms (3).

This phenomenon has long been known, especially in the era of thrombolysis, several studies have discussed the risk factors for acute reperfusion-related arrhythmias and the possible preventive solutions (e.g., beta-blockers and magnesium) in STEMI patients (4, 5).

Numerous angiographic and clinical parameters are known to predispose patients to reperfusion induced malignant arrhythmias associated with primary PCI, such as a culprit lesion in the left main artery, peak creatine kinase >3,000 IU/L, inferior wall STEMI, infarction due to dominant right coronary artery occlusion, pre-PCI thrombolysis, 0–1 TIMI flow and Killip class > 1 (1–3).

Reperfusion-induced ventricular arrhythmias strongly divide the medical community. There are contradictory data on long-term mortality, but most studies suggest that malignant ventricular arrhythmias associated with PCI do not increase mortality in the long term (2, 6). Nevertheless, numerous authors have shown that malignant arrhythmias associated with primary PCI significantly increase both in-hospital and 30-day mortality (7, 8).

Treatment of arrhythmias during primary PCI is consistent with the general management of VT or VF also accepted by the European Society of Cardiology (ESC) (9). Although the treatment algorithm is well-described, there is little data on the prevention of these conditions.

The pathomechanism is not fully understood, acute ischemia, anaerobic metabolism and acidosis, in the infarct-related area, presumably cause the activation of different types of potassium channels, responsible for repolarization, resulting in high potassium efflux from the cells. Furthermore, a catecholamine stress response caused by AMI through lipolysis, can lead to magnesium soap formation and resultant depletion. The intracellular magnesium loss caused by ischemia, leads indirectly to calcium influx and mitochondrial accumulation (10, 11). This cascade generates an electrolyte imbalance that triggers the electrical instability of the cell membrane (12, 13). Thus, restoring coronary blood flow is certainly beneficial for myocardial metabolism, but may further increase the risk of life-threatening arrhythmias due to pathophysiological abnormalities in membrane potential (14).

Magnesium is the second most important intracellular cation present with a concentration of 10–30 mM. Magnesium plays a key role in the synthesis and repair mechanisms of DNA, takes part-in numerous biochemical anabolic and catabolic processes, including protein synthesis and glycolysis since it is a cation, and is essential for the functioning of more than 800 enzymes (15). Furthermore, Mg^{2+} plays an important role in the regulation of many ion channels such as Na^{+}, K^{+}, and Ca^{2+} channels. It reduces the influx of potassium into the myocytes through the inhibition of late rectifier K^{+} channels. It can prolong both the atrial and ventricular refractory period, thus providing protection against proarrhythmic substrates. In the case of hypomagnesaemia, these protective mechanisms may be eliminated, which, in the case of a persistent condition, may contribute to the development of chronic cardiovascular diseases. In case of chronic magnesium deficiency, intacellular accumulation of Na^{+} and Ca^{2+} ions increase the risk of hypertension and coronary vasospasm. Through metabolic effects, Mg^{2+} deficiency increases the risk of type 2 diabetes or metabolic syndrome, because they are involved in the positive modulation of the GLUT-4 channel, enhancing insulin sensitivity (16–18).

Abbreviations: STEMI, ST-segment elevation myocardial infarction; PPCI, primary percutaneous coronary intervention; TSA, trial sequential analysis; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis; VT, ventricular tachycardia; EF, ejection fraction; IZWMSI, infarct zone wall motion index; WMD, weighted mean difference; OR, odds ratio.

In our case, perhaps the most important mechanism is, that Mg^{2+} can prevent intracellular Ca^{2+} accumulation, by inhibiting L-type Ca^{2+} channels, with a negative inotropic and anti-ischemic effect (1, 11, 19, 20), thus leading to the idea that Mg^{2+} supplementation can protect against the reperfusion-induced pathological ion cascade. Numerous experimental studies have shown that Mg^{2+} can inhibit the influx of Ca^{2+} into ischemic cells, thereby increasing the depolarization threshold of the cells and reducing their excitability (20, 21). Further, it can reduce vascular resistance, thus lessening the work of the heart (22).

The potential anti arrhythmic effect of magnesium, administered before opening the vessel is still debated in clinical practice.

In the 1990s and early 2000s, during the thrombolysis era, several studies investigated the magnesium effects in STEMI patients on the prevention of reperfusion induced arrhythmias, and did not find magnesium to be effective on the prevention of reperfusion arrhythmias, with the exception of the LIMIT-2 study (5). Given the more rapid vascular opening and flow growth associated with PCI, it is relevant to summarize this effect of magnesium in studies, conducted in the PPCI-era.

Primary PCI is now considered the gold standard of treatment for STEMI. There is limited data and evidence on the relationship between reperfusion arrhythmias and preventive magnesium administration during percutaneous coronary intervention. Therefore, our study aims to review the literature on the effects of magnesium on reperfusion arrhythmias in patients with STEMI undergoing primary PCI.

METHODS

The systematic-review theme is based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guideline (23), and meets all of its criteria. The PICO format was used: P(opulation)- patients with STEMI, I(ntervention)- patients undergoing magnesium treatment, C(ontrol)- patients who did not get magnesium, O(utcome): VT or VF, EF, IZWMSI, myocardial viability, mortality, hospitalization, etc.

Search Strategy

The article search was carried out in five databases: Pubmed, Embase, Web of Science, the WHO Global Health Library and Cochrane from inception to 20 June 2019 using the recommendations in the PRISMA statement (23). Two investigators conducted a comprehensive search with a combination of the following keywords: (myocardial infarction OR myocardial injury OR acute coronary syndrome OR acs OR stemi) AND magnesium. References in the articles that were found were screened for additional suitable publications. Our search identified a total of 7,295 articles in Embase, PubMed, Cochrane, Web of Science and the WHO Global Health Library databases.

Inclusion and Exclusion Criteria

Articles were selected if they involved a control group and a magnesium treatment group with patients suffering from STEMI, requiring primary PCI. The Zwolle Trial (24), the first study to investigate the efficacy of primary PCI in patients with AMI, was published in 1993, therefore, earlier publications were ignored. Conference abstracts were also included if they contained sufficient data. The language was not an exclusion criterion. Case reports, case series, duplicate reports, and non-human trials were excluded.

Selection Process

Records were managed with the EndNote X7.4 software (Clarivate Analytics, Philadelphia, PA, USA) to remove duplicates. The right or seemingly right articles were selected step by step, first by reading the title, and then by reading the abstract and finally the full text. The selection process, as well as the search, was carried out by two colleagues, based on the PRISMA guideline.

Data Extraction

Both numerical and textual data were collected on a 2016 Excel sheet (Office 365, Microsoft, Redmond, WA, USA). The following data were collected: author, date of publication, type of clinical trial, geographic location, number of subjects treated and controls, patient age, patient's history, infarction localization, baseline serum Mg (mg/dl), incidence of spontaneous recanalization, before and after echocardiographic parameters, such as the infarct zone wall motion score index (IZWMSI), coronarographic parameters including TIMI and Rentrop score, baseline hemodynamic parameters such as heart rate, blood pressure and left ventricular end-diastolic pressure, ejection fraction, cardiac output, drugs used during percutaneous transluminal coronary angioplasty (PTCA), magnesium dose, time of magnesium administration, creatine kinase peak (U/L), incidence of VT or VF, heart failure, cardiogenic shock, re-infarction, and deaths. The data were extracted by two colleagues independently and then confirmed by a third party.

Statistical Analysis

We used meta-analytical calculation tools to observe pooled effect sizes for the three studies we used meta-analitical calculation tools. In the case of VT, the odds ratio (OR) with a 95% confidence interval (CI) was calculated. In the case of the EF and IZWMSI, a weighted mean difference was conducted with a 95% CI, where the weights are based on the number of items, because the studies used the same scale as these variables. The random effects model developed by DerSimonian and Laird (25) was applied in all calculation. Heterogeneity was determined with Cochrane's Q and the I^2 statistics. Because of the small number of studies, the existence of publication bias was not investigated. The analysis was done with STATA software version 15.

In meta-analytical calculations, low-level methodological examinations and publication biases can lead to false *p*-values and thus to type 1 errors. To correct these distortions, the trial sequential analysis (TSA) method is used to compare studies with the same design. TSA is a randomized effect-based meta-analytical model to estimate the amount of data that can be used to make significant decisions about a given parameter. The relation between the cumulative Z curve and the trial sequential monitoring boundary shows the expressiveness of the results. If

the cumulative Z curve crosses the trial sequential monitoring boundary and the cumulative sample size of the analysis reaches the required sample size, firm evidence can be observed. For the analysis we used the TSA software tool from the Copenhagen Trial Unit, Center for Clinical Intervention Research, Denmark (version 0.9 beta, www.ctu.dk/tsa).

The points on the Z curve all indicate such data numbers. If the Z curve reaches the conventional boundary, the result of the statistical analysis is significant. If the Z curve reaches the futility area the result is not-significant and no data is expected to affect this in the future. If the Z curve does not affect either, the result is not significant, however, this may change in the future, depending on new data. The latter statement applies to all our results.

Quality Assessment

A risk of bias assessment was first performed at the individual study-level based on the Revised Cochrane risk-of-bias tool for randomized trials (RoB 2). From the individual studies, we chose the one with the highest risk of bias. Then we summarized overall RoB-assessment on the interventions at the comparison level with the same method.

Assessing of the Grade of Evidence

The GRADE system was used to assess the strength of evidence of our results. GRADE stands for Grades of Recommendation Assessment, Development, and Evaluation (26). GRADEpro Guideline Development Tool [Software]. McMaster University, 2020 (developed by Evidence Prime, Inc.) was used to evaluate the level of evidence.

RESULTS

Results of the Selection Process

Our search identified 4,127 articles in Embase, 1,621 in PubMed, 234 in the Cochrane database, 1,395 in Web of Science and 153 in the WHO Global Health Library. In the end three articles were eligible for the quantitative analysis with, 336 patients in total, 168 cases, and 168 controls. A further two articles provided results, but they were not suitable for meta-analytical calculations, as data from STEMI patients undergoing PCI or thrombolysis in these studies were aggregated and thus also excluded (27, 28). Due to the lack of publications, we attempted to save these articles and contact the authors via email to obtain the raw data, but were unsuccessful. Finally, we processed data from three articles (29–31) in more detail. The selection process is shown in the PRISMA flow chart, in **Figure 1**, and the main characteristics of the included studies are listed in **Table 1**.

Results of the Statistical Analysis

The three articles were comparable for a total of three parameters, processing data from a total of 336 patients.

For VTs, we used the odds ratio method because this is an event number, so we arrive at the approximate number of times that a given event occurs. The pooled result showed that the number of VTs non-significantly increased in the magnesium treated group, using 95% confidence (OR: 1.36; CI: 0.619; 2.986, $P = 0.263$). There was a low degree of heterogeneity across the studies included in the analysis (I^2: 25.2%; $P = 0.263$). A detailed result shown with the random effect model displayed in **Figure 2A**. Based on the TSA analysis, our result is not significant, however, this may change in the future with an adequate quantity of data (see **Figure 2B**).

The EF was comparable in all the three studies, which involved data on 336 patients, where employed the weighted mean difference method because the data used the same scale, so we were able to weight them based on the number of items. The weighted mean difference was non-significantly decreased in comparison with the placebo control group (WMD: 7.262, 95% CI: −0.238; −0.053; $P = 0.057$). There was a high degree of heterogeneity across the studies included in the analysis of the EF (I^2: 94.8%, $P = 0.000$). A detailed result shown with the random effect model is presented in **Figure 3A**. Based on the TSA analysis, our result is not significant, however, this may change in the future with an adequate amount of data (see in **Figure 3B**).

For the IZWMSI, we used the weighted mean difference method again for the same reason. The weighted mean difference was significantly decreased in the magnesium group (WMD: 0.384, 95% CI: −0.042; −0.811, $P = 0.015$). There was a high degree of heterogeneity across the studies included in the analysis (I^2: 76.0%; $P = 0.015$). A detailed result of the analysis shown with the random effect model is illustrated in **Figure 4A**. Based on the TSA analysis, our result is not significant, however, this may change in the future with an adequate amount of data (see in **Figure 4B**).

Two of the three articles (30, 32) stated other very important parameters, such as heart failure, cardiogenic shock, or death. Due to the low number of events of complications, a statistical analysis was not possible. These data are shown in **Table 1**, complete with the main characterisctics of the studies.

After performing the qualitative analysis (Rob2) and the GRADE method, all statistical results can be evaluated at a very low level of evidence.

The results of the statistical analysis are displayed in **Table 2**.

Findings from the quality assessment of the randomized controlled trials using the RoB-2 scoring system are presented in **Table 3**.

DISCUSSION

Restoration of blood flow, in the occluded coronary artery plays a crucial role in the management of acute myocardial infarction. Nowadays, the gold standard treatment of STEMI is PCI. However, in some cases, opening the vessel can lead to life-threatening arrhythmias. The potential preventive effect of magnesium on reperfusion arrhythmias has been a topic of concern to date. Several studies and reviews have reported these positive effects (28, 32). Over the past decades, numerous clinical studies have been conducted to clarify the issue and yielded conflicting results. Possible contradictions may be due to reasons such as heterogeneity of the examined population, differences in hemodynamic instability in patients, dose of magnesium, timing of administration, and use of different reperfusion techniques.

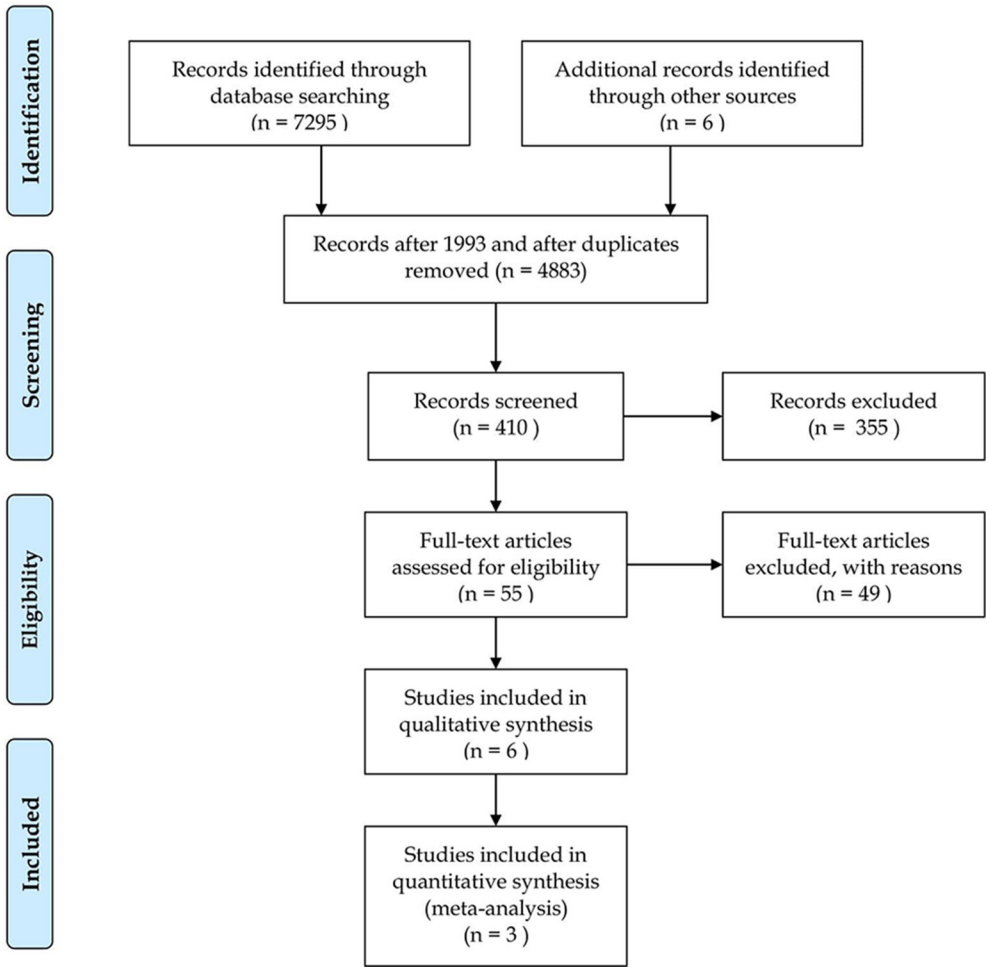
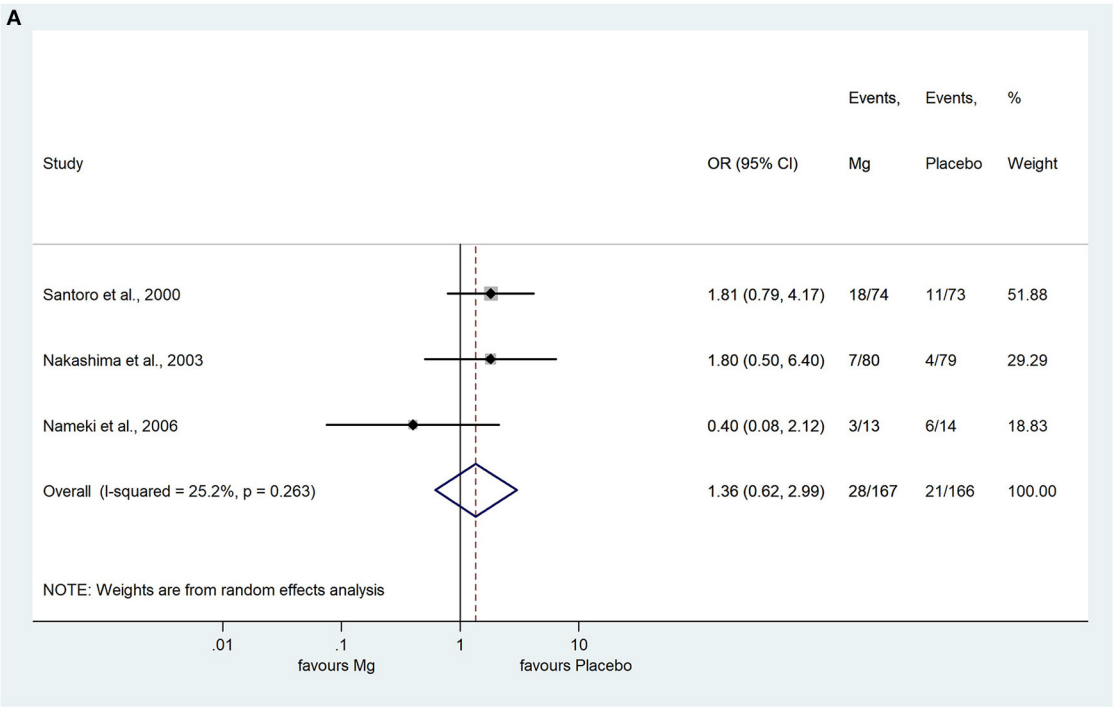


FIGURE 1 | Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) study selection flow diagram.

TABLE 1 | Characteristics of the included studies.

Design	Country	Patients		Statistically analyzed outcomes	Statistically not analyzed outcomes			Follow-up	Comments
					Heart failure	Cardiogenic shock	Deaths		
RCT	Italy	Mg group	75	VT, EF, IZWMSI	6	0	0	30 days	Patients with cardiogenic shock were excluded
		Placebo group	75		5	0	1		
RCT	Japan	Mg group	80	VT, EF, IZWMSI	18	10	1	21–28 days	After reperfusion, nicorandil infusion was started in both groups
		Placebo group	79		28	12	3		
RCT	Japan	Mg group	13	VT, EF, IZWMSI	N/A	N/A	N/A	28 days	After reperfusion, patients were maintained on 10,000–15,000 U/L heparin
		Placebo group	14		N/A	N/A	N/A		



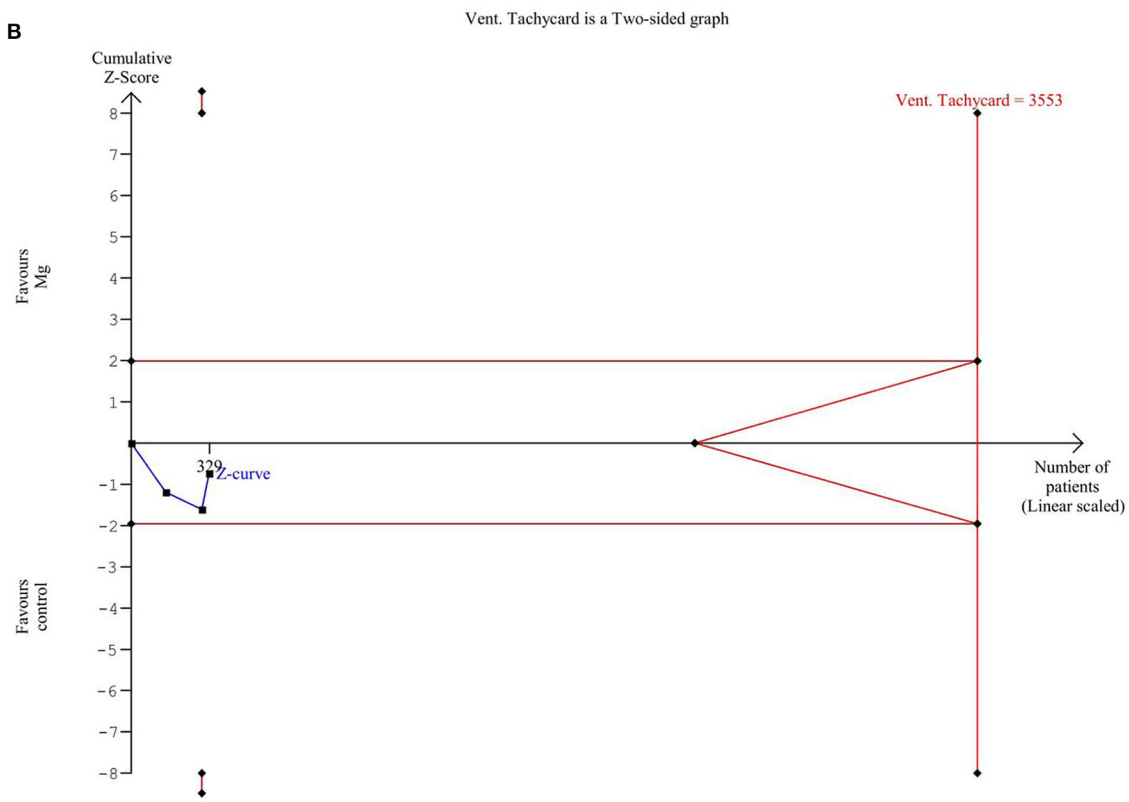


FIGURE 2 | (A) Forest plot with the odds ratio method, of the 3 studies concerning the number of reperfusion associated ventricular tachycardias (VT) showing a non-significant increase in the magnesium group. **(B)** Trial sequential analysis shows that the number of VTs is non-significantly lower in the placebo group. More importantly, the Z curve does not intersect either the conventional boundary or the futility area, therefore, the result is not significant and may be strongly influenced by additional data in the future.

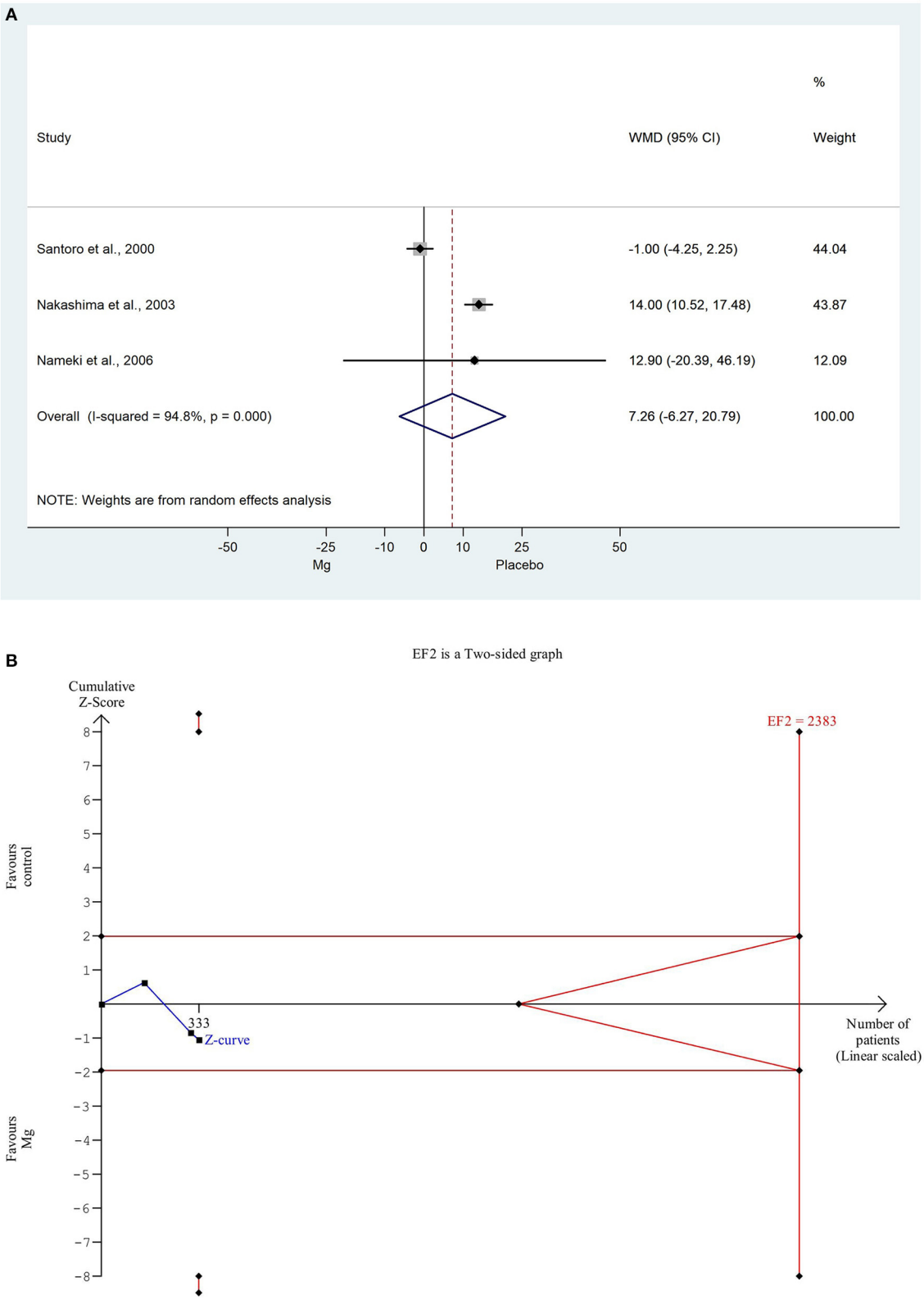
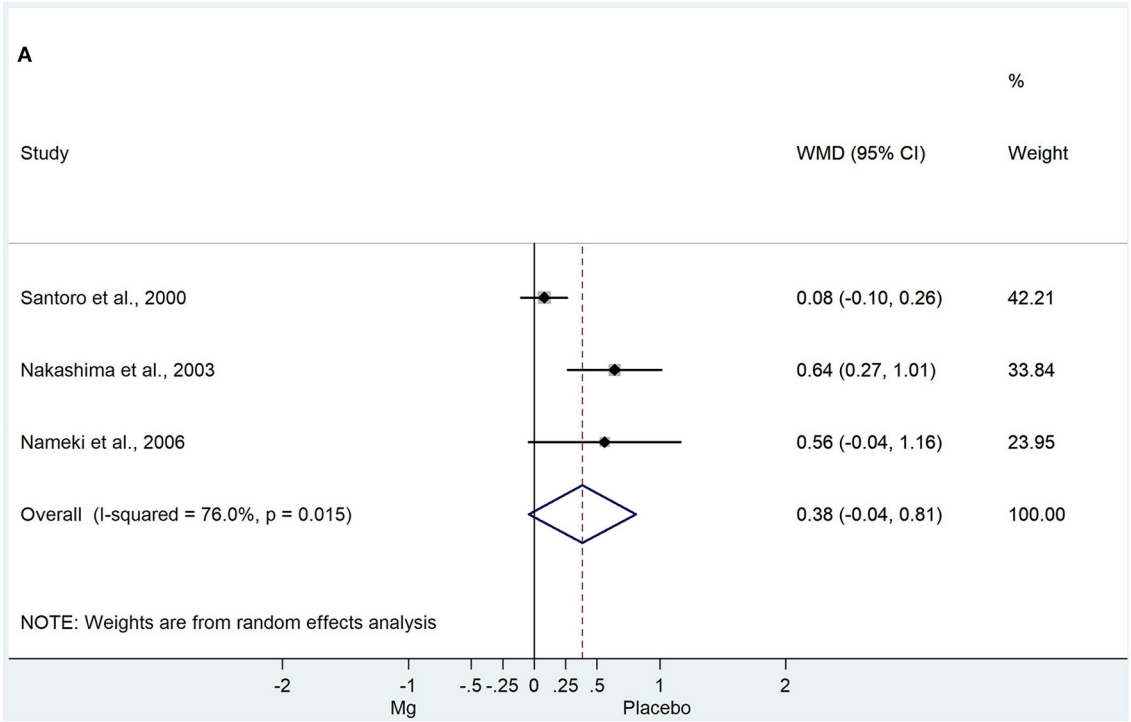


FIGURE 3 | (A) Forest plot with weighted mean difference method, of the 3 studies concerning the ejection fraction (EF) during hospitalization, showing a non-significant decrease in the magnesium group. **(B)** Trial sequential analysis shows that EF is non-significantly decreased in the magnesium group. More importantly, the Z curve does not intersect either the conventional boundary or the futility area, therefore, the result is not significant and may be strongly influenced by additional data in the future.



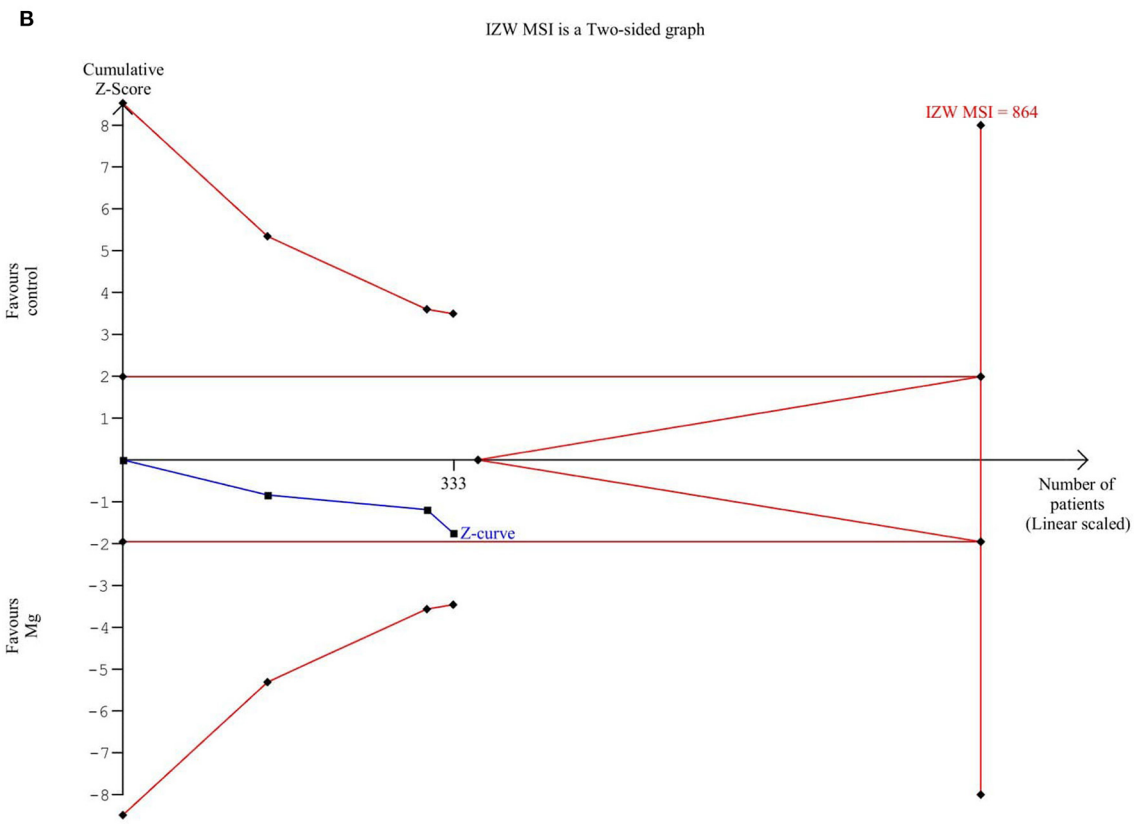


FIGURE 4 | (A) Forest plot with weighted mean difference method, of the 3 studies concerning the infact zone wall motion severity index (IZWMSI) during the hospitalization, showing a significant decrease in the magnesium group. **(B)** Trial sequential analysis shows that IZWMSI, in contrast to the weighted mean difference method, is non-significantly decreased in the magnesium group. More importantly, the Z curve does not intersect either the conventional boundary or the futility area, therefore, the result is not significant and may be strongly influenced by additional data in the future.

TABLE 2 | Summary of findings.

Outcomes	Results	Comments	Number of patients	Quality of evidence (GRADE)
VT	OR: 1.3	Non-significantly increased in the placebo group	336	Very low
	95% CI: 0.619; −2.986 $P = 0.263$			
EF	WMD: 7.262	Non-significantly improved in the placebo group	336	Very low
	95% CI: −0.238; 0.053; $P = 0.057$			
IZWMSI	WMD: 0.384	Non-significantly improved in the placebo group	336	Very low
	95% CI: −0.042; 0.811, $P = 0.015$			

VT, ventricular tachycardia; EF, ejection fraction; IZWMSI, infarct zone wall motion severity index; OR, odds ratio; WMD, weighted mean difference.

TABLE 3 | Revised Cochrane risk-of-bias tool for randomized trials (RoB 2).

	Item 1	Item 2	Item 3	Item 4	Item 5	Overall
Santoro et al. (29)	Yes	?	Yes	Yes	?	Low risk of bias
Nakashima et al. (31)	Yes	?	Yes	?	?	Intermediate risk of bias
Nameki et al. (30)	No	No	Yes	No	?	High risk of bias

Item 1, Randomization process; Item 2, Deviations from intended interventions; Item 3, Missing outcome data; Item 4, Measurement of the outcome; Item 5, Selection of the reported result.

Several meta-analyses have been conducted to investigate the association between non-PCI revascularization techniques induced arrhythmia and magnesium. In 1992, Horner et al. published a meta-analysis of eight studies, which contained data from 930 patients (33). All patients with acute myocardial infarction were revascularized with thrombolysis therapy. Their study showed that the periprocedural administration of magnesium significantly decreased ventricular and supraventricular reperfusion arrhythmias by 49 and 54%, respectively. The most recent meta-analysis in 2018 analyzed data from 22 articles and 6,061 patients (32). A major limitation of this study is that the data on patients treated with different revascularization techniques were pooled, thus potentially distorting the possible effects of magnesium. In any case, this meta-analysis also showed significant improvement in the magnesium group as regards the number of reperfusion arrhythmias. An interesting subgroup analysis was also performed in this analysis. In the magnesium group, patients who received magnesium $\geq$10 g, were separated, but higher efficacy was not demonstrated with higher doses of magnesium.

ISIS-4 (4) and LIMIT-2 (5), two monumental studies in the 1990-s, during the thrombolysis era, examined the effects of magnesium in patients with AMI, and produced conflicting results. ISIS-4 with 58,080 patients found no significant difference in 5-week mortality in the magnesium vs. placebo group. A major limitation of the study was that only stable patients were selected for the study, in contrast to the LIMIT-2 design, where there was no such exclusion criterion, which may be a major influencing factor as the control group had lower mortality in the former trial. Another possibility of bias in ISIS-4 is the use of inaccurate time windows. There was too much variance within the time of initiation of thrombolytic therapy, and, if magnesium was administered, this was 1–2 h after lysis therapy. This may also be the reason for this difference, as in the LIMIT-2 study involving 2,316 patients, a 24% relative reduction ($P = 0.04$, CI $= -1$; −43%) in in-hospital mortality was observed in the magnesium group (5).

Cardiac surgery, thrombolysis and PCI all have different effects on homeostasis. Previous meta-analyses (32, 33) ignored this distortion and worked with pooled data to investigate the potential preventive effects of magnesium on reperfusion arrhythmias. Therefore, the role of magnesium in PCI-related reperfusion arrhythmias remains questionable. Through the extensive literature research, we found five promising articles, three of which were suitable for statistical analysis (29–31). The other two (29, 30) were not used due to the pooled data problem, previously noted. One such study was the MAGIC trial (28), where 6,213 AMI patients were randomized for magnesium and

placebo. In the former group, 19 g magnesium was administered in 24 h. Patients over 65 years of age were revascularized, of whom 63% were treated with thrombolysis. Notably, magnesium treatment did not reduce either the number of VT's or mortality. On the other hand, in 1999, Shibata et al. reported 36 patients, revascularized with PCI or thrombolysis, in whom significantly less reperfusion arrhythmia was observed in the magnesium group (27).

The results from a register analysis in the United States were reported in 2001 by Ziegelstein et al. (34). One thousand three hundred twenty-six patients with AMI who underwent revascularization by various techniques received periprocedural magnesium. Patients with CABG or PCI had increased mortality in the magnesium group. No difference was found in the thrombolysis group compared to the placebo group.

From the three studies included in our analysis, Santoro et al. randomized 150 patients with STEMI and treated with PPCI for their research and examined the effects of magnesium (29). The infarct zone wall motion severity index was designated as the primary endpoint. A major limitation of thise study is that patients with a severe condition or cardiogenic shock were not selected. They found magnesium ineffective in terms of myocardial damage and short-term clinical outcome.

Nakashima et al. published a randomized study of 180 patients in 2001 (31). All STEMI patients were treated with PPCI. Ninety-one patients were administered a placebo, 89 were treated with magnesium, and additionally, both groups received nicorandil at a dose of 4 mg/h for 3 days after the intervention. It was demonstrated that magnesium sulfate administered before coronary intervention had a beneficial effect on left ventricular and microvascular function in STEMI patients.

A third study with a small number of patients was also selected. STEMI patients undergoing PPCI were divided into three groups (30). There were 13 patients in the magnesium group, 14 in the placebo group and 13 in the nicorandil group. The results of the first two of these groups were examined. All patients received 10–15 IU heparin and only LAD-affected STEMI patients were randomized. In 2006, Nameki et al. found that periprocedural magnesium was not effective compared to the placebo.

At the endpoints of our meta-analysis, magnesium was ineffective compared to placebo-controlled groups in STEMI patients undergoing primary PCI. At the same time, the TSA statistical method, which is a strength of our research, has shown for each endpoint that these results are not definitive and may be strongly influenced by newer data in the future.

Limitations

There are several limitations in our meta-analysis. In addition to the very few selected publications, a number of factors in all of the selected articles greatly weakens the value of the statistical analysis: the low number of patients, differences in hemodynamic stability among patient populations, heterogeneous data, varying doses of magnesium, and different definitions of VT.

Conclusion

Taking all of this into account, our statistical analysis and extensive literature review suggest that periprocedural intravenously administered magnesium seems to be ineffective in STEMI patients undergoing a percutaneous coronary intervention, however this result can only be expressed with a very low grade of evidence. Based on the TSA, our research highlights the need for further, well-designed studies on the effects of magnesium, on PCI-associated reperfusion-induced arrhythmias.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LS, IH, and IS designed the research and the study concept and wrote the article. LS and KS performed the data extraction. NF analyzed and interpreted the data. LS, DB, and MG performed the quality assessment. BM, BK, PH, and ZS supervised the study. IH, IS, and PH conducted a critical revision of the manuscript for important intellectual content. All authors granted final approval of the version of the article to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2020.608193/full#supplementary-material

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ORIGINAL RESEARCH
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Long-Term Effect of Salt Substitute on All-Cause and Cardiovascular Disease Mortality: An Exploratory Follow-Up of a Randomized Controlled Trial

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Background: Salt substitute, a strategy for salt reduction, has been shown to decrease blood pressure and the incidence of hypertension. However, whether its hypotensive effect will reduce long-term mortality remains unclear. Our study reported an exploratory follow-up of mortality outcomes from previous randomized controlled trial to assess the long-term effect of low-sodium salt on total and cardiovascular disease (CVD) mortality.

Methods: Participants who completed a previous 3-year double-blind randomized controlled trial were followed up from 2009 to 2019 to collect mortality data. Multivariable Cox regression models were used to evaluate the association between low-sodium salt intervention and all-cause and CVD mortality.

Results: Four hundred and forty participants completed the intervention trial, of which 428 participants had death outcome data recorded after 10 years follow-up: 209 in a salt substitute group and 219 in a normal salt group. Fifty participants died during follow-up, 25 died due to CVD. No significant differences in relative risks were found for all-cause mortality [HR = 0.81, 95% confidence interval (CI): 0.46–1.42] and CVD mortality (HR = 0.58, 95% CI: 0.26–1.32) in unadjusted analyses. After adjusted with age and alcohol drinking status, there were significant reductions for stroke mortality among all participants (HR = 0.26, 95% CI: 0.08–0.84) and for CVD mortality (HR = 0.38, 95% CI: 0.16–0.92) and stroke mortality (HR = 0.25, 95% CI: 0.08–0.82) among hypertensive participants.

Conclusions: Compared to normal salt, salt substitute might reduce the risk of CVD death, especially stroke among hypertensive patients. Our exploratory follow-up results provide potential evidence that low-sodium salt may be an accessible and effective strategy for prevention of CVD events, but definitive randomized controlled trials are warranted.

Keywords: salt substitute, all-cause mortality, hypertension, stroke mortality, CVD mortality

INTRODUCTION

According to the Global Burden of Disease (GBD) report, cardiovascular disease (CVD) is the leading cause of death globally, accounting for 17.8 million deaths in 2017 (1). Similarly, in China, the incidence and consequent mortality of CVD has been increasing (2). It is widely recognized that excessive sodium consumption increases blood volume and the resistance of peripheral vessels, resulting in raised blood pressure and CVD (3, 4). Salt is the main dietary source of sodium. Salt reduction is seen as the most cost-effective public health strategy for preventing hypertension and CVD in developed and developing countries (5, 6). In China, due in part to traditional dietary habits, salt consumption has been shown to be the highest in the world, with adults consuming on average over 10 grams of salt daily (12.9 g/d in 1992, 12 g/d in 2002, and 10.5 g/d in 2015) (7): over twice the WHO (World Health Organization) recommended limit (5 g/d) (8). It is therefore imperative to explore suitable strategies for salt reduction in China, without undue changes to dietary habits and culture.

Salt substitutes, as an existing salt reduction strategy available in industrialized China, are formulations where a proportion of the sodium is replaced with potassium or other element. Compared with normal salt with 100% sodium chloride, salt substitutes seek to decrease sodium intake without reducing the perceived total salt consumption, and thus avoid the inherently poor compliance typical of long-term behavioral intervention in salt restriction (9, 10). In our study, the salt substitute used comprised 65% sodium chloride, 25% potassium chloride, and 10% magnesium sulfate. Since 1986, 20 articles have reported the effects of salt alternatives, with results focusing primarily on blood pressure and the incidence of hypertension (11, 12). Data on the population effect of sodium consumption on CVD or death is limited.

Our previous randomized, double-blind, controlled study provided evidence that an appropriate salt substitute could lower blood pressure during a 3 year intervention (13, 14). Whether this hypotensive effects might prove durable and decrease long-term CVD mortality remains unclear. Here, we report a 10-year post-intervention follow-up study to explore the long term effects of salt substitute and its effect on total and CVD mortality.

METHODS

Study Design and Participants

Our study was an exploratory follow-up study with participants who completed the previous double-blind, randomized controlled trial. The previous trial explored the hypotensive effect of salt substitute. A detailed description of the previous trial has been reported previously (13, 14). Briefly, 200 families (462 participants) were randomized to an intervention (salt substitute) or control (normal salt) group in 2006. During intervention, participants were followed-up every 3 months to measure their systolic blood pressure (SBP) and diastolic blood pressure (DBP). Four hundred and forty participants completed the intervention in 2009. After completing the trial, participants were told the final results and both groups were recommended to reduce salt consumption to <6 g daily.

Subsequently, we have undertaken a 10-year observational study among the participants who completed the trial to examine the long-term effects of salt substitute on all-cause and CVD mortality. All surviving participants or relatives who provided information regarding deceased participants gave written informed consent. The institutional review board at the China Medical University approved the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in an *a priori* approval by the institution's human research committee.

Follow-Up and Assessment of Outcome

During the post-intervention follow-up, village health workers reviewed participants by telephone semi-annually. Multiple sources were analyzed to determine the time and cause of death, including medical records, death certificates and symptoms, as reported by a spouse, sibling, or child, were also cross-checked with the death registration system, where possible. Two physicians blinded to treatment, assessed the underlying cause of death, as obtained from death certificates, and assigned a code according to the International Classification of Disease, Tenth Revision (ICD-10). Causes of death were divided into two broad categories: CVD death (heart disease: I05-I09, I11, I20-I27, I30-I52; stroke: I60-I69) and non-CVD death (all other causes). The primary study endpoints were all-cause mortality and CVD mortality. The follow-up period started at the end of the intervention (April, 2009) lasting till death, loss to follow-up, or 30 April 2019, whichever occurred first.

Statistical Analysis

Data were presented as mean ± standard deviation (SD) for continuous variables and as number (n) and percentage (%) for categorical variables. T-test and Chi-square test were used to compare differences in baseline characteristics. Cause-specific mortality for all-cause, CVD, and non-CVD deaths were calculated as incidence density and cumulative incidence. The efficacy of salt substitution on mortality was evaluated using absolute risk reduction (ARR), relative risk reduction (RRR), and number-needed-to-treat (NNT). Multivariate Cox proportional hazards models were used to obtain hazard ratios (HRs) and 95% confidence intervals (95% CIs) for mortality, adjusted for baseline age and alcohol consumption. All analyses were undertaken using SPSS statistical software (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA). A 2-sided $P < 0.05$ was considered statistically significant.

RESULTS

Study Population and Participants Characteristics

Of the 462 participants in the 2006 study, 224 were assigned to the salt substitute group and 238 to the normal salt group, with 440 participants completing the 3-year intervention. The principal reasons for participants lost to follow-up were those who moved beyond observation and others reluctant to follow the prescribed

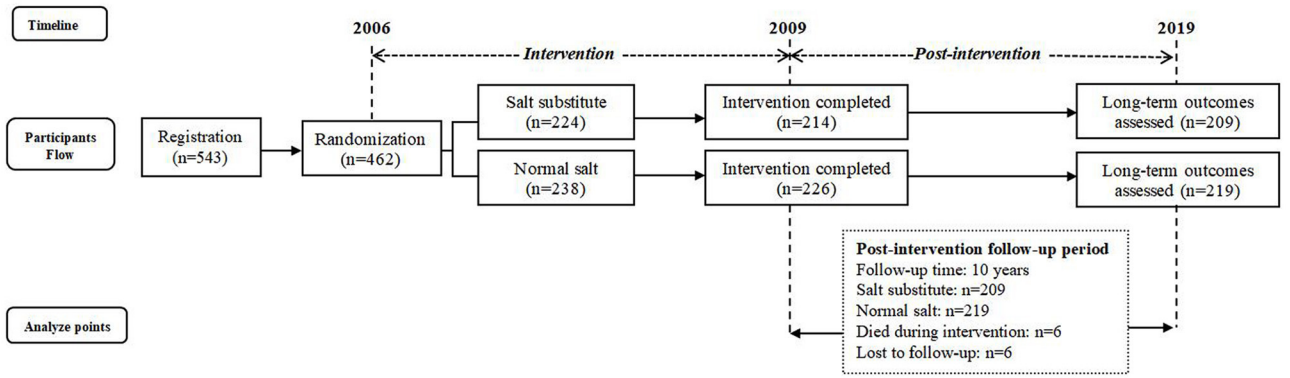

FIGURE 1 | Flow diagram of study design and participant follow-up.

schedule. As 6 participants died during intervention and 6 participants could not be traced, valid follow-up information relating to death outcomes was obtained from 428 participants only, 209 in the salt substitute group and 219 in normal salt group (**Figure 1**). Characteristics of participants at baseline (2006) and the end of the intervention (2009) are shown in **Table 1**. At baseline, no differences were seen in terms of age, gender, BMI, smoking, history of hypertension, history of CVD, medication use, SBP, DBP, or pulse between salt substitute and control groups, except for alcohol consumption ($P = 0.007$). At the end of intervention period, differences were shown in medications use ($P = 0.001$) and SBP level ($P = 0.049$), and the change in SBP and DBP during the intervention period (2006–2009) differ significantly ($P < 0.001$) by groups.

Cause-Specific Mortality in Salt Substitute Group and Normal Salt Group

During the follow-up period, 50 deaths (22 in the salt substitute group, 28 in the salt group) were recorded, as shown in **Table 2**. Approximately 50% of deaths were due to CVD (heart disease and stroke) and 28% from cancer. A smaller proportion of participants died of all-cause mortality and CVD in the salt substitute group than in the control group, despite the difference not being statistically significant (all-cause: $\chi^2 = 0.53$, $P = 0.467$, CVD: $\chi^2 = 1.75$, $P = 0.186$). A marginally significant difference in stroke mortality was seen in participants taking salt substitute vs. normal salt ($\chi^2 = 3.78$, $P = 0.052$). A substantial risk reduction for stroke mortality was observed in the salt substitute group, as estimated by ARR and RRR. NNT for salt substitute intervention was 29. The non-CVD incidence of death per 1,000 person-years was 6.55 in the experimental group and 5.85 in the control group.

Long-Term Effect of Salt Substitute Intervention Among All and Hypertensive Population

Participants in the salt substitute group had improved survival for all-cause mortality and CVD-related mortality than controls. This protective effect was more obvious in hypertensive patients (**Table 3** and **Figure 2**). In the multivariable regression adjusted for baseline age and alcohol consumption, salt substitute had an associated 57% (HR = 0.43, 95% CI = 0.18–1.02, $P = 0.056$; **Figure 2A**) and 62% (HR = 0.38, 95% CI = 0.16–0.92, $P = 0.032$;

TABLE 1 | Characteristics of study participants by group at baseline (2006) and end of 3-year intervention (2009).

	Salt substitute group	Normal salt group	*P*-value
Baseline (2006)			
Age (years)	45.4 ± 13.4	46.8 ± 13.2	0.283
Gender (male/female)	102/107	108/111	0.916
BMI (kg/m^2)	26.0 ± 3.9	26.7 ± 4.28	0.062
Cigarette smoking [*n* (%)]	92 (44.0%)	84 (38.4%)	0.234
Alcohol drinking [*n* (%)]	90 (43.1%)	67 (30.6%)	0.007
Hypertension [*n* (%)]	160 (76.6%)	162 (74.0%)	0.536
CVD [*n* (%)]	37 (17.7%)	26 (11.9%)	0.089
Medications [*n* (%)]	87 (46.8%)	89 (46.1%)	0.897
SBP	154.6 ± 28.2	149.7 ± 23.5	0.053
DBP	92.0 ± 14.5	89.6 ± 13.8	0.079
Pulse (bpm)	79.3 ± 11.0	81.2 ± 12.2	0.083
After intervention (2009)			
BMI (kg/m^2)	27.1 ± 3.7	26.52 ± 4.2	0.253
Cigarette smoking [*n* (%)]	64 (36.0%)	55 (29.6%)	0.194
Alcohol drinking [*n* (%)]	51 (29.1%)	47 (25.7%)	0.463
Hypertension [*n* (%)]	148 (82.7%)	159 (85.9%)	0.392
CVD [*n* (%)]	39 (21.8%)	33 (17.8%)	0.344
Medications [*n* (%)]	48 (26.8%)	81 (41.5%)	0.001
SBP	143.5 ± 21.4	148.4 ± 25.3	0.049
DBP	89.0 ± 12.8	91.2 ± 14.0	0.115
Pulse (bpm)	77.1 ± 10.7	77.0 ± 12.0	0.933
Change for blood pressure from 2006 to 2009 (mmHg)			
SBP	−14.3 ± 21.1	−5.4 ± 22.7	<0.001
DBP	−4.7 ± 12.0	−0.7 ± 13.4	<0.001

CVD, Cardiovascular disease; BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure.

TABLE 2 | Cause-specific mortality in groups and efficacy of salt substitute on mortality.

Cause of death	Salt substitute group			Normal salt group			ARR[a] (%)	RRR[b] (%)	NNT[c]
	Death	Cumulative incidence (%; 95% CI)	Incidence density [deaths per 1,000 person-years (95% CI)]	Death	Cumulative incidence (%; 95% CI)	Incidence density [deaths per 1,000 person-years (95%CI)]			
All-cause	22	10.53 (6.33 to 14.72)	11.08 (6.47 to 15.69)	28	12.79 (8.33 to 17.24)	13.66 (8.63 to 18.69)	−2.26	17.67	45
CVD	9	4.31 (1.53 to 7.08)	4.53 (1.58 to 7.49)	16	7.31 (3.83 to 10.78)	7.81 (3.99 to 11.62)	−3.00	41.04	34
Heart disease	5	2.39 (0.30 to 4.48)	2.52 (0.31 to 4.72)	4	1.83 (0.01 to 3.61)	1.95 (0.01 to 3.86)	0.56	30.60	179
Stroke	4	1.91 (0.04 to 3.79)	2.01 (0.04 to 3.99)	12	5.48 (2.44 to 8.52)	5.85 (2.55 to 9.16)	−3.57	65.15	29
non-CVD	13	6.22 (2.92 to 9.52)	6.55 (3.00 to 10.10)	12	5.48 (2.44 to 8.52)	5.85 (2.55 to 9.16)	0.74	13.50	136
Cancer	7	3.35 (0.89 to 5.81)	3.53 (0.92 to 6.13)	7	3.20 (0.85 to 5.54)	3.42 (0.89 to 5.94)	0.15	4.69	667
Diabetes mellitus	2	0.96 (−0.01 to 2.29)	1.01 (−0.01 to 2.40)	2	0.91 (−0.04 to 2.18)	0.98 (−0.38 to 2.33)	0.05	5.50	2,000
Accident	2	0.96 (−0.01 to 2.29)	1.01 (−0.01 to 2.40)	2	0.91 (−0.04 to 2.18)	0.98 (−0.38 to 2.33)	0.05	5.50	2,000
Others	2	0.96 (−0.01 to 2.29)	1.01 (−0.01 to 2.40)	1	0.46 (−0.44 to 1.36)	0.49 (−0.47 to 1.44)	0.50	108.70	200

CVD, Cardiovascular disease; ARR, Absolute risk reduction; RRR, Relative risk reduction; NNT, Number needed to treat.

[a] *Calculate as cumulative incidence of death in salt substitute group-cumulative incidence of death in normal salt group.*

[b] *Calculate as |ARR|÷cumulative incidence of death in normal salt group.*

[c] *Calculate as 1÷|ARR|.*

Figure 2C) lower risk of CVD death when compared to normal salt among all and hypertensive participants. Mortality risks from heart disease and stroke were lowered by 3 and 74% among all participants, 23 and 75% among hypertensive participants, while a statistically significant reduction was only found for stroke death (**Figures 2B,D**).

Measurements of Urinary Na^+, K^+, and Na^+/K^+ Ratios After Intervention

To evaluate treatment safety, spot urinary samples were collected and the concentration of Na^+ and K^+ at the end of intervention was measured. Urine data were available for 311 participants (161 salt substitute group, 150 normal salt group), as shown in **Figure 3**. The urinary K^+ excretion and Na^+/K^+ ratios were significantly different between groups after 3-year intervention, but no difference was identified for urinary excretion of Na^+.

DISCUSSION

The results of our previous RCT indicated salt substitute offered significant benefits for lowering SBP and DBP, with potential effects on hypertension (13, 14). However, the benefit of such intervention on cardiovascular disease and mortality was undetermined, necessitating a long-term follow-up study to evaluate the persistent effect of salt substitute. The results of our exploratory analysis following previous trial observed participants who received salt substitute had reduced total and CVD mortality, with larger effects observed in participants with baseline hypertension. Compared with the normal salt group, a statistically significant reduction (74%) in overall stroke mortality was observed, with reduced CVD (62%) and stroke mortality (75%) seen in hypertensive participants. This suggests that replacing normal salt with low-sodium salt lowers CVD and stroke mortality, particularly in individuals with hypertension.

Substantial evidence supports salt restriction as an effective non-pharmacological intervention at the population level for blood pressure management and improved long-term cardiovascular outcomes (11, 15–17). However, experience with behavior changing interventions shows even a moderate salt restriction for more than 6 months was hard to implement (9). Here, we used a commercially available salt substitute (18), which proved a pragmatic intervention strategy to reduce sodium intake (19). Hitherto, most salt substitution trials have evaluated blood pressure or hypertension as the primary outcome, with data concerning incidence, occurrence, and mortality of blood pressure-related cardiovascular events going unrecorded. Our study has reported the long-term effect of salt substitute on total and CVD mortality, indicating our study represents a significant advance.

Despite the long history of salt reduction research, associations between salt reduction and health outcomes remain at issue. In most studies, sodium intake was estimated form urinary sodium excretion. Follow-up of the famous TOHP (trials of hypertension prevention) report sodium reduction may reduce long-term CVD events (10–15 years after intervention) and all-cause mortality (23–26 years after intervention), suggesting a direct linear relationship between habitual sodium intake and total deaths (20–22). Meta-analysis and some observational studies reported increased CVD events at very low sodium intake, indicating a "J" or "U" shaped link between sodium intake and health outcomes (23–27). Results suggested an increasing CVD and mortality risk occurred at sodium intakes <3 g/day and >6 g/day (23), challenging the WHO's recommendation (<2 g/day of sodium, equivalent to 5 g salt/day), and querying the population-wide salt reduction policy to reduce blood pressure and CVD (8). However, methodological issues were readily apparent, mostly focusing on the use of suboptimal measurements (spot

TABLE 3 | Effect of salt substitute intervention on all-cause mortality and cardiovascular disease mortality among all and hypertensive participants.

Cause of death	HR_{rude}	*P*	$HR_{adjust1}$	*P*	$HR_{adjust2}$	*P*
All participants						
All-cause	0.81 (0.46–1.42)	0.463	0.80 (0.46–1.40)	0.434	0.75 (0.42–1.32)	0.311
CVD	0.58 (0.26–1.32)	0.194	0.48 (0.21–1.13)	0.094	0.43 (0.18–1.02)	0.056
Heart disease	1.28 (0.35–4.79)	0.707	1.05 (0.27–4.12)	0.945	0.97 (0.24–3.88)	0.961
Stroke	0.35 (0.11–1.07)	0.066	0.29 (0.09–0.94)	0.039	0.26 (0.08–0.84)	0.024
Hypertensive participants						
All-cause	0.73 (0.41–1.30)	0.284	0.71 (0.40–1.28)	0.252	0.68 (0.38–1.22)	0.195
CVD	0.49 (0.21–1.15)	0.103	0.41 (0.17–0.99)	0.049	0.38 (0.16–0.92)	0.032
Heart disease	0.98 (0.25–3.92)	0.976	0.79 (0.19–3.37)	0.750	0.77 (0.18–3.34)	0.726
Stroke	0.33 (0.11–1.02)	0.055	0.28 (0.09–0.91)	0.034	0.25 (0.08–0.82)	0.022

CVD, Cardiovascular disease; adjust1, Adjusted with baseline age; adjust2, Adjusted with baseline age and alcohol drinking status.

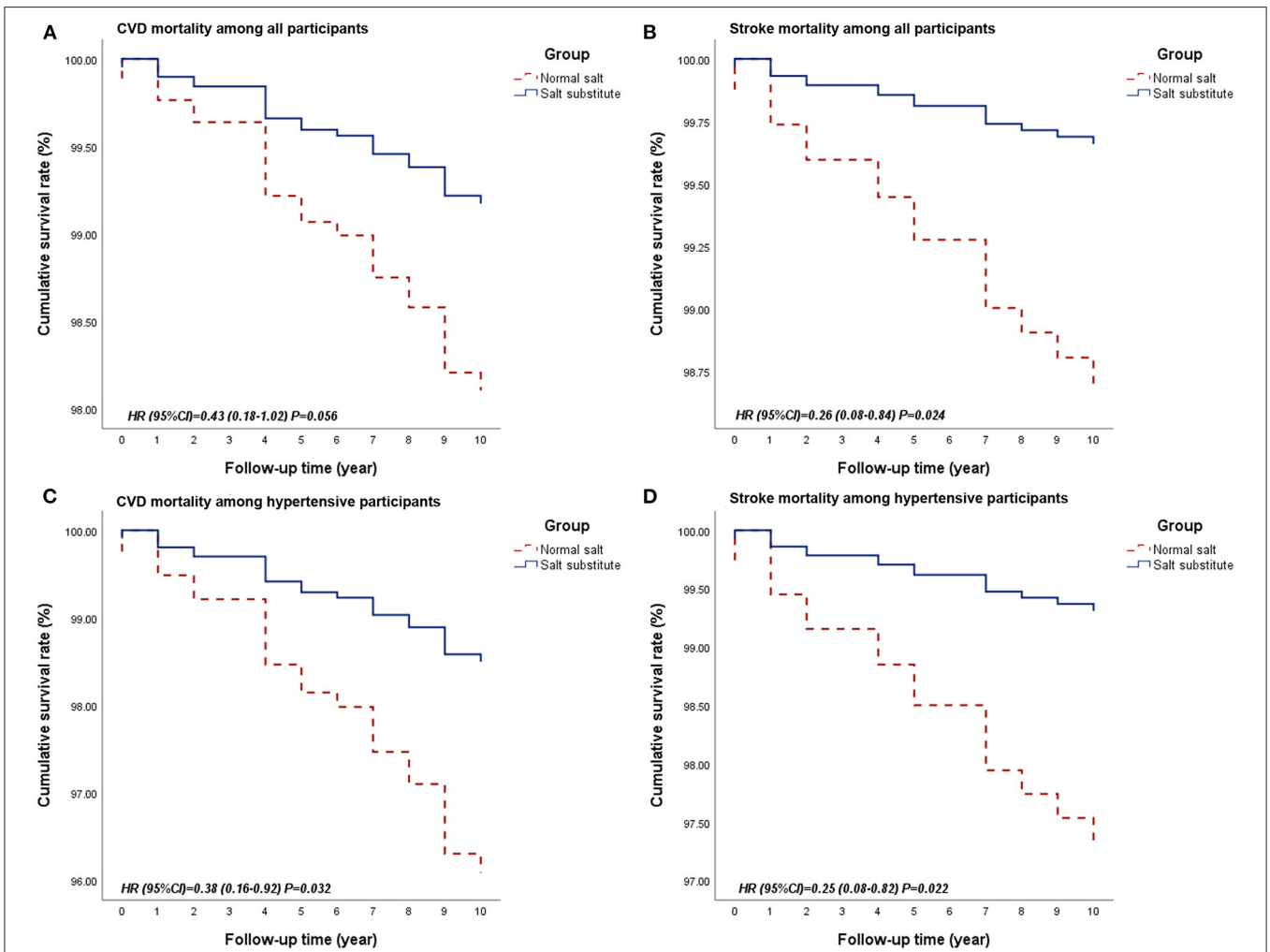

FIGURE 2 | Survival curves of CVD and stroke mortality between groups among all participants or hypertensive participants. (**A.** Survival curve for CVD mortality among all participants; **B.** Survival curve for stroke mortality among all participants; **C.** Survival curve for CVD mortality among hypertensive participants; **D.** Survival curve for stroke mortality among hypertensive participants.) CVD, Cardiovascular disease.

urine) to assess sodium consumption, as well as potential reverse causality in the studies, and many confounding factors (28, 29).

Stroke is strongly associated with blood pressure-lowering intervention (30), with salt reduction potentially reducing blood pressure, yet there is limited data from properly

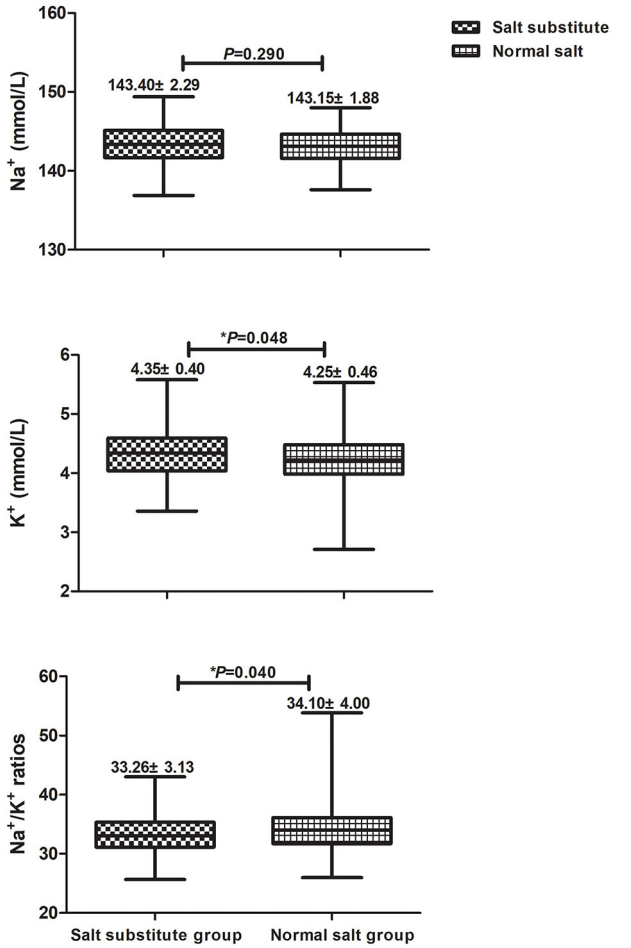

FIGURE 3 | Comparisons for urinary Na^+, K^+, and Na^+/K^+ ratios between salt substitute group and normal salt group.

conducted randomized trials evaluating the beneficial effects of salt substitution on stroke. Pan et al.'s research reported improved outcomes for stroke recovery after 6 months' intervention with salt substitute (31). However, the results were questioned due to weaknesses in trial methodology (32). Here, we found a considerable association between low-sodium salt and reduced stroke risk in our long-term cohort study. However, given blood pressure control level has a great impact on the stroke incidence and mortality, and we did not monitor the blood pressure level during follow-up period. Therefore, the statistical association should be interpreted cautiously and still need a finding deserving the further research. The ongoing Salt Substitute and Stroke Study (SSaSS) enrolled 20,996 patients at elevated risk of stroke across 600 villages in rural China. This is designed to assess effects of salt substitution on fatal and non-fatal stroke and other CVD events. The trial will complete shortly and should provide further information about the efficacy and safety of salt substitution, giving anticipated support to global sodium reduction strategies (33).

There is also evidence that a high-salt diet might be particularly harmful in hypertensive patients (34). The DASH (Dietary Approaches to Stop Hypertension) study indicated that salt reduction was beneficial in both hypertensive and non-hypertensive individuals, with the greatest blood pressure decrease found in hypertensive patients (35). An updated Cochrane review also indicated that cardiovascular mortality were reduced by decreasing salt intake among hypertensives, though not in the general populations (36), which again was consistent with our results. Together, these results support strongly the greater effects of salt reduction in hypertensive patients.

To date, there are few reports of long-term trials evaluating the effect of sodium on clinical outcomes, primarily due to logistic and feasibility considerations. The longest intervention period of sodium reduction trials was 36 months (21), and the longest salt substitute intervention period was 44 months (37). Such trials were too short to observe mortality or cardiovascular disease events in the general population. In 2016, Cook et al. reported the over 20-year post-trial follow-up results to evaluate the relationship between sodium reduction intervention and total mortality, and a non-significant 15% lower risk was observed among participants in intervention group compared with controls (21). Furthermore, non-significant risk reduction also reported on CVD mortality and events (36), which was inconsistent with our findings. There are two possible explanations. One possible reason for this inconsistency might be the participants involved in TOHP trial were pre-hypertensive adults, but over 70% participants were hypertensive patients with increased likelihood of CVD events in present study. Another possible reason is the different intervention. Compared with salt restriction intervention in TOHP trial, salt substitute intervention could not only reduce sodium intake, but also increase potassium intake simultaneously. Meta-analysis and RCTs both found potassium-enriched salt substitutes, compared with normal salt, reduced blood pressure level and risk of death from cardiovascular disease (4, 11, 37). Thus, the protective effect of CVD mortality was more remarkable in our study.

The observed association between sodium reduction and total or CVD mortality can be explained by the "programming" effect. Geleijnse's demonstrated that infants given low sodium formula during their first 6 months had lower SBP than controls after 15 years follow-up, despite no corresponding difference in their urinary sodium excretion (38). This is also seen in adults. Among participants in the Baltimore TOHP I study, a trend for lower blood pressure and reduced incidence of hypertension was observed in the reduced sodium group compared to controls, despite no difference in urinary sodium excretion (35). One explanation of this might be that even though sodium reduction was short in duration, blood pressure regulation was reset and subsequent structural or functional damage to cardiac and vascular systems was delayed due to this initial sodium reduction. An alternative reason might be that participants in the intervention group could well adopt salt reduction and live a healthier lifestyle once they knew the results of the RCT indicating that salt substitution reduced blood pressure. Since our study did not record changes in dietary or behavior after

intervention, further investigations are required to confirm or refute this hypothesis.

From the viewpoint of medical and financial demands on both government and patient, salt reduction is a cost-effective and promising strategy for reducing the burden of CVD. In China, driven by traditional cooking and eating habits, the long-term compliance with salt restriction is poor. Simply reducing the amount of salt consumed is not practical. The salt substitute we used comprised 65% NaCl, reducing the amount of sodium consumed by 35%, when compared to regular salt [100% NaCl]. Likewise, we found no statistically significant reduction in urinary Na^+ in our study. Possible explanations include: (1) our previous study was undertaken in the general population and lasted 3 years, so it was not possible to restrict food intake fully. Thus, concentrations of urinary Na^+ might be affected by other food contained large quantities of sodium, including *inter alia* monosodium glutamate, flour strings, and fermented bean curd. (2) The family baseline salt intake survey showed average salt consumption per participant to be 12.64 g/d (salt substitute group) and 9.36 g/d (normal salt group), indicating higher total salt consumption in the intervention group, possibly explaining why urinary Na^+ was slightly higher in this group. Moreover, our findings identified significant differences in K^+ excretion and Na^+/K^+ ratios, in line with expectations. Thus, substituting normal salt with potassium-enriched and low-sodium salt may prove effective in promoting healthier lifestyle among people without severely-impaired kidney function. Considering the results of urinary Na^+ and K^+, we make the assumption that the protective effect may primarily be due to the increase in potassium intake, because the sodium reduction achieved was moderate. Additional studies are encouraged to clarify whether the effect comes from lower sodium intake or from higher potassium intake.

Because the exploratory nature of present analysis, our previous trial was not originally designed to evaluate the impact of salt substitute intervention on survival outcome, the design of this follow-up study had flaws and statistical power was limited. First, our study did not evaluate sodium intake during the follow-up period, preventing identification of participants who continued to use salt substitutes or tried to restrict salt intake following our post-intervention recommendations. This means any observed benefits of low-sodium salt might result from other lifestyle change during follow-up. People who try one type of lifestyle intervention (sodium reduction) may be more motivated to try other health interventions, such as exercise, eating more fruit and vegetables, reducing smoking or alcohol consumption, etc. Unfortunately, such data could not be factored into our analysis. Secondly, because the information was collected by telephone interview, the blood pressure data, which have enormous implications for the progression of cardiovascular disease, were failed to collect in our follow-up study. Third, since over 70% of participants were hypertensive patients and the small number of deaths among those without hypertension, we could not evaluate properly if low-sodium salt reduced total and CVD mortality in the non-hypertensive population. Additionally, considering the low mortality level, we could only adjust baseline age and alcohol drinking status, which may affect the accuracy of our results.

CONCLUSIONS

Our results provide evidence that replacing normal salt with a low-sodium salt substitute could be a tractable and effective strategy to prevent CVD events. Salt substitute, which typically lowers blood pressure and prevents hypertension, may thus reduce CVD mortality, especially in hypertensive participants. Well-designed multi-center RCTs accessing large, properly-stratified patient populations will be required to elucidate properly and completely the effects of salt substitution on cardiovascular outcomes in the population.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board at the China Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BZ contributed to the conception and design of the work. HS, BM, and XW contributed to the acquisition, analysis, or interpretation of data for the work. HS drafted the manuscript. HW revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

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A U-Shaped Relationship Between Selenium Concentrations and All-Cause or Cardiovascular Mortality in Patients With Hypertension

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Background: Given the antioxidant activity of selenium, it has been reported benefits for blood pressure control and hypertension prevention, but few studies have investigated the association between serum selenium with mortality in hypertensive population.

Methods: All participants with hypertension aged ≥18 years at baseline were recruited from the National Health and Nutritional Examination Surveys (NHANES) 2003–2004, and followed for mortality through December 31, 2015. Subjects were categorized by quartiles of serum selenium (Q1: ≤124 μg/L, Q2: 125–135 μg/L, Q3: 136–147 μg/L, Q4: ≥148 μg/L). Multivariate Cox regression were implemented to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). Restricted cubic spline analysis and two-piecewise linear regression were used to evaluate the relationship of serum selenium with mortality. Survival curves were used to depict cause-specific mortalities.

Results: A total of 929 participants (52.53% were male) were eligible for the current study with the average age of 63.10 ± 12.59 years. There were 307 deaths occurred including 56 cardiovascular death events during the mean follow-up time of 121.05 ± 40.85 months. A U-shaped association was observed between serum selenium and all-cause or cardiovascular mortality. In fully adjusted model, comparisons among quartiles revealed that risks of all-cause [HR (95%CI), 0.57 (0.39–0.81)] and cardiovascular death [HR (95%CI), 0.33 (0.13–0.86)] were lower in Q3. The nadir mortality of all-cause and cardiovascular was occurred at the serum selenium level of 136 μg/L and 130 μg/L, respectively.

Conclusion: Serum selenium concentration showed a U-shaped association with all-cause and cardiovascular mortality.

Keywords: selenium, hypertension, all-cause mortality, cardiovascular mortality, risk factors

INTRODUCTION

Selenium is one of the essential trace element for human beings that play a role via selenoproteins (1). It has for long been suggested as a protective factor for cancer, critical illness, diabetes, and cardiovascular disease through functions of antioxidant, anti-inflammation and reducing platelet aggregation (2, 3). Given oxidative stress is showed to be a primary contributor to hypertension development (4), sufficient selenium may benefit blood pressure control and hypertension prevention (5, 6). Deficient or excessive concentration of serum selenium has been reported closely correlated to elevated prevalence of hypertension (6–8), especially for subjects in the selenium-replete region (9). Because of the different contents of selenium in the environment, the concentration of selenium varies greatly worldwide. Compared with Europe, the United States demonstrated a higher level of selenium in soil and food intake (10). As a consequence, the average serum selenium concentration in most American adults was above 95 ng/mL (11), while Europeans ranged from 50 to 90 ng/mL (12).

Published literatures presented controversial conclusions of the efficacy of selenium concentration on mortality. Several studies reported a negative correlation between selenium status and risk of all-cause or cardiovascular death (13–15). However, the Third National Health and Nutrition Examination Survey (NHANES III; 1988–1994) reported that no association was found between serum selenium and cardiovascular mortality (16). Another prospective study conducted in China showed that no association was observed between serum selenium and overall mortality (17). To our knowledge, there was no investigation about the relationship of selenium status with mortality in hypertensive population. Thus, this study sought to evaluate the association between serum selenium concentration with all-cause and cardiovascular mortality in patients with hypertension.

MATERIALS AND METHODS

Study Population

Data for the current study were abstracted from the NHANES database, which aimed at evaluating the health and nutritional status in the US population. It was conducted by the National Center for Health Statistics (NCHS) within the United States Centers for Disease Control and Prevention (CDC). The study survey was administrated through face-to-face interviews and physical examination in the mobile examination center (MEC), and the resulting data release by a 2-year cycle. Further information about NHANES was available on the website (https://www.cdc.gov/nchs/nhanes/index.htm).

In total, 7,564 participants were enrolled from NHANES 2003–2004. Subjects younger than 18 years of age (n = 1,944), without hypertension (n = 3,538), had missing data on selenium (n = 471), blood pressure (n = 669), height and weight (n = 10), or lost to follow-up (n = 3) were excluded. Thus, 929 participants were included in the final list (**Figure 1**). The survey was approved by the Institutional Review Board of the CDC (ethical approval code: Protocol #98-12). Signed informed consent was obtained from each subject.

Assessment of Serum Selenium

Measurements of serum selenium was obtained at baseline. Blood drawn must be performing in the fasting state. After centrifugation, serum specimens should be stored at 4°C for transport and frozen at −20°C or at −70°C until time for analysis. Inductively Coupled Plasma-Dynamic Reaction Cell-Mass Spectrometry (ICP-DRC-MS) method was used to detect serum selenium concentration at the Trace Elements Laboratory, State of New York Department of Health, Wadsworth Center. More detail about the laboratory analyses procedures were described elsewhere (18).

Covariate Data Collection

Information on sex, age, race, alcohol consumption, education level, smoking behavior, marital status, history of cardiovascular disease and cancer, medication history (including antihypertensive drugs, hypoglycemic agents, antiplatelet drugs and lipid-lowering drugs) were obtained through face-to-face questionnaire. Hypertension was defined as the examined systolic blood pressures (SBP) ≥ 130 mmHg or/and diastolic blood pressure (DBP) ≥ 80 mmHg (19), confirmed to be taking antihypertensive medications, or self-reported history of hypertension. Diabetes was defined as fasting blood glucose (FBG) ≥ 126 mg/dL, self-report, hemoglobin A1c(HbA1C) ≥6.5% (20), or using hypoglycaemic drugs. SBP, DBP, height and weight were measured in accordance with laboratory procedures in MEC. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared (kg/m^2) (21). Total cholesterol (TC, mg/dL), high-density lipoprotein cholesterol (HDL-C, mg/dL), and C-reactive protein (CRP, mg/dL) were assessed under standardized procedure and protocol. The estimated glomerular filtration rate (eGFR, mL/min/1.73 m^2) was calculated using the Chronic Kidney Disease Epidemiology equation (22).

Outcomes

The primary outcome of the current study was all-cause and cardiovascular mortality. Mortality data were obtained from the NHANES Public-use Linked Mortality Files through December 31, 2015, provided through the CDC. All-cause mortality was defined as death resulting from any cause. Cardiovascular mortality was considered when I00-I09, I11, I13, I20-I51, or I60-I69 were recorded as the underlying cause of death, based on the International Classification of Diseases (ICD-10) codes.

Statistical Analysis

Participants were categorized by serum selenium quartiles (Q1: ≤124 μg/L, Q2: 125–135 μg/L, Q3: 136–147 μg/L, Q4: ≥148 μg/L). Descriptive data are expressed as mean ± standard deviation (SD) for continuous variables or as proportions for categorical variables. The one-way ANOVA and chi-square were used to assess differences among selenium quartiles or mortality and survival group. Cox proportional hazard regression models were performed to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for all-cause and cardiovascular mortality. Crude analysis adjust for none; adjusted model adjust for: age, sex, and race; fully adjusted model adjust for: age,

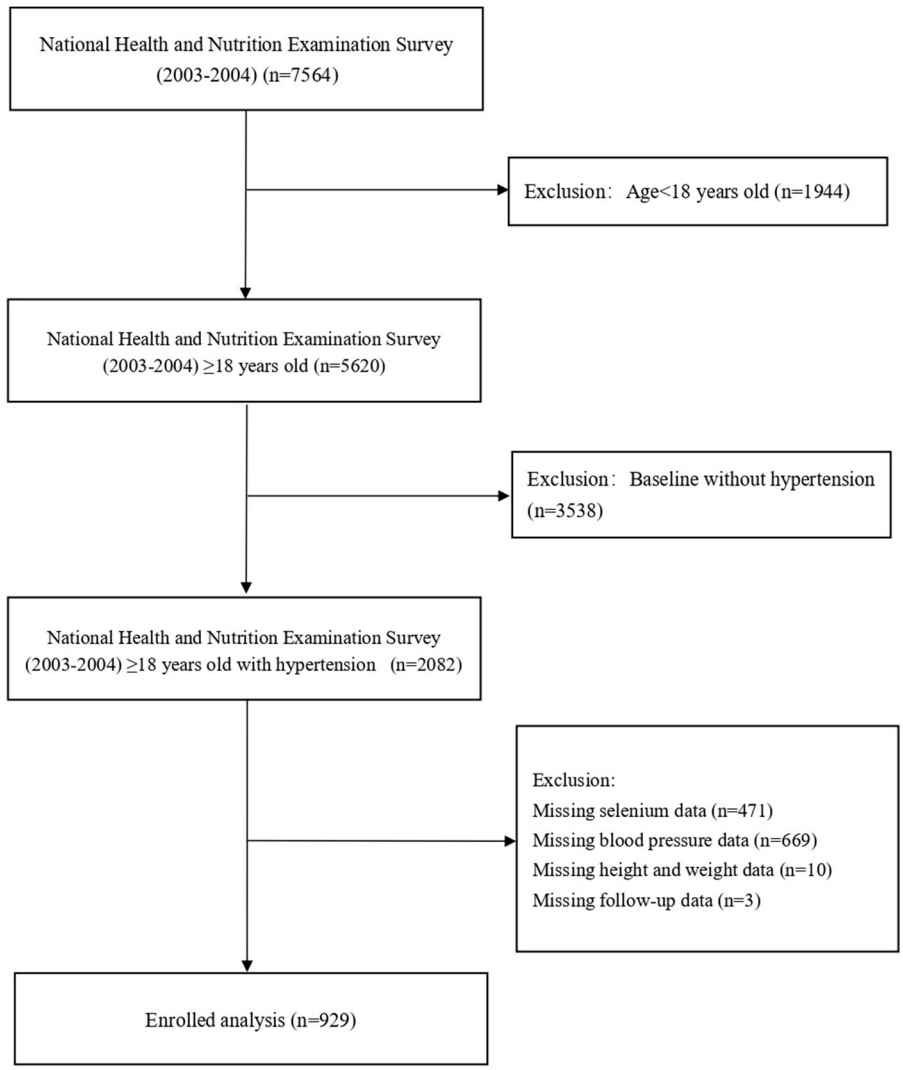

FIGURE 1 | Study cohort.

sex, race, education level, marital status, smoking, BMI, SBP, TC, HDL-C, CRP, alcohol consumption, eGFR, comorbidities (cardiovascular disease, diabetes, and cancer), and medication use (lipid-lowering drugs, hypoglycemic agents, antiplatelet drugs, and antihypertensive drugs). Restricted cubic spline analysis was performed to examine the non-linearity of serum selenium and mortality. Three knots (10th, 50th, 90th percentiles of serum selenium distribution, corresponding to 115,135, and 159 μg/L, respectively) were used for restricted cubic spline modeling, with the lowest level of risk as the reference value. If a non-linear correlation was detected, a recursive algorithm was conducted to calculate the inflection point and a two-piecewise Cox proportional hazards model on both sides of the inflection point was then performed. Threshold level was defined by choosing the inflection point with maximum model likelihood, along with a log-likelihood ratio test to examine the statistical difference with one-line Cox proportional hazards model (23). Survival probability of serum selenium quartiles was presented by Kaplan–Meier curves and the log-rank test. All analyses were performed with R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Baseline Demographic and Clinical Parameters

The current study enrolled 929 participants with mean age of 63.10 ± 12.59 years, 488 (52.53%) of them were males. The mean level of selenium was 136.78 ± 20.32 μg/L. Participants with higher selenium levels were more prone to be male, diabetics, having hypoglycemic agents, elevated TC, and reduced CRP. During the mean follow-up time of 121.05 ± 40.85 months, 307 (33.05%) deaths were recorded including 56 (6.03%) cardiovascular deaths (**Table 1**). Participants in survival group

TABLE 1 | Baseline demographic and clinical parameters among participants by selenium quartiles.

	Total	Q1	Q2	Q3	Q4	*P*-value
Number	929	246	231	223	229	
Age, years	63.10 ± 12.59	62.33 ± 12.56	63.96 ± 13.09	62.37 ± 12.08	63.79 ± 12.58	0.331
Body mass index, kg/m^2	29.79 ± 5.79	30.19 ± 6.67	30.17 ± 5.78	29.35 ± 5.00	29.43 ± 5.46	0.221
Systolic blood pressure, mmHg	127.56 ± 17.66	127.22 ± 18.78	126.08 ± 16.99	128.42 ± 18.60	128.60 ± 16.06	0.386
Diastolic blood pressure, mmHg	70.60 ± 16.37	69.91 ± 17.87	68.82 ± 16.95	72.30 ± 15.45	71.49 ± 14.78	0.099
Total cholesterol, mg/dL	203.18 ± 43.45	198.33 ± 49.27	197.28 ± 41.55	207.07 ± 38.65	210.58 ± 41.78	0.001
HDL cholesterol, mg/dL	52.69 ± 15.50	54.20 ± 16.20	51.45 ± 15.55	52.50 ± 14.91	52.50 ± 15.20	0.275
C-reactive protein, mg/L	0.77 ± 1.71	1.11 ± 2.55	0.70 ± 1.19	0.67 ± 1.33	0.58 ± 1.24	0.003
Alcohol consumption, gm	8.65 ± 27.02	8.51 ± 28.82	8.43 ± 29.72	9.76 ± 25.98	7.94 ± 23.00	0.910
Selenium, μg/L	136.78 ± 20.32	115.49 ± 7.40	129.96 ± 3.09	140.72 ± 3.46	162.68 ± 19.39	<0.001
eGFR, mg/min/1.73 m^2	72.34 ± 20.40	73.20 ± 20.46	72.73 ± 21.53	71.94 ± 19.26	71.39 ± 20.30	0.779
Sex, *n* (%)						0.015
Male	488 (52.53)	108 (43.90)	125 (54.11)	124 (55.61)	131 (57.21)	
Female	441 (47.47)	138 (56.10)	106 (45.89)	99 (44.39)	98 (42.79)	
Education level, *n* (%)						0.582
Less than high school	306 (32.97)	84 (34.29)	80 (34.63)	75 (33.63)	67 (29.26)	
High school or above	622 (67.03)	161 (65.71)	151 (65.37)	148 (66.37)	162 (70.74)	
Marital status, *n* (%)						0.175
Married	380 (40.95)	115 (46.75)	91 (39.39)	83 (37.39)	91 (39.74)	
Other	548 (59.05)	131 (53.25)	140 (60.61)	139 (62.61)	138 (60.26)	
Smoking, *n* (%)						0.616
No	394 (42.41)	97 (39.43)	96 (41.56)	98 (43.95)	103 (44.98)	
Yes	535 (57.59)	149 (60.57)	135 (58.44)	125 (56.05)	126 (55.02)	
Race, *n* (%)						0.052
White	541 (58.23)	145 (58.94)	120 (51.95)	128 (57.40)	148 (64.63)	
Other races	388 (41.77)	101 (41.06)	111 (48.05)	95 (42.60)	81 (35.37)	
Diabetes, *n* (%)						0.007
No	673 (72.44)	196 (79.67)	166 (71.86)	161 (72.20)	150 (65.50)	
Yes	256 (27.56)	50 (20.33)	65 (28.14)	62 (27.80)	79 (34.50)	
Cardiovascular disease, *n* (%)						0.109
No	811 (88.25)	208 (85.60)	193 (85.78)	202 (90.99)	208 (90.83)	
Yes	108 (11.75)	35 (14.40)	32 (14.22)	20 (9.01)	21 (9.17)	
Cancer, *n* (%)						0.711
No	791 (85.51)	210 (86.42)	197 (85.28)	185 (83.33)	199 (86.90)	
Yes	134 (14.49)	33 (13.58)	34 (14.72)	37 (16.67)	30 (13.10)	
Antihypertensive drugs, *n* (%)						0.143
No	309 (33.26)	92 (37.40)	64 (27.71)	73 (32.74)	80 (34.93)	
Yes	620 (66.74)	154 (62.60)	167 (72.29)	150 (67.26)	149 (65.07)	
Lipid-lowering drugs, *n* (%)						0.821
No	679 (73.09)	183 (74.39)	167 (72.29)	166 (74.44)	163 (71.18)	
Yes	250 (26.91)	63 (25.61)	64 (27.71)	57 (25.56)	66 (28.82)	
Hypoglycemic agents, *n* (%)						0.005
No	777 (83.64)	219 (89.02)	194 (83.98)	188 (84.30)	176 (76.86)	
Yes	152 (16.36)	27 (10.98)	37 (16.02)	35 (15.70)	53 (23.14)	
Antiplatelet drugs, *n* (%)						0.645
No	893 (96.12)	238 (96.75)	219 (94.81)	216 (96.86)	220 (96.07)	
Yes	36 (3.88)	8 (3.25)	12 (5.19)	7 (3.14)	9 (3.93)	
Cardiovascular disease mortality, *n* (%)						0.034
No	873 (93.97)	227 (92.28)	221 (95.67)	216 (96.86)	209 (91.27)	
Yes	56 (6.03)	19 (7.72)	10 (4.33)	7 (3.14)	20 (8.73)	

(Continued)

TABLE 1 | Continued

	Total	Q1	Q2	Q3	Q4	*P*-value
All-cause mortality, *n* (%)						0.019
No	622 (66.95)	152 (61.79)	149 (64.50)	167 (74.89)	154 (67.25)	
Yes	307 (33.05)	94 (38.21)	82 (35.50)	56 (25.11)	75 (32.75)	

N, number; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate.
Values are mean ± standard deviation or n (%).
P-value by one-way analysis of variance (ANOVA) test for continuous data and chi-square test for categorical variables among selenium quartiles.

TABLE 2 | Baseline demographic and clinical parameters among participants between mortality and survival group.

	Survival	Total death		*P*-value
		Cardiovascular death	Non-cardiovascular death	
Number	622	56	251	
Age, years	58.91 ± 11.13	72.29 ± 11.55	71.45 ± 10.88	<0.001
Body mass index, kg/m^2	30.18 ± 5.90	28.71 ± 5.95	29.08 ± 5.37	0.014
Systolic blood pressure, mmHg	127.99 ± 17.22	126.95 ± 21.66	126.63 ± 17.77	0.568
Diastolic blood pressure, mmHg	73.39 ± 15.68	64.14 ± 17.26	65.12 ± 16.12	<0.001
Total cholesterol, mg/dL	206.60 ± 41.45	205.43 ± 54.12	194.22 ± 44.54	<0.001
HDL cholesterol, mg/dL	53.16 ± 14.97	54.57 ± 15.38	51.09 ± 16.70	0.13
C-reactive protein, mg/L	0.67 ± 1.41	1.26 ± 3.59	0.93 ± 1.72	0.011
Alcohol consumption, gm	8.32 ± 22.01	6.51 ± 21.36	9.94 ± 37.37	0.61
Selenium, μg/L	137.08 ± 17.50	138.95 ± 28.73	135.54 ± 24.25	0.426
eGFR, mg/min/1.73 m^2	76.79 ± 18.21	60.38 ± 18.82	63.95 ± 22.15	<0.001
Sex, *n* (%)				<0.001
Male	300 (48.23)	41 (73.21)	147 (58.57)	
Female	322 (51.77)	15 (26.79)	104 (41.43)	
Education level, *n* (%)				<0.001
Less than high school	180 (28.94)	26 (46.43)	100 (40.00)	
High school or above	442 (71.06)	30 (53.57)	150 (60.00)	
Marital status, *n* (%)				<0.001
Married	209 (33.66)	34 (60.71)	137 (54.58)	
Other	412 (66.34)	22 (39.29)	114 (45.42)	
Smoking, *n* (%)				0.002
No	286 (45.98)	14 (25.00)	94 (37.45)	
Yes	336 (54.02)	42 (75.00)	157 (62.55)	
Race, *n* (%)				<0.001
White	334 (53.70)	41 (73.21)	166 (66.14)	
Other races	288 (46.30)	15 (26.79)	85 (33.86)	
Diabetes, *n* (%)				<0.001
No	478 (76.85)	33 (58.93)	162 (64.54)	
Yes	144 (23.15)	23 (41.07)	89 (35.46)	
Cardiovascular disease, *n* (%)				<0.001
No	566 (91.44)	39 (72.22)	206 (83.74)	
Yes	53 (8.56)	15 (27.78)	40 (16.26)	
Cancer, *n* (%)				<0.001
No	550 (88.71)	46 (82.14)	195 (78.31)	
Yes	70 (11.29)	10 (17.86)	54 (21.69)	
Antihypertensive drugs, *n* (%)			<0.001	
No	235 (37.78)	15 (26.79)	59 (23.51)	
Yes	387 (62.22)	41 (73.21)	192 (76.49)	

(Continued)

TABLE 2 | Continued

	Survival	Total death		*P*-value
		Cardiovascular death	Non-cardiovascular death	
Lipid-lowering drugs, *n* (%)				0.07
No	469 (75.40)	37 (66.07)	173 (68.92)	
Yes	153 (24.60)	19 (33.93)	78 (31.08)	
Hypoglycemic agents, *n* (%)				0.004
No	538 (86.50)	44 (78.57)	195 (77.69)	
Yes	84 (13.50)	12 (21.43)	56 (22.31)	
Antiplatelet drugs, *n* (%)				<0.001
No	604 (97.11)	48 (85.71)	241 (96.02)	
Yes	18 (2.89)	8 (14.29)	10 (3.98)	

N, number; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate.
Values are mean ± standard deviation or n (%).
P-value: One-way analysis of variance (ANOVA) test for continuous data and chi-square test for categorical variables.

TABLE 3 | The association of selenium levels with all-cause mortality and cardiovascular mortality.

	Crude analysis HR (95%CI) *P*-value	Adjusted model HR (95%CI) *P*-value	Fully adjusted model HR (95%CI) *P*-value
All-cause mortality			
Selenium per 10 μg/L increment	0.98 (0.92, 1.04) 0.4502	0.95 (0.90, 1.01) 0.0842	0.96 (0.90, 1.03) 0.2486
Selenium quartiles			
Q1	1.0	1.0	1.0
Q2	0.88 (0.65, 1.18) 0.3826	0.75 (0.56, 1.01) 0.0555	0.74 (0.54, 1.03) 0.0715
Q3	0.60 (0.43, 0.83) 0.0024	0.56 (0.40, 0.78) 0.0006	0.57 (0.39, 0.81) 0.0020
Q4	0.83 (0.61, 1.12) 0.2238	0.72 (0.53, 0.97) 0.0333	0.74 (0.53, 1.04) 0.0802
p for trend	0.0574	0.0110	0.0419
Cardiovascular disease mortality			
Selenium per 10 μg/L increment	1.05 (0.93, 1.18) 0.4572	1.01 (0.90, 1.14) 0.8215	1.03 (0.90, 1.17) 0.6882
Selenium quartiles			
Q1	1.0	1.0	1.0
Q2	0.53 (0.25, 1.14) 0.1046	0.42 (0.20, 0.91) 0.0279	0.38 (0.16, 0.90) 0.0276
Q3	0.38 (0.16, 0.90) 0.0281	0.35 (0.15, 0.82) 0.0166	0.33 (0.13, 0.86) 0.0226
Q4	1.10 (0.58, 2.05) 0.7761	0.94 (0.50, 1.76) 0.8500	0.94 (0.46, 1.90) 0.8582
p for trend	0.9070	0.8987	0.9652

HR, hazard ratio; CI, confidence interval. Ref, reference. Crude analysis adjust for none; Adjusted model adjust for: age, sex, and race; Fully adjusted model adjust for: age, sex, race, education level, marital status, smoking, body mass index, systolic blood pressure, total cholesterol, high density lipoprotein cholesterol, C-reactive protein, alcohol consumption, estimated glomerular filtration rate, comorbidities (cardiovascular disease, diabetes, and cancer), and medication use (lipid-lowering drugs, hypoglycemic agents, antiplatelet drugs, and antihypertensive drugs).
P-value by Cox proportional hazard regression models.

were on average younger in age, higher proportion being female, lower prevalence of smoking, diabetes, cancer, and cardiovascular disease, and had higher TC, DBP, eGFR, BMI, and education level, lower CRP level than participants in other two groups (cardiovascular death group and non-cardiovascular death group) (**Table 2**).

Association of Serum Selenium With All-Cause and Cardiovascular Mortality

The results of the Cox regression analysis were listed in **Table 3**. Taking the lowest quartiles (Q1) as reference, the full-adjusted HRs for all-cause mortality among groups (Q2, Q3, and Q4) were 0.74 (95%CI, 0.54–1.03), 0.57 (95%CI, 0.39–0.81), and 0.74 (95%CI, 0.53–1.04), respectively. For cardiovascular mortality, these numbers were 0.38 (95%CI, 0.16–0.90), 0.33 (95%CI, 0.13–0.86), and 0.94 (95%CI, 0.46–1.90), respectively. Thus, risk for all-cause or cardiovascular death by categorized selenium level suggesting a U-shaped association, which was lowest in Q3, and the beneficial effects could be attenuated in Q4.

The Analyses of U-Shaped Relationship

As depicted in **Figure 2**, results of restricted cubic spline regression found a U-shaped relationship between serum

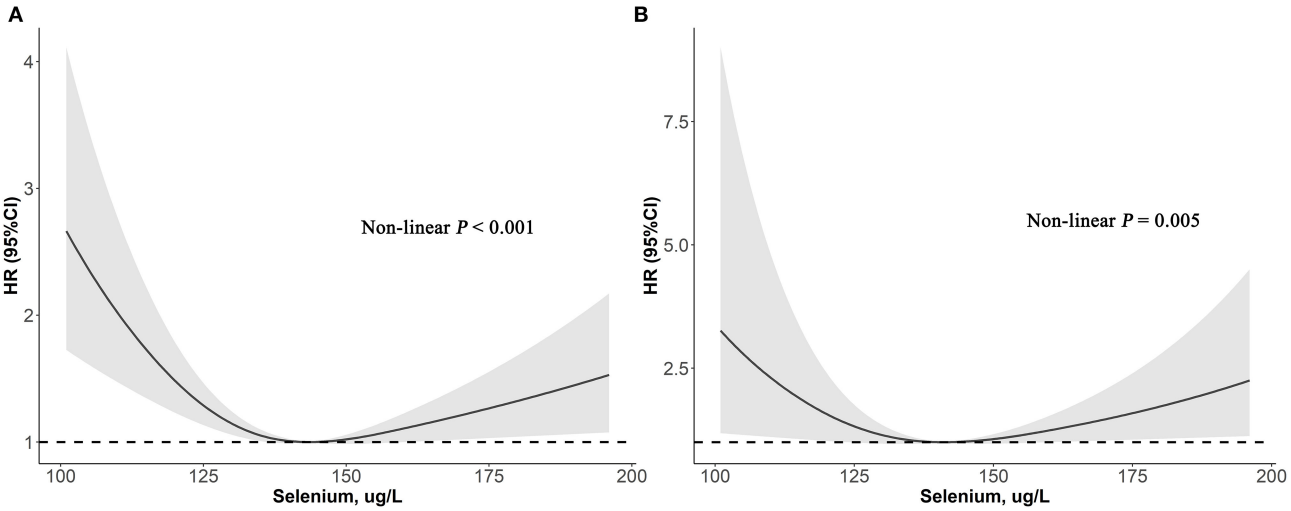

FIGURE 2 | Association of selenium levels with the all-cause **(A)** and cardiovascular mortality **(B)** performed by restricted cubic spline analysis.

TABLE 4 | The results of two-piecewise linear regression model between selenium levels and all-cause or cardiovascular mortality.

	All-cause mortality HR (95% CI) *P*-value	Cardiovascular mortality HR (95% CI) *P*-value
Cutoff value, μg/L	136	130
<Cut-off value (per 10 μg/L increment)	0.75 (0.66, 0.85) <0.0001	0.60 (0.42, 0.86) 0.0051
>Cut-off value (per 10 μg/L increment)	1.08 (1.01, 1.16) 0.0207	1.15 (1.02, 1.31) 0.0255
P for log likelihood ratio test	<0.001	0.004

HR, hazard ratio; CI, confidence interval.
Two-piecewise linear regression model were adjusted for age, sex, race, education level, marital status, smoking, body mass index, systolic blood pressure, total cholesterol, high density lipoprotein cholesterol, C-reactive protein, alcohol consumption, estimated glomerular filtration rate, comorbidities (cardiovascular disease, diabetes, and cancer), and medication use (lipid-lowering drugs, hypoglycemic agents, antiplatelet drugs, and antihypertensive drugs).
P-value by two-piecewise Cox proportional hazards model.

selenium with all-cause and cardiovascular mortality (Non-linear $p < 0.001$ and Non-linear $p = 0.005$, respectively). Furthermore, the two-piecewise linear regression revealed that the risk of all-cause mortality decreased up to a minimum at the selenium level of 136 μg/L, and then increased with higher selenium level [HR (95%CI): 0.75 (0.66–0.85), 1.08 (1.01–1.16) per 10 μg/L selenium increment in less or more than 136 μg/L, respectively]. Similarly, risk of cardiovascular death fell with rising serum selenium up to 130 μg/L, then ascended with greater level of selenium [HR (95%CI): 0.60 (0.42–0.86), 1.15 (1.02–1.31) per 10 μg/L selenium increased in less or more than 130 μg/L, respectively] (**Table 4**). Therefore, a U-shaped association between serum selenium and all-cause or cardiovascular mortality was noted, and the nadir mortality was occurred at the serum selenium level of 136 and 130 μg/L, respectively.

Survival Analysis

Kaplan-Meier survival curves of all patients stratified by selenium were demonstrated in **Figure 3**. It showed that participants in Q3 had the lowest risk of all-cause or cardiovascular mortality (Log-rank $P = 0.023$ and Log-rank $P = 0.028$, respectively).

DISCUSSION

This study found that the higher the selenium concentration, it more likely to accompanied by higher TC, and diabetes. Meanwhile, a U-shaped relationship between serum selenium with all-cause and cardiovascular mortality was observed in patients with hypertension, and the optimal cut-off value was 136 and 130 μg/L, respectively.

It has been showed that the relationship between selenium and death has been draw different conclusions in different populations. The AtheroGene study (24) followed up 879 participants with acute coronary syndrome during 6.1 years of median follow-up in Koblenz, Germany. In their research, the HR (95%CI) was 0.38 (0.16–0.91) for cardiovascular mortality comparing the highest (>84.4 μg/L) with the lowest (<64.0 μg/L) tertile of serum selenium, demonstrated a negative trend across the tertiles. Another prospective study of 347 community-dwelling older conducted in Italy found that after 10 years of follow-up time, compared with low serum selenium (≤105.3 μg/L), participants with high serum selenium (>105.3 μg/L) had a lower risk of all-cause death [HR(95%), 0.71(0.54–0.92)] (13). Interestingly, the two studies mentioned above were conducted in Europe, where the selenium intake and average serum selenium levels were lower than the United States (11, 12). Thus, their results for the lower level of selenium related to a higher risk of all-cause or cardiovascular death could compatible with our findings. However, a cohort study followed up with 1,103 adults during 15 years in China showed that no association was observed between serum selenium and total death (17), which

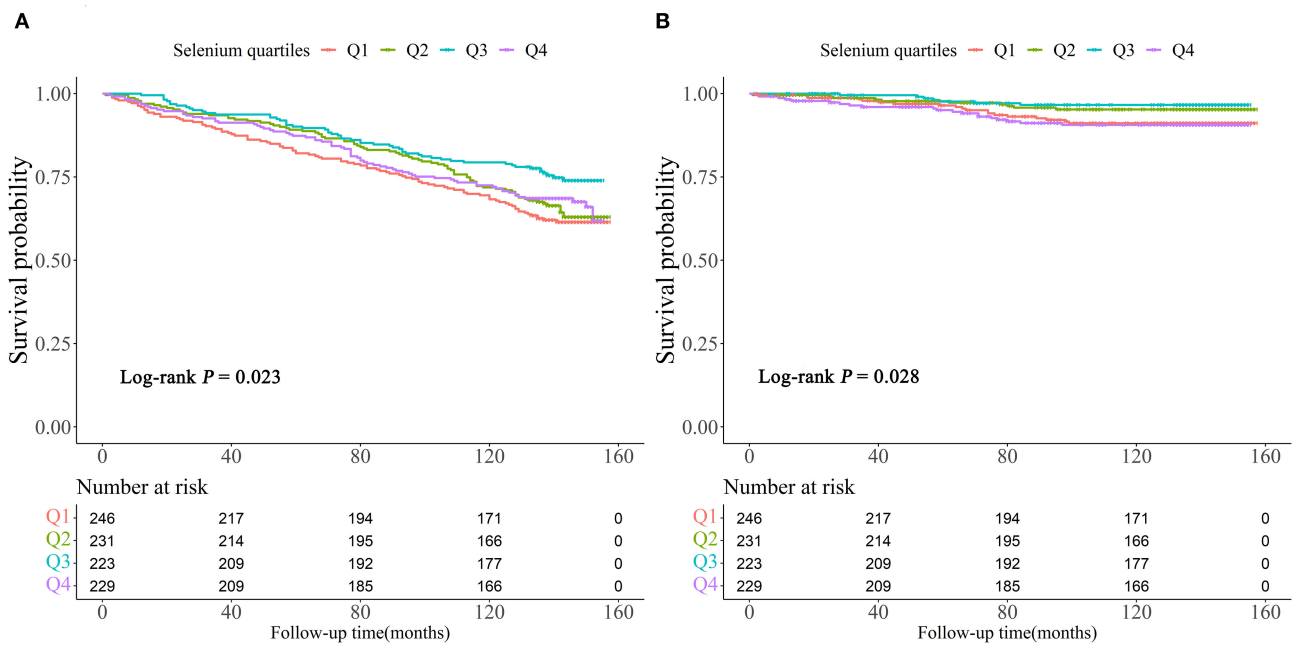

FIGURE 3 | Kaplan-Meier survival curve for all-cause **(A)** and cardiovascular mortality **(B)**.

was inconsistent with our finding. The reason for discrepancy may due to different adjustment factors. The previous study only adjusted for sex, age, smoking, drinking, and serum cholesterol, whereas our study adjusted for more potential confounding variables, such as anthropometric and demographic features, CRP, eGFR, comorbidities, and medication use.

The results of our study showed that too low and too high level of serum selenium may be linked to elevated all-cause and cardiovascular mortality were partially consistent with several previous research. The NHANES III (1988–1994) study (16) found that a U-shaped relationship was confirmed between serum selenium and all-cause mortality, instead, no association was observed between serum selenium and cardiovascular mortality. This discrepancy could be explained by the different characteristics of study population and detection methods. In the present research, the study population comprised people with hypertension were found to be older than the previous study involved a general population. Moreover, the ICP-DRC-MS method was used to assay selenium in our research, which reaching a high degree of precision demonstrated by a lower inter-assay coefficient of variation than the traditional method in the previous research. Li et al. (25) reported that the non-linear association was found between serum selenium and risks of all-cause death, however, the relationship between serum selenium with cardiovascular mortality was markedly in females only. The disparate results could be explained by the application of different versions and the range of ICD code, which may impact the recording of cardiovascular mortality. Death from cardiovascular disease was determined by ICD-10 (version presently applied, I00-I09, I11, I13, I20-I51, or I60-I69) in the current study, whereas previous studies used a broader set of ICD codes to define cardiovascular mortality, which may overestimate the prevalence of cardiovascular death.

Several potential mechanisms underlying our findings explained below. During the hypertensive state, bioavailability of antioxidants decreases and excessive reactive oxygen species (ROS) production eventually led to oxidative stress, cellular and tissue damage (26). Selenium is known to defense against oxidative stress through selenoproteins [including glutathione peroxidases (GPx) and thioredoxin reductases (TrxR)] (27). Low level of selenium could be limiting the synthesis of selenoproteins, leading to blunted effect of antioxidant and anti-inflammatory (11) and resulting in elevated risk of death (28). Nevertheless, with increasing selenium concentration, the GPx activity increases directly until reaching a plateau (29), and depletion of selenoproteins may occur when selenium oversupply resulting in health problem (30). In animal model, wistar rats with selenium supplement for 85 days showed elevation of blood pressure, indicating that longer-term selenium supplement may affect cardiovascular health (31). Furthermore, excessive exposure of selenium may also relate to harmful effects (32), including preventing proper protein folding (33), inducing the unfolded protein response (UPR) (34), causing production of superoxide and angiogenesis damage (30, 35).

The strengths of our study are the large sample size from a nationally representative population with strict adherence to protocol, and the use of a national register for identification of deaths. However, some limitations still exist. First, cardiovascular mortality is a complex phenomenon, and although we have adjusted for most relevant confounders, we cannot rule out residual or unknown confounding factors. Second, the exclusion of these patients who had missing data on selenium,

blood pressure, height and weight may lead to selection bias. Third, serum selenium was only measured at baseline, and selenium exposure may have changed over time, which might have resulted in misclassification. Forth, some factors may affect selenium metabolism including supplement intake, living environment and dietary behaviors were not collected in our study.

In conclusion, serum selenium concentrations had a U-shaped relationship with all-cause and cardiovascular mortality in patients with hypertension, and the cut-off values were showed to be 136 and 130 μg/L, respectively. This suggested that too low or too high concentration of serum selenium might be concomitant with poor outcomes. The exact reaction mechanism of selenium in human in biological systems require further study.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhanes/index.htm.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Centers for Disease Control and Prevention. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-qF, Y-qH, KL, and Q-hT: conceptualization and methodology. Y-qH, Q-hT, LL, and X-cL: formal analysis. Y-qH, J-yC, and Y-qF: supervision and validation. Q-hT and KL: writing and revision. All authors contributed to the article and approved the submitted version.

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Anemia of Chronic Disease in Patients With Cardiovascular Disease

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Objective: Anemia is often found in patients with coronary artery disease (CAD) or acute coronary syndrome (ACS) and related to disease severity. Our study investigated the relationship between anemia, iron homeostasis and inflammation in CAD and examined their influence on the outcome of patients.

Patients and Methods: Markers of immune activation (neopterin, interleukin [IL]-12, IL-6, high sensitive C-reactive protein (hsCRP), fibrinogen, serum amyloid A [SAA]) and iron metabolism (ferritin, transferrin saturation, hemoglobin) were determined in 2,082 patients (68.7 % men, median age 63 years) from the Ludwigshafen Risk and cardiovascular Health (LURIC) cohort. Patients were followed-up for a median of 9.81 years.

Results: 960 patients (46.1 %) presented with chronic CAD, 645 patients (31.0 %) had an ACS, and 477 patients (22.9 %) presented with no CAD in coronary angiography (CAG). Anemia (n = 357, 17.1 %) was associated with disease severity (reflected by more progressed stenosis in CAG, CCS, and NYHA classes, and a lower LV-EF), a higher cardio-cerebrovascular event rate and higher levels of inflammatory markers. Interestingly, anemia was only predictive for an adverse outcome in patients with elevated inflammatory markers. Accordingly, anemia of chronic disease (ACD) was associated with a higher cardio-cerebrovascular event-rate in the subsequent 2 years as compared to patients with other types of anemia or without anemia (14.3 vs. 6.1 vs. 4.0%, $p < 0.001$).

Conclusions: This study confirms that anemia and immune activation are strongly related to cardiovascular disease progression and an adverse outcome. Our data suggest that the association of anemia with disease severity and outcome might mainly be due to underlying inflammation.

Keywords: anemia, anemia of chronic disease, iron deficiency, immune activation, cardiovascular disease, outcome

INTRODUCTION

Anemia and iron deficiency (ID) are frequently encountered in patients with coronary artery disease (CAD) and acute coronary syndrome (ACS) and are both related to a poor prognosis (1, 2), a reduced functional capacity and a reduced health-related quality of life of patients (3, 4). Anemia can be the consequence of absolute ID, which is mostly due to chronic blood loss, causing iron deficiency anemia (IDA). However, in many patients enhanced formation of pro-inflammatory cytokines including interleukin 1 (IL-1), tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ) leads to the development of functional iron deficiency (ID) and anemia of chronic disease (ACD) (5). These pro-inflammatory cytokines suppress renal erythropoietin production, but also directly inhibit erythropoiesis in the bone marrow (6–8). In addition, cytokines like interleukin 6 (IL-6) or interleukin 10 (IL-10) and crucially also the iron master regulator hepcidin increase iron uptake and inhibit iron export from macrophages (5). Iron restriction within macrophages limits iron availability for erythropoiesis finally leading to anemia (9).

IFN-γ, which orchestrates T-helper cell type 1 (Th1) immune responses, has diverse effects on erythropoiesis and the progression of atherosclerosis. IFN-γ increases iron uptake and decreases iron release into macrophages thus reducing iron availability for erythropoiesis (10). Moreover, IFN-γ inhibits the proliferation and life span of erythroid progenitor cells (6, 11), the production of erythropoietin (12) and its receptors on erythroid progenitor cells (13). While changes in iron homeostasis occur immediately (10, 14), inhibition of erythropoiesis and consequently anemia occur only after chronic exposure to IFN-γ (11). IFN-γ further enhances immune effector pathways and biochemical pathways in human monocytes/macrophages thus enforcing the progression of atherosclerosis: IFN-γ enforces the formation of reactive oxygen species, promotes antigen presentation (15) and stimulates the production of neopterin in macrophages (16). Previous studies have already described a significant correlation between increased neopterin concentrations and anemia (17–20). Finally, higher neopterin concentrations have also been related with a poor prognosis in patients with cardiovascular disease (21–24).

As both, immune activation and anemia appear to be linked with the outcome of patients with cardiovascular disease, we wanted to investigate whether patients with ACD or multifactorial anemia differed regarding their risks for further cardiovascular events. Therefore, the objective of this study was to specify the cause of anemia in patients with CAD and ACS more precisely. Furthermore, we wanted to investigate whether the underlying cause of anemia (inflammation, ID or multifactorial anemia) has an impact on the prognosis of patients.

MATERIALS AND METHODS

Study Population

We analyzed data of 3,316 subjects from Ludwigshafen Risk and Cardiovascular Health (LURIC) study. Subjects were hospitalized for coronary arteriography (CAG) between June 1997 and January 2000 (25) because of chest pain and/or non-invasive test results consistent with myocardial ischemia. Additional inclusion criteria were available CAG, German ancestry and clinical stability except for acute coronary syndrome (ACS). Exclusion criteria were defined as any acute illness (other than ACS), any chronic non-cardiac diseases, or malignancy within the last 5 years. For further analysis, we included 2,082 patients with available neopterin concentrations. (Flow-chart of patients selection see **Figure 1**).

The study conformed to the principles outlined in the Declaration of Helsiniki and was approved by the ethics committee at the Landesärztekammer Rheinland-Pfalz. All patients gave written informed consent to participate in this study.

Angiography

Five experienced angiographers analyzed the angiograms and estimated maximal luminal narrowing by visual analysis. The three major coronary arteries were divided into 15 coronary arterial segments (26). Subjects were defined to have a clinically relevant CAD, if they presented with at least one stenosis (visible luminal narrowing) of $\geq$20 % in at least one of 15 coronary segments.

Outcome Analysis

Patients were followed up over a 10-year-period. The event-free survival was defined as the period of time between the first hospitalization and patients' death. Information about patients' vital status was obtained from local community registries. Death certificates were reviewed to classify deceased patients according to whether they died from cardio-cerebrovascular or non-cardio-cerebrovascular events. Death from cardio-cerebrovascular events included sudden cardiac death (SCD), fatal myocardial infarction (MI), congestive heart failure (CHF), death after intervention to treat CAD, fatal stroke or other death due to coronary heart disease. The classification was performed by two experienced physicians who were given the death certificates without any other data of the study participants. In case of a disagreement in classification of cause of death, the final decision was made by one of the principal investigators of the LURIC study after discussion.

Measurements

Laboratory measurements were performed at the baseline visit before CAG was performed (27). In addition, blood samples were stored at -80°C for later specific analyzes. Standard laboratory methods have been described elsewhere (25).

Hemoglobin concentrations (n = 2,082) were detected with the cyanmethemoglobin method (Technicon H1, Technicon GmbH, Bad Vilbel, Germany and Advia 120 Bayer Diagnostics, Tarrytown, USA). Transferrin (n = 2,082) was measured with an immunoturbidimetry and serum iron (n = 2,082) with a colorimetric ferrozine assay on a Hitachi 7171 analyzer (Roche, Mannheim, Germany). Transferrin saturation (TSAT) was defined as followed: (serum iron/5.5)/(transferrin/100) x 3.98. Ferritin concentrations (n = 2,082) were measured with an electrochemiluminescence enzyme immunoassay on

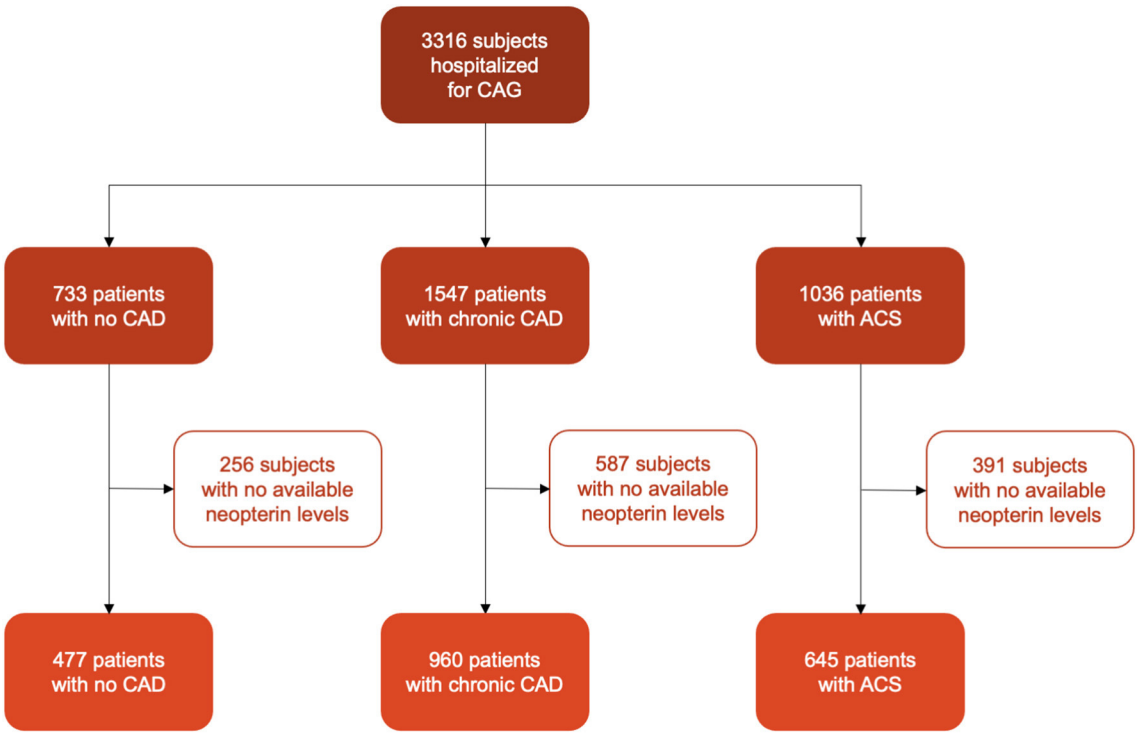

FIGURE 1 | Flowchart of patients' selection.

an Elecsys 2010 Roche autosampler (Roche, Mannheim, Germany). sTfR-Ferritin-index was calculated as quotient of sTfR concentration/log(ferritin concentrations).

Serum neopterin concentrations (n = 2,082) were measured with a radioimmunassay (BRAHMS Diagnostica, Hennigsdorf, Germany) with a sensitivity of 1 nmol/L neopterin and an interassay coefficient of variation ranging from 3.9 to 8.2% (28). We calculated the ratio of neopterin/eGFR since neopterin is excreted by the kidney and was shown to be higher/accumulate in patients with reduced kidney function (24, 29). The high-sensitive C-reactive protein (hsCRP; n = 2,082) was quantified by immunonephelometry (N High Sensitive CRP, Dade Behring, Marburg, Germany). IL-6 concentrations (n = 2,079) were detected by ELISA (R&D Systems Inc. USA). IL-12 concentrations (n = 1,809) were also measured by ELISA (BioSource Europe SA Nivelles, Belgium). Fibrinogen levels (n = 2,080) were measured with the Clauss method (STA fibrinogen/STA Stago, Stago Diagnostica/Roche, Mannheim, Germany). Serum amyloid A (n = 2,082) was measured using nephelometry (Behring Nephelometer II Analyzer).

Creatinine was measured with the method of Jaffé at 37°C with a Hitachi 717 autoanalyzer and commercial kits (Roche Diagnostics, Mannheim, Germany) (30). Estimated glomerular filtrations rate (eGFR) was defined as followed (CKD-EPI equation): eGFR (mL/min/1.73 m^2) = 141 x min[serum creatinine (mg/dL)/κ or 1]$^{\alpha}$ x max[serum creatinine (mg/dL)/κ or 1]$^{-1,209}$ x 0.993^{Age} (x 1.018 if female) (x 1.159 if black); κ is 0.7 for females and 0.9 for males; $^{\alpha}$ x is −0.329 for females and −0.411 for males; min indicates the minimum of serum creatinine (mg/dL)/κ or 1; max indicated the maximum of serum creatinine (mg/dL)/κ or 1. N-terminal pro brain natriuretic peptide (NT-proBNP) was measured by ElectroChemiLuminescence (ECL) on an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany).

In course of the CAG the following measurements were obtained: left ventricular ejection fraction (LV-EF, n = 895), mean pulmonary artery pressure (mean PAP, n = 644), pulmonary capillary wedge pressure (PCWP, n = 626) and cardiac index (CI, n = 474).

Classifications

CAD was classified into minor disease with a stenosis of 20–49%, one vessel disease (1VD), two vessel disease (2VD) and three vessel disease (3VD). ACS was scored in no ACS, unstable angina pectoris, non-ST elevation myocardial infarction (NSTEMI) and ST elevation myocardial infarction (STEMI). Angina pectoris complaints were classified according to the Canadian Cardiovascular Society (CCS) grading scale into Class I (ordinary physical activity does not cause angina), Class II (slight limitation of ordinary activity), Class III (marked limitation of ordinary physical activity) and Class IV (angina syndrome at rest) (31). Anemia was defined according to the criteria of the World Health Organization (WHO): hemoglobin <130 g/L in men and hemoglobin <120 g/L in women—severe anemia is defined as hemoglobin <80 g/L, moderate anemia is defined as hemoglobin 80−109 g/L and mild anemia is defined as

hemoglobin 110–129 g/L in men and 110–119 g/L in women (32). We further subdivided anemic patients into those with anemia of chronic disease (ACD; TSAT <20% with ferritin >100 μg/L), iron deficiency anemia (IDA; TSAT <20% with ferritin <30 μg/L), ACD/IDA (TSAT <20% with ferritin 30–100 μg/L) or multifactorial anemia (TSAT >20%) (5). ID without anemia was defined as ferritin <100 μg/L plus TSAT <20% (absolute or true ID) or serum ferritin between 100–300 μg/L plus TSAT of <20% (functional ID) (33).

Statistical Analysis

We used the Shapiro-Wilk test to test for Gaussian distribution. Parameters are depicted as *n* (%) or medians (25, 75th percentile), because they showed a skewed distribution. Mann-Whitney-*U* test, Kruskal-Wallis test or Pearson chi-square tests were used to test for significant differences among groups. Proportional hazard regression analysis was used to analyze the effects of risk factors on an adverse outcome. Multivariate proportional hazard regression analysis was stratified for sex and adjusted for established risk factors in patients with CAD including age, BMI, pack years, type 2 diabetes, hypertension, LDL, HDL, and triglyceride levels. Spearman rank correlation tests were performed to correlate continuous variables. All tests were two tailed and *p*-values <0.05 were regarded as statistically significant. We performed Bonferroni correction to address type I errors in univariate analyses. Statistical analysis was performed using SPSS Statistics Version 26.0 for Macintosh (IBM Corporation, Armonk, NY, USA).

RESULTS

Baseline Characteristics

We analyzed a data set comprising 2,082 patients (1,430 men and 652 women) in whom all preselected parameters were available with a median age of 63.4 years (56.2–70.6 years): 960 patients with chronic CAD (723 men and 237 women) and a median age of 64.5 years (58.0–71.1 years), 645 patients with ACS (475 men and 170 women) and a median age of 63.8 years (56.3–71.1 years), and 477 patients without CAD (232 men and 245 women) and a median age of 60.6 years (51.8–67.5 years; $p < 0.001$). The median follow-up was 9.81 years (8.58–10.53 years). By then 382 patients (18.4 %) died from cardio-cerebrovascular (CCV) disease and 218 patients (10.5%) from non-CCV diseases, while 12 patients were lost to follow-up. Baseline characteristics of patients with or without anemia are listed in **Table 1**.

At least one inflammatory marker (CRP or neopterin) was elevated in 988 patients (47.5 %) and the prevalence of that finding was significantly higher in CAD and ACS patients as compared to controls [177 patients without CAD (37.1%), 402 patients with chronic CAD (41.9%), 409 patients with ACS (63.4%)]. Among the specific immune biomarkers, 196 patients (9.4%) had only elevated neopterin levels (≥8.7 nmol/L), 494 patient (23.7%) had only elevated hsCRP levels (≥0.5 mg/dL) and 298 patients (14.3%) had both, elevated neopterin and hsCRP levels.

Anemia Is Frequently Encountered and Related to Disease Severity and Immune Activation

Anemia was found in 357 patients (17.1%): 41 patients without CAD (8.6%), 155 patients with chronic CAD (16.1%) and 161 patients with ACS (25.0%, $p < 0.001$). Grading of anemic patients showed that most patients presented with a mild anemia ($n = 288$, 13.8%), while 68 patients (3.3%) had a moderate and only one patient a severe anemia. The prevalence of anemia tended to be higher in women than in men (19.3 vs. 16.2%, $p = 0.075$); yet, in patients under the age of 55 years significantly more women were anemic (19.4 vs. 9.6%, $p = 0.006$).

Anemia was related to a more progressed CAD, higher CCS and NYHA classes, a significantly higher mean PAP and PCWP, and a significantly lower systolic BP, diastolic BP, eGFR and LV-EF. Anemic patients also had significantly higher neopterin, hsCRP, IL-6, IL-12, fibrinogen and serum amyloid A (SAA) levels, as well as a higher neopterin/eGFR ratio compared to non-anemic patients independent of sex and age (**Table 1**). Accordingly, hemoglobin levels correlated with neopterin, hsCRP, IL-6, IL-12, fibrinogen and SAA levels as well as with the neopterin/eGFR ratio independent of sex and age in patients with chronic CAD and ACS (**Table 2**).

However, there were gender differences regarding inflammatory parameters and anemia: higher neopterin, hsCRP and IL-6 concentrations were positively related to anemia severity especially in men, while IL-12 rose with anemia severity especially in women (**Figure 2**). In fact, IL-12 levels were significantly higher in women compared to men in general (72.69 vs. 62.03 ng/L, $p < 0.001$) and were correlated with hemoglobin concentrations only in women (**Table 2**). **Table 2** shows correlations of inflammatory parameters with hemoglobin concentrations for the total population and separately for men and women.

Anemia Classification According to Iron Deficiency in Terms of Disease Severity and Immune Activation

We then studied patients according to the different types of anemia. Anemia of chronic disease (ACD) was present in 119 patients (5.7%; 84 men [5.9%] and 35 women [5.4%]), while 52 patients had ACD + IDA (2.5%; 29 men [2.0%] and 23 women [3.5%]). IDA was found in 28 patients (1.3%; 15 men [1.0%] and 13 women [2.0%]), and 158 patients had multifactorial or unclassified anemia (7.6%; 103 men [7.2%] and 55 women [8.4%]).

Patients with ACD presented with a more progressed CAD and also with a higher CCS. Patients with ACD also differed from patients with IDA regarding laboratory parameters and also vital parameters (see **Table 3**). Patients with ACD ($n = 119$) had significantly higher neopterin, hsCRP, IL-6, IL-12, fibrinogen and SAA levels compared to non-anemic patients ($n = 1,725$). Interestingly, neopterin and IL-12 levels did not significantly differ among patients with ACD, ACD + IDA ($n = 52$) or IDA ($n = 28$; **Figures 3A,B**), while patients with ACD had significantly higher hsCRP, IL-6, fibrinogen and SAA levels compared to

TABLE 1 | Baseline characteristics.

	Total population	No anemia	Anemia	Significance
	n = 2,082	*n* = 1,725	*n* = 357	
	Median (IQR) or %	Median (IQR) or %	Median (IQR) or %	*p*-Value
Demographics and comorbidities				
Age [years]	*63.4 (70.6–56.2)*	*62.6 (69.6–55.7)*	*68.3 (74.2–60.3)*	*<0.001*
Sex [men]	68.7%	69.5%	64.7%	0.075
BMI [kg/m^2]	*27.1 (29.6–24.7)*	*27.2 (29.8–24.9)*	*26.1 (28.9–23.8)*	*<0.001*
Hypertension	52.0%	53.3%	45.4%	0.006
Atrial fibrillation	11.5%	11.3%	12.5%	0.495
Diabetes mellitus Type 2	*16.6%*	*15.2%*	*23.2%*	*<0.001*
Smoking	63.0%	62.6%	64.4%	0.741
Pack Years	9 (30–0)	9 (30–0)	8 (30–0)	0.527
Lipid lowering therapy	*49.8%*	*47.9%*	*58.5%*	*<0.001*
Clincial characteristics				
Heart rate [bpm]	68 (76–61)	68 (76–61)	68 (77–62)	0.111
Syst. BP [mmHg]	*140 (155–123)*	*140 (156–123)*	*136 (151–119)*	*<0.001*
Diast. BP [mmHg]	*80 (88–73)*	*81 (89–74)*	*75 (83–68)*	*<0.001*
CAD classification				*<0.001*
no CAD	*22.9%*	*25.3%*	*11.5%*	–
minor CAD	*10.7%*	*10.7%*	*10.9%*	–
one vessel disease	*19.5%*	*19.6%*	*19.3%*	–
two vessel disease	*18.5%*	*17.8%*	*22.1%*	–
three vessel disease	*28.3%*	*26.7%*	*36.1%*	–
CCS grading scale				*<0.001*
CCS class I	*38.8%*	*38.6%*	*39.5%*	–
CCS class II	*34.4%*	*36.3%*	*25.5%*	–
CCS class III	*15.5%*	*15.0%*	*18.2%*	–
CCS class IV	*11.3%*	*10.1%*	*16.8%*	–
NYHA class				0.006
NYHA class I	52.6%	54.0%	46.2%	–
NYHA class II	29.4%	29.2%	30.5%	–
NYHA class III/IV	18.0%	16.9%	23.2%	–
LV-EF [%]	*64 (73–47)*	*65 (74–49)*	*55 (70–37)*	*<0.001*
mean PAP [mmHg]	18 (25–15)	18 (24–15)	20 (30–15)	0.025
PCWP [mmHg]	10 (15–8)	10 (15–8)	12 (19–9)	0.021
CI [L/min/m^2]	2.44 (3.00–2.10)	2.40 (3.00–2.10)	2.60 (3.10–2.10)	0.588
Laboratory testing (serum)				
eGFR [mL/min/1.73 m^2]	*83.85 (96.54–70.14)*	*84.97 (97.51–72.00)*	*77.83 (89.92–59.92)*	*<0.001*
Cholesterol [mg/dL]	*206 (237–178)*	*211 (240–183)*	*182 (209–159)*	*<0.001*
LDL [mg/dL]	*116 (138–95)*	*119 (141–98)*	*104 (124–84)*	*<0.001*
HDL [mg/dL]	*37 (45–31)*	*38 (45–32)*	*34 (43–29)*	*<0.001*
Triglycerides [mg/dL]	*143 (198–107)*	*145 (202–110)*	*132 (174–98)*	*<0.001*
hsCRP [mg/dL]	*0.33 (0.83–0.13)*	*0.29 (0.69–0.12)*	*0.74 (2.08–0.26)*	*<0.001*
Neopterin [nmol/L]	*6.93 (8.56–5.67)*	*6.77 (8.28–5.57)*	*8.05 (11.55–6.57)*	*<0.001*
Neopterin/eGFR ratio	*0.083 (0.122–0.061)*	*0.079 (0.111–0.059)*	*0.106 (0.187–0.074)*	*<0.001*
IL-6 [ng/L]	*3.12 (6.08–1.74)*	*2.85 (5.34–1.67)*	*5.17 (10.09–2.61)*	*<0.001*
IL-12 [ng/L]	*65.33 (104.93–38.50)*	*63.19 (101.97–37.33)*	*75.70 (122.75–45.74)*	*<0.001*
Fibrinogen [mg/dL]	*376 (451–319)*	*368 (438–316)*	*431 (527–359)*	*<0.001*
SAA [mg/L]	*5.15 (12.40–2.90)*	*4.70 (9.50–2.80)*	*9.40 (39.90–3.80)*	*<0.001*
TSAT [%]	*25.22 (32.85–18.67)*	*26.41 (33.96–20.15)*	*18.74 (25.78–12.84)*	*<0.001*
Ferritin [ng/mL]	156 (274–89)	160 (276–91)	144 (256–78)	0.025
Hepcidin [ng/mL]	6.57 (10.16–4.03)	6.57 (9.87–4.11)	6.64 (11.83–3.28)	0.638

(Continued)

TABLE 1 | Continued

	Total population	No anemia	Anemia	Significance
	n = 2,082	*n* = 1,725	*n* = 357	
	Median (IQR) or %	Median (IQR) or %	Median (IQR) or %	*p*-Value
sTfR [mg/L]	1.26 (1.53–1.06)	1.25 (1.52–1.06)	1.26 (1.64–1.05)	0.435
Ferritin-index	0.581 (0.737–0.469)	0.579 (0.728–0.472)	0.593 (0.809–0.457)	0.192
Hemoglobin [g/L]	*13.90 (14.90–12.90)*	*14.20 (15.10–13.40)*	*11.80 (12.40–11.20)*	*<0.001*
Vitamin B12 [ng/L]	347 (473–259)	347 (467–264)	344 (515–237)	0.716
Folic acid [μg/L]	*8.00 (10.40–6.10)*	*8.20 (10.50–6.20)*	*7.40 (9.70–5.30)*	*<0.001*

Parameters from 2,082 patients are listed as median (IQR) or n (%) for the total patients' cohort and for patients with or without anemia. Mann-Whitney U test and the Pearson chi-square test was used for comparisons between the two groups. Bonferroni correction was used to address type I errors for multiple testing thus p-Values < 0.00128 were suggested as statistically significant.

BMI, body mass index; CAD, coronary artery disease; CCS, Canadian Cardiovascular Society; NYHA, New York Heart Association; Syst. BP, systolic blood pressure; Diast. BP, diastolic blood pressure; LV-EF, left ventricular ejection fraction; mean PAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; CI, cardiac index; eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein; HDL, high density lipoprotein; hsCRP, high-sensitive C-reactive protein; IL-6, interleukin 6; IL-12, interleukin 12; SAA, serum amyloid A; TSAT, transferrin saturation; sTfR, soluble transferrin receptor.

TABLE 2 | Spearman-rank correlations with hemoglobin.

	Total population	Men	Women
	n = 2,082	*n* = 1,430	*n* = 652
hsCRP [mg/dL]	−0.226*	−0.253*	−0.182*
Neopterin [nmol/L]	−0.164*	−0.175*	−0.136*
Neopterin/eGFR ratio	−0.195*	−0.176*	−0.132*
IL-6 [ng/L]	−0.176*	−0.232*	−0.159*
IL-12 [ng/L]	−0.140*	−0.070	−0.188*
Fibrinogen [mg/dL]	−0.164*	−0.209*	−0.143*
SAA [mg/L]	−0.233*	−0.217*	−0.136*
TSAT [%]	0.351*	0.357*	0.307*
Ferritin [ng/mL]	0.188*	0.074	0.034
Hepcidin [ng/mL]	0.051	−0.014	0.027
sTfR [mg/L]	0.084*	0.105*	0.069
Ferritin-index	−0.018	0.057	0.058
Vitamin B12 [ng/L]	0.031	0.078	0.020
Folic acid [μg/L]	0.047	0.120*	0.023

Spearman-rank correlation analyses of hemoglobin with different parameters from 2,082 patients are listed for the total patients' cohort and separated for sex. Bonferroni correction was used to address type I errors for multiple testing thus p-Values < 0.00119 were suggested as statistically significant (*); hsCRP, high-sensitive C-reactive protein; IL-6, interleukin 6; IL-12, interleukin 12; SAA, serum amyloid A; TSAT, transferrin saturation; sTfR, soluble transferrin receptor.

patients with IDA (**Figures 3C,D**). The latter finding was less pronounced in women compared to men.

Most of the patients with multifactorial anemia had a normocytic, normochromic anemia ($n = 136$, 86.1%) and presented with elevated neopterin and/or hsCRP levels ($n = 96$, 60.8%), which are hallmarks of ACD.

Impact of Anemia and Immune Activation on Cardio-Cerebrovascular Mortality

In univariate Cox regression analysis, the presence of anemia was linked to an almost 2-fold higher CCV mortality (HR1.914 [95%CI 1.521–2.408]). In addition, multivariate Cox regression analysis stratified for sex and adjusted for established risk factors in patients with CAD including age, BMI, LDL, HDL, triglycerides, pack years, hypertension, or type 2 diabetes confirmed that the presence of anemia was linked to a significantly higher CCV mortality (HR 1.348 [95%CI 1.052–1.727]). Further classification of anemic patients showed that those with ACD (with and without concomitant IDA) had the highest CCV event rate within the following 2 years (ACD 14.3%; ACD + IDA 11.4%; IDA 3.6%; multifactorial anemia 4.5%; no anemia 4.0%). Interestingly, after 10 years of follow-up mortality rates did not differ between patients with ACD and anemia of other cause (**Figure 4A**).

In patients with multifactorial anemia those with elevated inflammatory markers also had a significantly higher CCV mortality (HR 2.095 [95%CI 1.014–4.332]). Especially, patients with elevated neopterin concentrations (HR 2.137 [95%CI 1.120–4.079]) were at a higher risk to die by a CCV event.

Finally, we studied the impact of inflammation on the outcome of CAD patients and investigated whether inflammatory markers were linked to the presence of anemia. Cox-regression analysis revealed a significant interaction of neopterin and hemoglobin (neopterin x hemoglobin: $p = 0.031$; neopterin/eGFR ratio x hemoglobin: $p = 0.024$). Accordingly, Kaplan-Meier curves show that patients with elevated neopterin levels (or an elevated neopterin/eGFR ratio to account also for renal function) had a significantly higher CCV mortality rate independent of concomitant anemia. However, anemia further increased the risk for subsequent events in patients with elevated neopterin levels (HR 1.487 [95%CI 1.069–2.069], **Figure 4B**) or with an elevated neopterin/eGFR ratio (HR 1.737 [95%CI 1.346–2.241], **Figure 4C**). Interestingly, in patients with a low neopterin/eGFR ratio (defined by the median), anemia was not related to a significantly higher CCV event rate **Figure 4C**).

DISCUSSION

Our findings confirm that anemia is related to an adverse outcome of patients with cardiovascular disease (34, 35). We

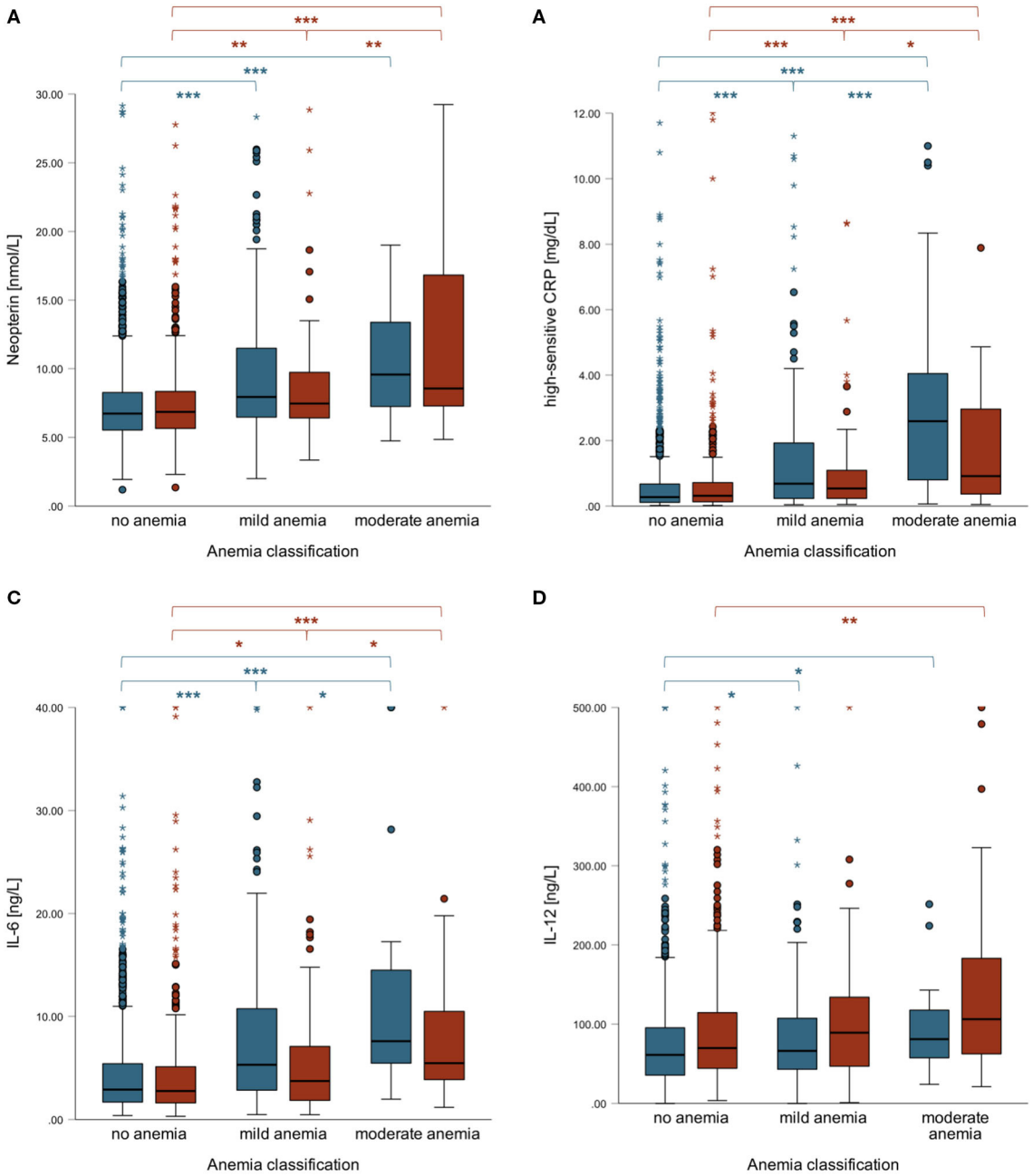

FIGURE 2 | Boxplots of neopterin **(A)**, hsCRP **(B)**, IL-6 **(C)** and IL-12 levels **(D)** in men (blue) and women (red) according to anemia severity. Men with mild or moderate anemia had significantly higher IL-6 levels compared to women with mild ($p = 0.007$) or moderate anemia ($p = 0.060$). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

could show for the first time that the underlying cause of anemia also has a significant impact on the prognosis of patients: ACD, which is caused by immune activation, is associated with a significantly higher risk for cardio-cerebrovascular events in the subsequent 2 years when compared to patients with IDA or multifactorial anemia. Our data further indicate that inflammation is the underlying cause for the development of anemia in a high percentage of patients with CAD. Therefore, it might be useful to differentiate between patients with ACD, IDA or multifactorial anemia to better predict their risks to die within the next years and to choose the best therapy option.

Anemia was linked to CAD severity and predictive for all-cause mortality—similar to elevated neopterin levels. However, anemia was only predictive for an adverse outcome in patients with elevated inflammatory markers (and a higher neopterin/eGFR ratio, respectively). Thus, our data suggest that

TABLE 3 | Baseline characteristics within the ACD/IDA classification.

	no anemia	ACD	ACD + IDA	IDA	MfA	Sig.
	n = 1,725	*n* = 119	*n* = 52	*n* = 28	*n* = 158	
	Median or %	Median or %	Median or %	Median or %	Median or %	*p*-Value
Demographics and comorbidities						
Age [years]	*62.63*	*68.14*	*70.90*	*69.04*	*67.52*	*<0.001*
Sex [men]	69.5%	70.6%	55.8%	53.6%	65.2%	0.071
BMI [kg/m^2]	*27.18*	*26.10*	*26.00*	*26.71*	*26.09*	*<0.001*
Hypertension	*53.3%*	*34.5%*	*51.9%*	*67.9%*	*37.5%*	*<0.001*
Atrial fibrillation	11.3%	11.9%	18.4%	14.3%	10.9%	0.617
Diabetes mellitus Type 2	*15.2%*	*24.4%*	*25.0%*	*25.0%*	*21.5%*	*0.006*
Smoking	62.6%	68.9%	53.8%	57.2%	65.8%	0.311
Pack Years	9.0	12.5	0.9	1.2	10.0	0.171
Lipid lowering therapy	*47.9%*	*65.5%*	*55.8%*	*50.0%*	*55.7%*	*0.002*
Clinical characteristics						
Heart rate [bpm]	*67.67*	*71.00*	*67.67*	*70.67*	*66.00*	*0.013*
Syst. BP [mmHg]	*140.00*	*129.00*	*141.50*	*145.00*	*137.33*	*<0.001*
Diast. BP [mmHg]	*81.33*	*71.33*	*74.33*	*80.00*	*76.33*	*<0.001*
CAD classification						*<0.001*
no CAD	*25.3%*	*8.4%*	*9.6%*	*17.9%*	*13.3%*	–
minor CAD	*10.7%*	*6.7%*	*7.7%*	*14.3%*	*14.6%*	–
one vessel disease	*19.6%*	*17.6%*	*23.1%*	*21.4%*	*19.0%*	–
two vessel disease	*17.8%*	*28.6%*	*15.4%*	*10.7%*	*21.5%*	–
three vessel disease	*26.7%*	*38.7%*	*44.2%*	*35.7%*	*31.6%*	–
CCS grading scale						*<0.001*
CCS class I	*38.6%*	*42.0%*	*34.6%*	*50.0%*	*37.3%*	–
CCS class II	*36.3%*	*18.5%*	*26.9%*	*21.4%*	*31.0%*	–
CCS class III	*15.0%*	*16.8%*	*21.2%*	*21.5%*	*17.7%*	–
CCS class IV	*10.1%*	*22.7%*	*17.3%*	*7.1%*	*13.9%*	–
NYHA class						*0.003*
NYHA class I	*54.0%*	*52.1%*	*36.5%*	*39.3%*	*46.2%*	–
NYHA class II	*29.2%*	*26.1%*	*36.5%*	*17.9%*	*34.2%*	–
NYHA class III/IV	*16.9%*	*21.8%*	*26.9%*	*42.9%*	*19.6%*	–
LV-EF [%]	*65*	*42*	*46*	*64*	*60*	*<0.001*
mean PAP [mmHg]	18	21	21	26	19	0.084
PCWP [mmHg]	10	13	12	15	11	0.058
CI [L/min/m^2]	2.40	2.60	2.65	3.00	2.30	0.527
Laboratory testing (serum)						
eGFR [mL/min/1.73 m^2]	*84.97*	*77.83*	*73.90*	*74.15*	*80.81*	*<0.001*
Cholesterol [mg/dL]	*211*	*177*	*180*	*200*	*186*	*<0.001*
LDL [mg/dL]	*119*	*101*	*102*	*109*	*105*	*<0.001*
HDL [mg/dL]	*38*	*31*	*36*	*43*	*36*	*<0.001*
Triglycerides [mg/dL]	*145*	*138*	*119*	*119*	*134*	*0.001*
hsCRP [mg/dL]	*0.29*	*1.93*	*0.79*	*0.52*	*0.43*	*<0.001*
Neopterin [nmol/L]	*6.77*	*8.40*	*8.05*	*8.40*	*7.42*	*<0.001*
Neopterin/eGFR ratio	*0.079*	*0.118*	*0.110*	*0.115*	*0.093*	*<0.001*
IL-6 [ng/L]	*2.85*	*8.28*	*6.42*	*4.06*	*3.41*	*<0.001*
IL-12 [ng/L]	*63.19*	*74.76*	*81.40*	*89.71*	*69.41*	*0.001*
Fibrinogen [mg/dL]	*368*	*514*	*432*	*387*	*393*	*<0.001*
SAA [mg/L]	*4.70*	*35.00*	*9.70*	*6.15*	*5.70*	*<0.001*
TSAT [%]	*26.41*	*13.53*	*14.97*	*10.49*	*26.70*	*<0.001*
Ferritin [ng/mL]	*160*	*196*	*65*	*15*	*167*	*<0.001*
Hepcidin [ng/mL]	*6.57*	*9.10*	*4.62*	*0.49*	*7.28*	*<0.001*
sTfR [mg/L]	*1.25*	*1.30*	*1.41*	*2.10*	*1.17*	*<0.001*

(Continued)

TABLE 3 | Continued

	no anemia	ACD	ACD + IDA	IDA	MfA	Sig.
	n = 1,725	*n* = 119	*n* = 52	*n* = 28	*n* = 158	
	Median or %	Median or %	Median or %	Median or %	Median or %	*p*-Value
sTfR-ferritin-index	*0.579*	*0.531*	*0.773*	*1.869*	*0.530*	*<0.001*
Hemoglobin [g/L]	*14.20*	*11.60*	*11.80*	*11.50*	*11.90*	*<0.001*
Vitamin B12 [ng/L]	347	339	357	363	341	0.896
Folic acid [μg/L]	*8.20*	*6.80*	*7.55*	*8.45*	*7.65*	*<0.001*

Parameters from 2,082 patients are listed as median (IQR) or n (%) for patients within the ACD/IDA classification. Kruskal-Wallis test and the Pearson chi-square test was used for comparisons between all subgroups. Bonferroni correction was used to address type I errors for multiple testing thus p-Values < 0.00128 were suggested as statistically significant. MfA, multifactorial anemia; IDA, iron deficiency anemia; ACD, anemia of chronic disease; Sig., significance; BMI, body mass index; CAD, coronary artery disease; CCS, Canadian Cardiovascular Society; NYHA, New York Heart Association; Syst. BP, systolic blood pressure; Diast. BP, diastolic blood pressure; LV-EF, left ventricular ejection fraction; mean PAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; CI, cardiac index; eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein; HDL, high density lipoprotein; hsCRP, high-sensitive C-reactive protein; IL-6, interleukin 6; IL-12, interleukin 12; SAA, serum amyloid A; TSAT, transferrin saturation; sTfR, soluble transferrin receptor.

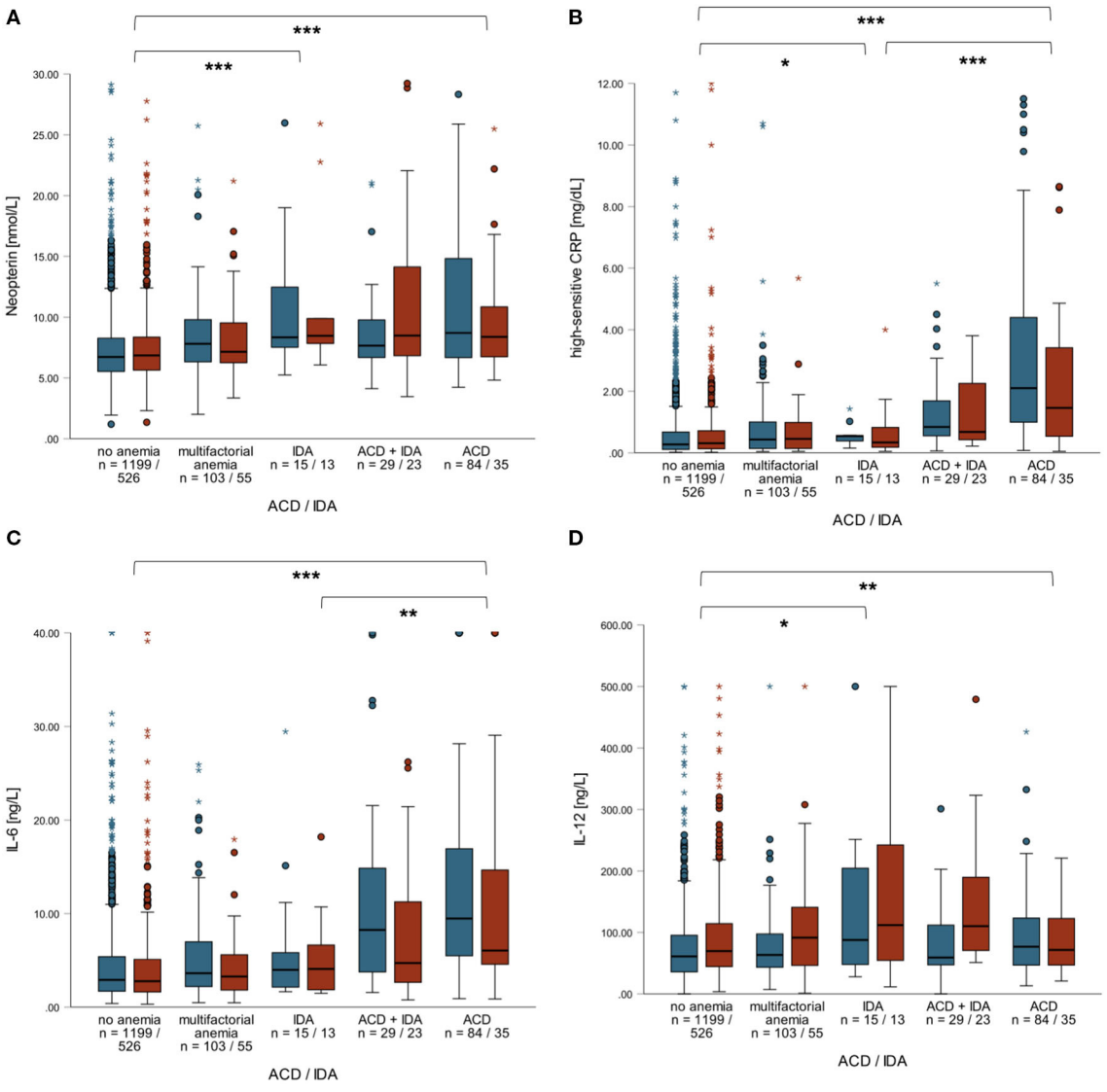

FIGURE 3 | Boxplots of neopterin **(A)** hsCRP **(B)**, IL-6 **(C)** and IL-12 levels **(D)** in men (blue) and women (red) with or without ACD and/or IDA or multifactorial anemia. Neopterin and IL-12 levels did not significantly differ between patients with ACD, ACD+IDA or IDA independent of sex. Contrary, IL-6 and hsCRP levels were significantly higher in patients with ACD compared to patients with IDA or ACD + IDA (especially in men). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

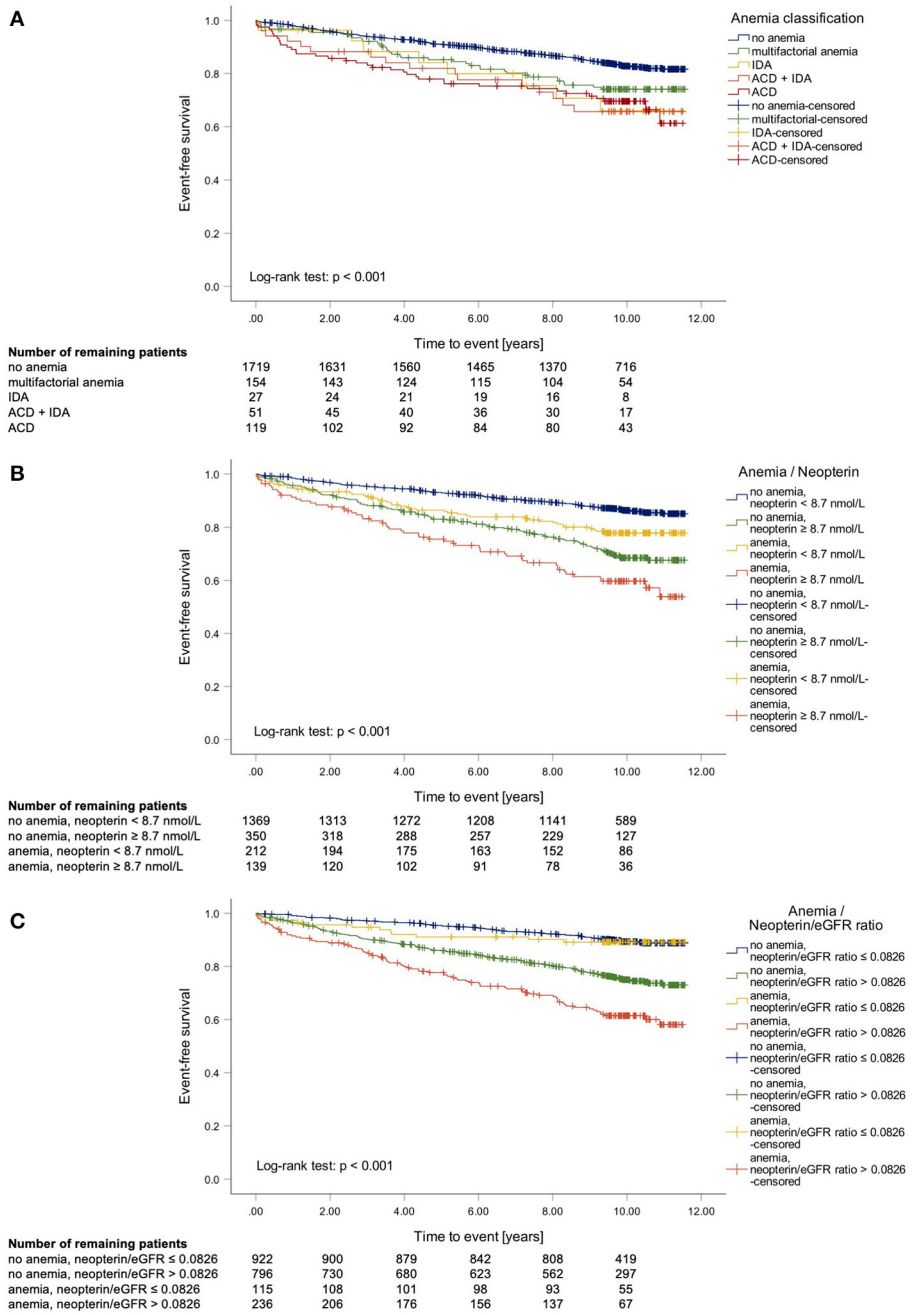

FIGURE 4 | Kaplan-Meier curves of cardio-cerebrovascular (CCV) mortality in terms of the anemia subdivision into anemia of chronic disease (ACD) and iron deficiency anemia (IDA) **(A)** and Kaplan-Meier curve of CCV mortality in terms of anemia and elevated neopterin levels [≥8.7 nmol/L; **(B)**] or respectively neopterin/eGFR ratio [>0.0826; **(C)**].

anemia reflects disease severity and predicts outcome mainly because it is strongly associated with inflammation. Since also decreasing renal function during aging can contribute to the development of anemia, we also accounted for this point by calculating the neopterin/eGFR ratio. Recently, advanced inflammation and reduced kidney function were shown to contribute to a poor clinical course in patients with chronic heart failure (35).

In line with this hypothesis, patients with ACD also had a poorer prognosis than subjects with multifactorial anemia—pointing to the crucial relationship between anemia, iron homeostasis and inflammation in subjects with cardiovascular diseases (36). In our study population, more than 80% of patients with multifactorial anemia had a normocytic, normochromic anemia and presented with elevated inflammatory markers which are both typical for ACD. However, according to established

classifications (9, 33), those patients were not classified as having ACD because TSAT was above 20%. Still, the finding that ferritin levels were similar to ACD subjects in patients with multifactorial anemia indicates that chronic inflammation might also contribute importantly to the development of anemia in these patients. Also, the fact that patients with elevated inflammatory markers had a higher CCV risk in this subgroup of patients suggests that the inflammatory process may underlie CAD progression as well as the development of anemia. Higher TSAT in patients with multifactorial anemia may also result from low transferrin levels as a consequence of inflammation (thereby masking the ACD phenotype) (37).

The hypothesis that inflammation contributes to the development of anemia in patients with cardiovascular disease, is also supported by a recently published *post hoc* analysis of the CANTOS trial (38). In patients with previous myocardial infarction rising hsCRP concentrations were related to an increasing incidence rate of anemia. In patients receiving canakinumab—an antibody that is targeting IL-1β—hsCRP and IL-6 levels decreased while hemoglobin levels increased concomitantly in patients with baseline anemia (38). Furthermore, a reduced incidence of anemia could be demonstrated in patients treated with canakinumab compared to patients receiving placebo (38). This indicates that anti-inflammatory strategies can improve anemia most likely by ameliorating inflammation-driven disturbances of iron homeostasis and cytokine mediated effects on erythropoiesis (37).

Unfortunately, the CANTOS trial did not investigate different anemia types, which might in fact provide interesting new results. Still, also other data support our hypothesis, that inflammation is causally involved in CAD progression and anemia development: Anti-inflammatory treatment with colchicine reduces the secondary attack rate in CAD (39), and ameliorates anemia in CAD as shown in Familial Mediterranean fever (40).

In our population, patients with ACD had significantly higher hsCRP, IL6, fibrinogen and SAA levels when compared to patients with IDA or multifactorial anemia. Furthermore, ACD patients had the most advanced disease progression and the poorest short-time outcome. ACD is characterized by iron restriction by macrophages (5), while IDA is characterized by decreased tissue iron stores (41). Both are resulting in decreased erythropoiesis over time due to reduced iron availability (5, 41). Iron was shown earlier to reduce the efficiency of the IFN-γ signal in monocytes, thus also decreasing neopterin production (42) and cellular Th1 immune response (43). Therefore, in patients with IDA, low iron availability may enhance Th1 immune response.

Finally, we could also show some significant differences in immune activation between anemic men and women. Anemic men (especially male patients with ACD) had higher neopterin, hsCRP and IL-6 levels compared to anemic women, while anemic women had higher IL-12 levels compared to anemic men. The above-mentioned mechanisms of inflammation affecting erythropoiesis might not be as distinctive in women as in men. Since women have a higher prevalence of anemia especially before menopause because of their menstrual bleeding, they might have some sort of protection mechanism preventing an additional decrease of erythropoiesis due to inflammation. Actually, IL-12 was shown to enhance erythropoiesis *in vitro* (44), suggesting that higher IL-12 levels observed in women may counteract the inflammatory burden on erythropoiesis.

Limitations

Neopterin levels were not available for all patients that were initially included in the study, which is why the findings made in this study do not allow unrestricted generalization to CAD patients in general. Also, the fact that data concerning concomitant erythropoietin therapy or blood transfusion were not available is a limitation of this study. Unfortunately, we do not have follow-up data regarding anemia and inflammation of patients—these data would certainly provide very interesting information.

CONCLUSION

This study confirms that anemia is common and strongly related to immune activation in patients with coronary artery disease. Specifically, the combination of anemia and inflammation is associated with a worse prognosis and an increased risk of cardio-cerebrovascular death in patients with CAD. Actually, anemia was only predictive for further cardio-cerebrovascular events in patients with elevated inflammatory markers. We could also show differences in immune activation between anemic men and women.

In summary, our data suggest that the association of anemia with disease severity and outcome might mainly be due to underlying inflammation; additionally, advanced renal dysfunction should also be taken into account.

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 at the Landesärztekammer Rheinland-Pfalz. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WM: conceptualization and project administration. DF, TG, KK, WM, and GW: methodology. LL: software, formal analysis, and visualization. KK, LL, and GW: validation. DF, TG, MK, WM, and HS: investigation, resources, and data curation. KK and LL: writing—original draft preparation. DF, TG, MK, WM, HS, and GW: writing—review and editing. KK: supervision. All authors have read and agreed to the published version of the manuscript.

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Association Between Serum Calcium and the Prevalence of Hypertension Among US Adults

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Background: Hypertension is a significant risk factor of cardiovascular diseases, posing a serious threat to global health. Calcium plays an important role in regulating body homeostasis. The association of calcium with hypertension remains uncertain in the general population.

Methods and Results: Cross-sectional data from the 2007–2018 National Health and Nutrition Examination Survey (NHANES) were analyzed. Adjusted multivariable logistic regression analysis and restricted cubic spline were used to investigate the association of serum calcium with the prevalence of hypertension. A total of 26,778 participants were included. The increase in calcium levels showed a positive association with the prevalence of hypertension in all three models with ORs of 1.347 (1.249–1.454), 1.522 (1.401–1.654), and 1.438 (1.306–1.583). The further subgroup analysis demonstrated a robust trend across all categories by sex, age, race, BMI, and eGFR. The restricted cubic spline plot exhibited an S-curve relationship between calcium and hypertension.

Conclusion: Our cross-sectional study demonstrated a positive association between higher serum calcium level and the prevalence of hypertension. Our findings highlighted serum calcium level in hypertensive patients.

Keywords: association, serum calcium, hypertension, multivariable logistic regression, S-curve

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INTRODUCTION

Hypertension is a common medical condition defined as systolic blood pressure (BP) ≥140 mmHg and/or diastolic BP ≥90 mmHg (1). The prevalence of hypertension has been consistently increasing in the past decades, especially in low- and middle-income countries, posing a serious threat to global health (2). Growing epidemiological evidence has confirmed the substantial influence of lifestyle interventions on blood pressure, including physical exercise, dietary patterns, and body weight management beyond genetic endowment (3).

As one of the most abundant mineral elements broadly involved in diverse body activities, the role of calcium in hypertension has received much attention as well as other cations. Nevertheless, unlike the normal recognized recommendation of sodium restriction and potassium intake for the dietary prevention of hypertension, previous studies that examined the association between serum calcium and hypertension have shown contradictory results (4–6). Some studies reported calcium to be associated with a higher risk of hypertension, whereas some shown an inverse or null effect. Most evidence from previous studies lack strong credibility due to the small sample size, incomplete adjustment of confounding factors, or excessive experimental extrapolation (5, 7).

Approximately 50% of serum calcium is in the ionized form, while 40% in the bound form mainly to albumin, and 10% is bound to anions (8). Serum total calcium is the total sum of three forms and is least affected by physiological changes or varieties in measurement. Therefore, total serum calcium is routinely used in clinical practice to represent calcium status in the human body (9). In this context, by using data from a large representative US population, we performed a cross-sectional study to investigate the association between calcium and the prevalence of hypertension.

MATERIALS AND METHODS

Data Source and Study Population

Our study was a cross-sectional study. National Health and Nutrition Examination Survey (NHANES) is a public database recording the health and nutritional status among the US population and is published every 2 years (https://www.cdc.gov/nchs/nhanes/index.htm). A sample of subjects was selected and interviewed by using a stratified cluster sampling method to ensure the representativeness. We selected available data from the year cycle 2007–2008, 2009–2010, 2011–2012, 2013–2014, 2015–2016, 2017–2018. Individuals with full information on body measures, blood pressure, medical conditions, diabetes, smoking, alcohol consumption, dietary interview, and standard biochemistry profile were included. Based on previous literature (10, 11), the exclusion criteria were as followed: (1) pregnant individuals, (2) age <18 or ≥ 80, (3) estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73m^2, and (4) participants without calcium or phosphorus record. Notably, we excluded subjects with CKD [eGFR < 60 mL/min/1.73m^2 (12)] to eliminate possible effects of CKD on calcium (13) and blood pressure (14). National Center for Health Statistics Research Ethics Review Board approved this study, and informed consent was obtained from all participants.

The Definition of Hypertension

Blood pressure measurements were taken using a mercury sphygmomanometer according to standardized blood pressure measurement protocols recommended by American Heart Association at that time. After 5 min of seated rest, trained clinicians measured the blood pressure and repeated three times at the interval of 30 s. The mean of all available measurements was recorded. Hypertension was defined as (1) average systolic blood pressure ≥ 140 mmHg, (2) average diastolic blood pressure ≥ 90 mmHg, (3) current use of anti-hypertensive medications, (4) subjects with a self-reported hypertension (15–17). Moreover, we performed a sensitivity analysis by using a new cut-off value of 130/80 mmHg according to the American Heart Association (18).

Covariates

Covariates related to hypertension were selected and controlled based on previously published studies (16, 19). We obtained age, sex (male and female), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American, other Hispanic, and other races), and education levels (below high school, high school, and above high school) from the demographic questionnaire. Self-reported diabetes history (yes/no), smoking status (smoked at least 100 cigarettes in life or not), sodium intake, and alcohol consumption were acquired from dietary questionnaire, whereas total calcium (mg/dl), triglycerides (mg/dl), total cholesterol (mg/dl), albumin (g/L), serum phosphorus (mg/dl), and creatinine (mg/dl) were obtained from laboratory tests. Individuals smoking more than 100 cigarettes during their lifetime were considered smokers (20), and participants consuming at least 12 alcohol drinks per year were considered alcohol users (21). Body mass index (BMI) was calculated by dividing weight in kilograms by the square of their height in meters (kg/m^2). Estimated glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) equation incorporating age, sex, race, and serum creatinine in the equation (22).

Statistical Analysis

Continuous variables were represented as mean $\pm$ standard deviation (normal distribution), median with interquartile range (skewed distribution), or percentages (categorical variables). Comparisons between the hypertensive and non-hypertensive groups were performed using the chi-square test (categorical variables), one-way ANOVA test (normal distribution), or Kruskal-Wallis test (skewed distribution). Importantly, to maximize statistical power and minimize bias caused by missing data, we applied multivariate multiple imputation strategies based on five replication and Markov chain Monte Carlo method to fill missing covariates (23, 24).

Total calcium was categorized into quartiles, and the lowest quartile was set as the reference group. Multivariable logistic regression analysis was used to estimate the correlation between total calcium and hypertension. Three multivariable adjustment models were used: crude model (model 1) adjusted for age and gender; model 2 additionally adjusted for race/ethnicity, education levels, BMI, diabetes history, smoking status, alcohol consumption, sodium intake, triglycerides, and total cholesterol levels; model 3 adjusted for variables in model 2 plus albumin, serum phosphorus, and eGFR. The odds ratios (ORs) with 95% confidence intervals (CIs) were calculated accordingly. Subgroup analyses were performed to examine whether the associations of total calcium and hypertension were consistent across categories of sex (male and female), age (18–80), race (Non-Hispanic White, Non-Hispanic Black, Mexican American, other Hispanic, and Other-race including Multi-racial), BMI (underweight, normal weight, overweight, class I obese, class II obese, and class III obese), and eGFR (<133 ml/min/1.73 m^2, and ≥ 133 ml/min/1.73 m^2) using fully adjusted model 3. To further illustrate the correlation between total calcium and hypertension, we also used a restricted cubic spline with five knots located at the 5th, 27.5th, 50th, 72.5th, and 95th percentiles to flexibly model the underlying relationship. The median total calcium was chosen as the reference.

Moreover, we performed Spearman correlation analysis to assess the correlation of total calcium and systolic/diastolic blood pressure. Receiver operating characteristic curve (ROC curve) was drawn to show identification ability of calcium in the prevalence of hypertension. We divided participants into a

training set and a testing set randomly at a ratio of 7:3. The training set was used to create a predictive model, whereas the testing set was used to evaluate the model performance by area under the curve (AUC), sensitivity, specificity, positive predictive value, and negative predictive value. A sensitivity analysis using the cut-off value of 130/80 mmHg was also performed to validate the association between total calcium and hypertension.

All statistical analysis was performed by R software version 3.6.1 (version 3.6.0; The R Foundation for Statistical Computing). P-value < 0.05 was considered statistically significant for all analyses.

RESULTS

Characteristics of Study Population

We initially included 34,573 subjects with data from NHANES 2007–2018. After excluding participants who were pregnant ($n = 372$), age <18 or >80 ($n = 2168$), eGFR < 60 ml/min/1.73 m^2 or missing ($n = 3,536$), and those with other missing values for covariates, a total of 26,778 participants were ultimately included for further analysis. In the overall population, the prevalence of hypertension was 49.6%, the median age was 46 (33–60) years, and the median total calcium was 9.4 mg/dl. There were significant differences between the hypertensive and non-hypertensive groups in age, sex, race/ethnicity, education levels, triglycerides, total cholesterol, eGFR, total calcium, diabetes mellitus, BMI, drinking, and smoking (all $P < 0.05$). Hypertensive participants tended to have higher level of triglycerides, cholesterol, and BMI, lower eGFR, more probability of co-existed diabetes mellitus, smoking, and drinking behaviors than non-hypertensive participants. The general baseline characteristics of all subjects in this study are presented in **Table 1**.

Association of Calcium With Hypertension

The results of multivariable logistic regression analysis are reported in **Table 2**. When treating total serum calcium as a continuous variable, the increase in serum total calcium showed a positive association with the prevalence of hypertension in all three regression models. When fully adjusted for age, gender, race/ethnicity, education levels, BMI, diabetes history, smoking status, alcohol consumption, sodium intake, triglycerides, total cholesterol levels, albumin, eGFR, and serum phosphorus, increased calcium was significantly associated with the prevalence of hypertension (OR, 1.438; 95%CI, 1.306–1.583). When using the lowest quartile of total calcium as a reference, individuals in the second to the fourth quartile had a higher risk of hypertension after fully adjusting for potential confounding factors. The ORs with 95% CIs for hypertension across increasing quartiles were 1.088 (1.008, 1.175), 1.228 (1.129, 1.336), and 1.432 (1.309, 1.567) in fully adjusted model.

In **Figure 1**, we used restricted cubic spline to assess the relationship of total calcium and hypertension, and an S-shaped correlation was displayed. When setting the median calcium level as a reference, the prevalence of hypertension was increased in individuals with a higher serum total calcium.

Additionally, we performed Spearman correlation analysis to assess the association between varieties and systolic/diastolic blood pressure. To eliminate possible effects of anti-hypertensive medications, Spearman correlation analysis was performed, respectively. As shown in **Figure 2**, blood pressure was positively associated with BMI, triglycerides, and cholesterol. However, no direct correlation was found between total calcium and systolic pressure/diastolic pressure both in participants with and without anti-hypertensive medications.

Receiver operating characteristic curve was drawn to show identification ability of calcium in patients with hypertension. As shown in **Supplementary Figure 1**, serum calcium showed a poor identifying performance of the prevalence of hypertension, with an AUC of 0.53, sensitivity of 0.23, specificity of 0.81, positive predictive value of 0.55, and negative predictive value of 0.52 evaluated by the testing set.

Subgroup Analysis

Subgroup analysis was subsequently performed based on categories of sex, age, race, BMI, eGFR. The association between serum calcium and hypertension remained significant across categories of sex, age, race, BMI, and eGFR. As shown in the forest plot (**Figure 3**), patients who were female, older, Non-Hispanic, other Hispanic, or multiracial, had a higher BMI were more disposed to hypertension.

To explore gender difference in the association of calcium with hypertension, we performed a subgroup analysis by sex and age (**Table 3**). Total serum calcium was found to be positively correlated to the prevalence of hypertension in both genders aged over 40. Conversely, there was no significant association of calcium with hypertension in young participants. When quartering total serum calcium level and using the lowest quartile of total calcium as a reference, a robust trend of greater prevalence of hypertension was observed with the increase in total serum calcium from the second to the fourth quartile among all age categories. Interestingly, the association of calcium with hypertension seemed to be stronger among females aged 40–60 compared with males at the same age. The ORs with 95% CIs for hypertension was 1.557 (1.282–1.894) for females and 1.24 (1.005–1.53) for males aged 40–60, respectively.

Sensitivity Analysis

When the cut-off value was set as 130/80 mmHg, the prevalence of hypertension was 49.9%. Similarly, the ORs with 95% CIs for hypertension was 1.36 (1.26–1.468) in crude model (model 1), 1.544 (1.421–1.678) in model 2, and 1.457 (1.323–1.605) in model 3, respectively.

DISCUSSION

In our large, cross-sectional study among US adults, we observed a significant positive association of serum calcium level with the prevalence of hypertension. The association was independent of age, gender, race/ethnicity, education levels, BMI, diabetes history, smoking status, alcohol consumption, sodium intake,

TABLE 1 | Baseline characteristics of study population.

	Overall	Non-hypertensive	Hypertensive	*P*
Number	26,778	13,495	13,283	
Age	46.0 [33.0, 60.0]	38.0 [28.0, 50.0]	55.0 [43.0, 64.0]	<0.001
Gender (Female/Male)	13,526/13,252 (50.5/49.5)	7,325/6,170 (54.3/45.7)	6,201/7,082 (46.7/53.3)	<0.001
Race (%)				<0.001
Non-Hispanic White	10,483 (39.1)	5,328 (39.5)	5,155 (38.8)	
Non-Hispanic Black	5,524 (20.6)	2,200 (16.3)	3,324 (25.0)	
Mexican American	4,367 (16.3)	2,421 (17.9)	1,946 (14.7)	
Other Hispanic	3,005 (11.2)	1,605 (11.9)	1,400 (10.5)	
Other Races	3,399 (12.7)	1,941 (14.4)	1,458 (11.0)	
Education (%)				<0.001
Below high school	6,330 (23.6)	2,905 (21.5)	3,425 (25.8)	
High school	6,026 (22.5)	2,874 (21.3)	3,152 (23.7)	
Above high school	14,422 (53.9)	7,716 (57.2)	6,706 (50.5)	
Triglycerides (mg/dl)	121.0 [80.0, 188.0]	106.0 [71.0, 164.0]	138.0 [92.0, 210.0]	<0.001
Cholesterol (mg/dl)	190.0 [164.0, 218.0]	187.0 [163.0, 213.0]	194.0 [167.0, 223.0]	<0.001
eGFR (ml/min/1.73 m^2)	114.2 [91.4, 143.1]	118.7 [97.2, 145.0]	109.2 [86.1, 140.6]	<0.001
Total calcium (mg/dl)	9.4 [9.2, 9.6]	9.4 [9.1, 9.6]	9.4 [9.2, 9.6]	<0.001
Diabetes = No/Yes (%)	23,629/3,149 (88.2/11.8)	12,838/657 (95.1/4.9)	10,791/2,492 (81.2/18.8)	<0.001
BMI (kg/m^2)	28.5 [24.7, 33.2]	26.7 [23.4, 30.9]	30.3 [26.5, 35.1]	<0.001
Drinking = No/Yes (%)	24,109/2,669 (90.0/10.0)	12,272/1,223 (90.9/9.1)	11,837/1,446 (89.1/10.9)	<0.001
Smoking = No/Yes (%)	15,028/11,750 (56.1/43.9)	8,136/5,359 (60.3/39.7)	6,892/6,391 (51.9/48.1)	<0.001

BMI, body mass index; eGFR, estimated glomerular filtration rate. Data are presented as percentages for categorical variables or median with interquartile range for continuous variables with skewed distribution.

TABLE 2 | Association of calcium with hypertension.

	Crude model (Model 1)		Model 2		Model 3	
	Odds ratio	*P*-value	Odds ratio	*P*-value	Odds ratio	*P*-value
Total calcium	1.347 (1.249–1.454)	<0.001	1.522(1.401–1.654)	<0.001	1.438 (1.306–1.583)	<0.001
Q1	Ref.		Ref.		Ref.	
Q2	1.054 (0.982–1.132)	0.145	1.112 (1.032–1.198)	0.005	1.088 (1.008–1.175)	0.03
Q3	1.164 (1.08–1.254)	<0.001	1.271 (1.175–1.376)	<0.001	1.228 (1.129–1.336)	<0.001
Q4	1.361 (1.263–1.466)	<0.001	1.506 (1.39–1.632)	<0.001	1.432 (1.309–1.567)	<0.001

Crude model (Model 1): We adjusted for age, and gender. Model 2: We adjusted for age, gender, race/ethnicity, education levels, BMI, diabetes history, smoking status, alcohol consumption, sodium intake, triglycerides, and total cholesterol levels. Model 3: We adjusted for age, gender, race/ethnicity, education levels, BMI, diabetes history, smoking status, alcohol consumption, sodium intake, triglycerides, total cholesterol levels, albumin, eGFR, and serum phosphorus.

triglycerides, total cholesterol levels, albumin, eGFR, and serum phosphorus. When serum calcium level was analyzed as a categorical variable, the prevalence of hypertension for subjects in the highest calcium quartile was 1.45 times that of those in the lowest quartile. The association was robust in further subgroup analysis stratified by sex, age, race, BMI, and eGFR.

The relation between calcium intake and hypertension has long been a subject of debate since the 1980s. Most previous studies assessing the effects of calcium on blood pressure established a statistical correlation between low calcium level and a higher risk of elevated blood pressure (25–27). Moreover, numerous randomized controlled trials of calcium and vitamin D supplementation corroborated the antihypertensive role of calcium. Meta-analyses of randomized controlled trials demonstrated that calcium supplementation could decrease systolic blood pressure, with a mean difference of 2.5 mmHg (95% CI = 0.6–4.5) and 1.4 mmHg (95% CI= 0.72–2.15) in hypertensive and normotensive individuals, respectively (28, 29). Possible mechanisms may be that low plasmatic calcium concentration could stimulate the release of parathyroid hormone (PTH) (30) and parathyroid hypertensive factor (31), foster the synthesis of calcitriol (32), and activate RAAS system (33), which in return increase intracellular calcium concentration and lead to vasoconstriction, vascular resistance, and high blood pressure (34). Apart from this, both angiotensin II and PTH could provoke aldosterone secretion from adrenal gland (35), which could upregulate epithelial sodium channels (ENaC) in the principal cells of the collecting duct in

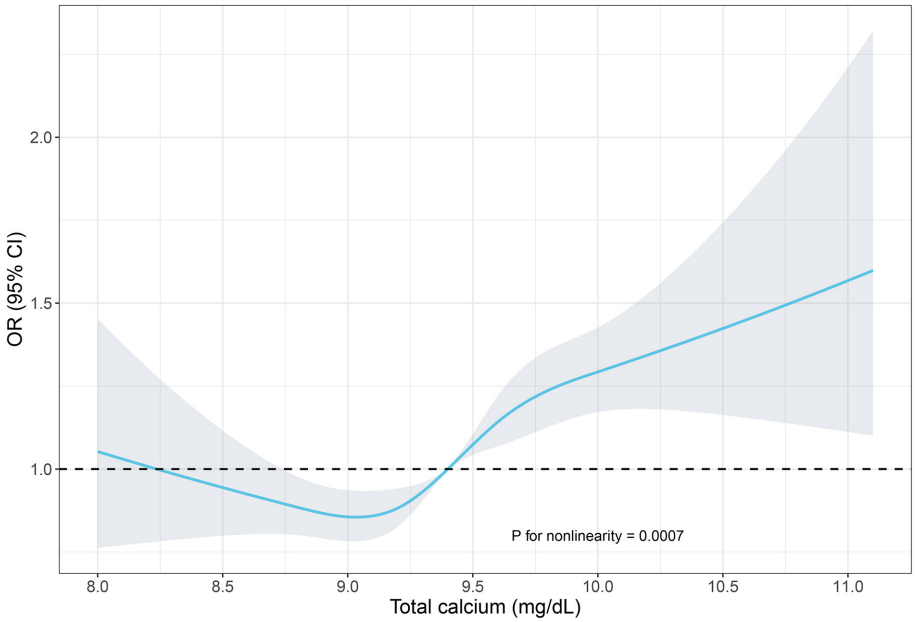

FIGURE 1 | Restricted cubic spline of the association between serum total calcium and hypertension. The association was adjusted for age, gender, race/ethnicity, education levels, BMI, diabetes history, smoking status, alcohol consumption, sodium intake, triglycerides, total cholesterol levels, albumin, eGFR, and serum phosphorus. The median total calcium (9.4 mg/dl) was chosen as a reference. The plot showed a reduction of the risk within the lower range of serum calcium, which reached the lowest risk around 9.1 mg/dl and then increased thereafter. OR, odds ratio; CI, confidence intervals.

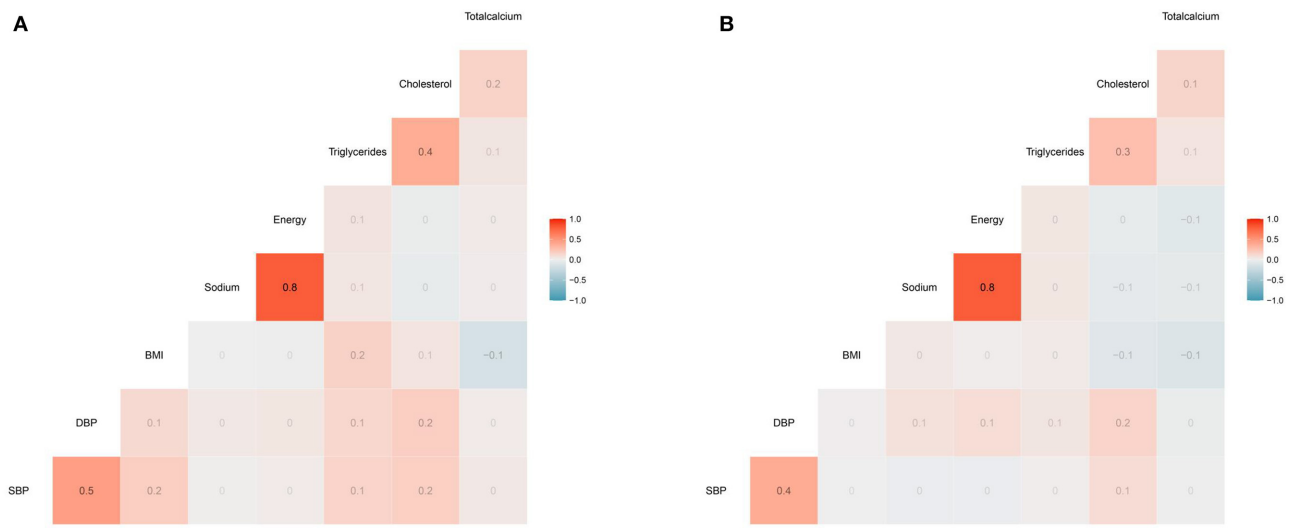

FIGURE 2 | The heatmap of the correlation between covariates and SBP/DBP using the Spearman correlation analysis among participants **(A)** with anti-hypertensive medications **(B)** without anti-hypertensive medications. No direct correlation was found between total calcium and systolic pressure/diastolic pressure both in participants with and without anti-hypertensive medications. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index.

the kidney, increase apical membrane permeability for Na+, facilitate Na+, and water reabsorption and thus, increase blood pressure (36). Collectively, the calcium deficiency hypothesis of hypertension populated and calcium supplementation was advocated in lowering blood pressure and delaying the onset of hypertension.

Nevertheless, some critics on the calcium deficiency hypothesis of hypertension arose with time, and the most quoted query came from Kaplan et al. (7). Kaplan stated that the theoretical construct was based on inconclusive extrapolation of experimental evidence obtained from epidemiologic, biochemical, and hemodynamic. Calcium

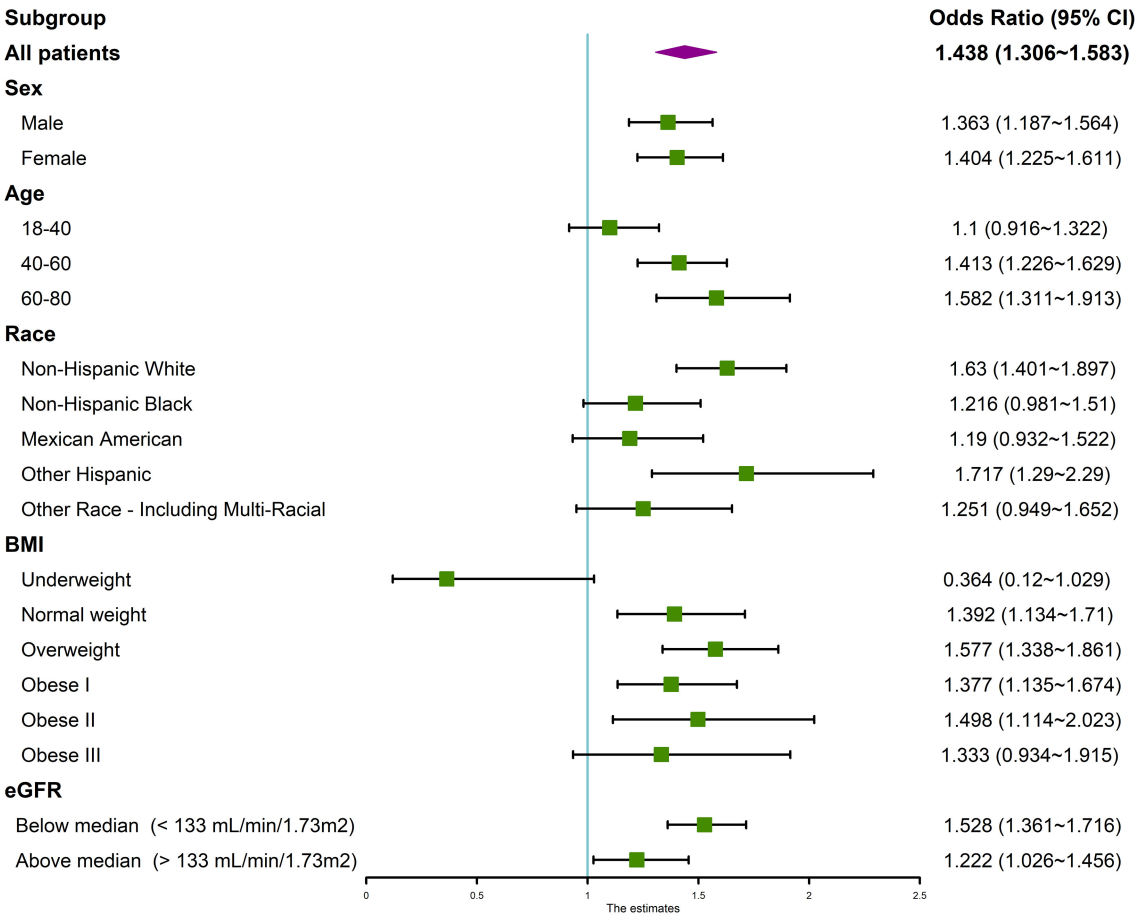

FIGURE 3 | Forest plot of subgroup analysis of the association between serum calcium and hypertension. We used the ORs to evaluate the association between per 1 mg/dl increase in serum calcium with the prevalence of hypertension in the subgroup of sex, age, race, BMI, and eGFR. The association between serum calcium and hypertension remained significant across categories. The association was adjusted for age, gender, race/ethnicity, education levels, BMI, diabetes history, smoking status, alcohol consumption, sodium intake, triglycerides, total cholesterol levels, albumin, eGFR, and serum phosphorus. OR, odds ratio; CI, confidence intervals.

TABLE 3 | Association of calcium with hypertension by sex and age using fully adjusted model.

		Model 3 odds ratios (ORs) with 95% confidence intervals (CIs)			
	Total calcium	**Q1**	**Q2**	**Q3**	**Q4**
Male	1.363 (1.187–1.564)				
18–40	1.157 (0.905–1.48)	Ref.	0.968 (0.795–1.177)	0.904 (0.739–1.106)	1.076 (0.872–1.328)
40–60	1.24 (1.005–1.53)	Ref.	1.04 (0.885–1.223)	1.133 (0.949–1.354)	1.303 (1.073–1.583)
60–80	1.598 (1.218–2.102)	Ref.	1.197 (0.969–1.481)	1.495 (1.179–1.903)	1.594 (1.23–2.074)
Female	1.404 (1.225–1.611)				
18–40	0.985 (0.745–1.304)	Ref.	0.981 (0.806–1.193)	1.028 (0.813–1.295)	1.144 (0.873–1.494)
40–60	1.557 (1.282–1.894)	Ref.	1.051 (0.897–1.232)	1.248 (1.041–1.497)	1.547 (1.277–1.875)
60–80	1.579 (1.213–2.062)	Ref.	1.207 (0.952–1.534)	1.541 (1.196–1.991)	1.528 (1.188–1.97)

Model 3: We adjusted for age, gender, race/ethnicity, education levels, BMI, diabetes history, smoking status, alcohol consumption, sodium intake, triglycerides, total cholesterol levels, albumin, eGFR, and serum phosphorus.

deficiency should not be accepted as a mechanism responsible for hypertension, whereas excessive calcium or vitamin D supplementation should be used with caution. With the improvement of experimental design, expansion of sample size, and enhanced control of heterogeneity between trials, the role of calcium in the prevention and treatment of hypertension

has again been controversial, and no specific consensus has been reached.

Our research was consistent with several previous studies examining the association of serum calcium and hypertension (5, 37–40). Charumathi and his associates documented that elevated serum total calcium levels were positively associated with hypertension after adjustments of serum albumin, 25(OH)D, serum phosphorus, and other confounders in a cross-sectional study of 12,405 US adults. Similarly, a study in the rural area of Northeast China aimed to assess the association of serum calcium and hypertension among adolescents aged 12–17 years showed that the multivariable ORs of hypertension among adolescents with serum calcium levels ≥2.53 mmol/L in comparison with serum calcium levels ≤2.37 mmol/L was 1.89 (1.41–2.53; P <0.001) (5). Moreover, higher serum calcium levels were also positively associated with an average increase in SBP [4.22 (2.74–5.83; $P < 0.001$)] and DBP [2.23 (1.00–3.46; $P < 0.001$)], respectively. Kesteloot and his colleagues demonstrated that higher serum total calcium was positively associated with hypertension in both men and women after adjusting for serum creatinine and other confounders in a cross-sectional study of 4,167 men and 3,891 women in Belgium (39). Several longitudinal researches have also observed this association. XiaoYan Wu et al. conducted a prospective population-based study including 8,653 subjects with an average follow-up of 5.3 years in China (38). The results showed that the odd ratios for incident hypertension had a consistently increasing trend with increasing serum calcium concentration quartiles, indicating serum calcium level a significant risk of hypertension [1.37 (1.10, 1.70); 1.45 (1.17, 1.81); 2.18 (1.77, 2.68) for quartile 2, 3, and 4; quartile 1 set as a reference]. Cheng-Wai Chou and coworkers performed both cross-sectional analysis and longitudinal analysis among 27,364 community-dwelling participants in Taiwan during the period 2010–2016. They suggested that both serum calcium or albumin-corrected calcium was associated with an increased risk of hypertension (40).

Contrary to the positive correlation between serum calcium and hypertension at the clinical level, discrimination analysis using ROC curve failed to show a good predictive performance of hypertension by serum calcium with an AUC of 0.53 evaluated in the testing set. Interestingly, a satisfactory specificity of 0.81 was found, indicating that high serum calcium level might be a prominent phenotype of hypertension. Similarly, there was no apparent correlation witnessed by Spearman correlation analysis. As a matter of fact, hypertension is a long-term, systematic result of multiple factors, including genetic susceptibility, external environmental interference, sympathetic nervous system, hormones change, vascular abnormalities, etc. (41). So far, no single determining factor or biomarker of hypertension have been found (42). The multifactorial nature of pathogenesis of hypertension as well as lack of adjusted covariates in statistical analysis might partly explain for such confounding findings.

The mechanisms underlying the observed association between serum calcium and hypertension remain uncertain. Serum calcium may directly affect vasoconstriction by the influx of calcium into the smooth muscle of the artery, which enhances muscle contracture, increases vascular resistance, and therefore, leads to the development of hypertension (43). Additionally, calcium could indirectly promote hypertension by alteration in the extracellular binding of calcium (44), inducing insulin resistance (38), and interacting with other cations, especially sodium and potassium (45). Another possible mechanism lies in that subtle alterations in intracellular calcium may affect the secretion and action of hormones, such as the pressor action of catecholamines, angiotensin II, or aldosterone, which may target the blood pressure control centers and increase blood pressure levels (6). Moreover, calcium could mediate PTH release and several previous studies have found a positive correlation between PTH concentration and blood pressure (46, 47).

In our study, we observed a slightly stronger association between calcium and the prevalence of hypertension in females than males. A subsequent logistic regression analysis by sex and age found that the OR for hypertension was much greater in females aged 40–60 compared to males at the same age, which might explain for overall gender variance. To date, there was no report of such interesting findings in previous large, cross-sectional study. When we try to understand gender variance in the association between calcium and the prevalence of hypertension in middle-aged participants, we may inevitably encounter the influence of menopause in women. Menopause is a unique and natural process in women's lives during which sex hormones fluctuate and vast changes in metabolic state occur. It has been well-confirmed by epidemiological data that women had an obvious increase in blood pressure during menopause vs, age-matched men, which contributed to a greater prevalence of hypertension among women aged over 65 years in the US (48). The reduction of estrogen, a protective anti-hypertensive factor, may reduce the release of nitric oxide (NO), promote vascular remodeling process, increase endothelial dysfunction, and lead to the progression of hypertension (49, 50). It is reasonable to speculate that the metabolic state disparity between the two genders during such unique process of women would lead to variance in the association between calcium and hypertension. Although the beneficial role of adequate calcium has been addressed regarding bone metabolism, obesity, and even hypertension in middle-aged women (51, 52), a rising body of evidence linking calcium supplementation with adverse cardiovascular events such as coronary artery calcification, and cardiovascular mortality has risen to be a cause for concern (4). Our large, cross-sectional study suggested that total serum calcium might be a more significant risk factor for hypertension in middle-aged females. Given that calcium is only available to the body through dietary sources, cautions should be raised to blinded calcium supplementation especially in such populations. More specific basic and clinical researches are needed in the future.

There are several limitations to our study. First, though a large study sample was assessed, extensive international research composed of diverse ethnicities and regions was required to establish the association between calcium and hypertension better. Second, even if we have searched the

literature and tried our best to adjust for potential confounders, unknown and complex confounders may exist, such as plasma renin activity (53), aldosterone (54), and anti-hypertensive medications interfering with our results. Third, concentrations of total calcium might be influenced by nutrition intake or alterations in biological factors (55, 56). For instance, menopausal status, use of calcium supplements, and use of osteoporosis medications may affect serum calcium level and cause bias. In addition, calcium was measured at baseline or at the initiation of the observation period without taking the changes in concentration into consideration in our study. Last but not least, the cross-sectional nature of the study limits making causal inferences.

CONCLUSION

Serum total calcium levels were found to be positively associated with the prevalence of hypertension in a representative sample of US adults.

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

WS, Y-QX, and X-QK conceived and designed the study. J-YS and H-lL analyzed the data. YH wrote the paper. All authors provided critical revisions of the manuscript and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.719165/full#supplementary-material

Supplementary Figure 1 | The ROC curve of the model performance in the testing set. All individuals were randomly divided into a training set and testing set at a ratio of 7:3. We used the training set to create a predictive model, whereas the testing set was used to evaluate the model performance. Serum calcium showed a poor identifying performance of the prevalence of hypertension, with an AUC of 0.53, sensitivity of 0.23, specificity of 0.81, positive predictive value of 0.55, and negative predictive value of 0.52. AUC: area under the curve.

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REVIEW
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Iron in Cardiovascular Disease: Challenges and Potentials

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Iron is essential for many biological processes. Inadequate or excess amount of body iron can result in various pathological consequences. The pathological roles of iron in cardiovascular disease (CVD) have been intensively studied for decades. Convincing data demonstrated a detrimental effect of iron deficiency in patients with heart failure and pulmonary arterial hypertension, but it remains unclear for the pathological roles of iron in other cardiovascular diseases. Meanwhile, ferroptosis is an iron-dependent cell death that is distinct from apoptosis, necroptosis, and other types of cell death. Ferroptosis has been reported in several CVDs, namely, cardiomyopathy, atherosclerotic cardiovascular disease, and myocardial ischemia/reperfusion injury. Iron chelation therapy seems to be an available strategy to ameliorate iron overload-related disorders. It is still a challenge to accurately clarify the pathological roles of iron in CVD and search for effective medical intervention. In this review, we aim to summarize the pathological roles of iron in CVD, and especially highlight the potential mechanism of ferroptosis in these diseases.

Keywords: iron, cardiovascular disease, iron overload, ferroptosis, iron chelation

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INTRODUCTION

Iron is an essential mineral nutrient involved in numerous biologic processes, namely, heme synthesis, iron-dependent catalytic reaction, DNA synthesis, and mitochondrial respiration (1). Iron metabolism is complex and has received much attention. Iron homeostasis is maintained by elaborate mechanisms involving iron consumption, uptake, transfer, and storage (1). Inappropriate iron overload or deficiency correlates to a wide range of cardiovascular diseases (CVDs). Iron deficiency can impair cardiomyocyte mitochondrial function and energy supplement, leading to cardiac dysfunction (2). An excess amount of iron can also be toxic by producing hydroxyl radicals *via* the Haber–Weiss–Fenton reactions, causing oxidative damage to cellular components like lipids, proteins, and DNA (3). Moreover, iron-mediated cell death, namely, ferroptosis, has recently been reported to induce cardiomyocyte damage and plays an important role in CVD (4).

In 1981, Jerome Sullivan proposed the iron hypothesis to explain the sex differences in the risk of heart disease and the lower incidence of CVD in premenopausal women (5). Since then, it has been investigated for decades to untangle the connection of iron–heart diseases. Numerous epidemiological studies have indicated that body iron levels are associated with CVD. To date, it is definite that iron deficiency is prevalent in patients with heart failure (HF) or pulmonary arterial hypertension (PAH), and intravenous iron supplements can improve the quality of life of these patients and reduce the associated risk of hospitalization (2, 6). However, the clinical observation regarding the iron status and atherosclerotic cardiovascular disease (ASCVD) is still controversial despite some studies supporting that elevated iron store positively correlates with the incidence of coronary artery disease (CAD). Besides, iron status in other CVD remains unclear and it is a challenge to clarify their relation.

The precise mechanisms of iron homeostasis in the cardiovascular system are distinct and complicated. In this review, we aim to summarize the pathological roles of iron in CVD, and especially highlight the potential mechanism of ferroptosis in these diseases.

IRON HOMEOSTATIC REGULATION: THE BASICS

Systemic Iron Homeostasis

The adult body contains about 3–5 g of total iron, with two-thirds in the form of hemoglobin and myoglobin. The rest of iron is almost bound to ferritin, a specialized cytoplasmic iron storage protein. Only about 0.1% of total body iron constitutes extracellular iron. Normally, senescent or damaged erythrocytes are phagocytized by macrophages in the spleen and other organs and release their iron into circulation, which can be recycled for heme synthesis in the bone marrow (7). Except for cyclic utilization of erythrocyte iron, dietary iron intake is an important way for the iron supply to replenish body iron loss *via* gut mucosa (8).

Dietary iron is absorbed by gut mucosa cells *via* two distinct mechanisms based on heme and inorganic forms of iron (9). The heme form of iron is absorbed in the apical membrane of epithelial cells *via* a specific heme transporter, heme carrier protein 1 (HCP1). The heme can be degraded by heme oxygenase-1 (HO-1) to release ferrous iron (Fe^{2+}), carbon monoxide, and biliverdin. The absorption of inorganic ferric ion (Fe^{3+}) requires two key steps: the conversion of insoluble Fe^{3+} to absorbable Fe^{2+} by cytochrome b reductase 1 (DCYTB) and following transportation of Fe^{2+} by divalent metal transporter protein 1 (DMT-1) across the membrane. Internalized Fe^{2+} enters the cytosolic labile iron pool (LIP). Extra iron can be stored as ferritin or exported through the basolateral membrane by ferroportin (FPN), the only known iron export protein. The exported Fe^{2+} is undergone re-oxidation to Fe^{3+} by membrane-bound hephaestin and binds to transferrin (Tf) for long-distance delivery. Circulating Tf-bound iron can be internalized into the cells of peripheral tissues *via* binding to its receptor, transferrin receptor 1 (TfR1) (10).

Circulating iron levels are predominantly regulated by the transmembrane protein FPN. Hepcidin is a peptide hormone released mainly by the liver, effectively preventing cellular iron efflux *via* promoting FPN internalization and degradation. When hepcidin is transcriptionally downregulated in the condition of enhanced erythropoiesis or iron deficiency, more iron is released into circulation from intestinal epithelial cells, macrophages, and hepatocytes (7). High levels of serum iron and chronic inflammatory states can lead to increased levels of hepcidin. The hepcidin-FPN axis tightly regulates systemic iron homeostasis to meet body requirements (7).

Cellular Iron Metabolism

Iron homeostasis in the body is regulated at both systemic and cellular levels. The Tf-bound iron binds to TfR1 and is internalized by endocytosis, while the uptake of non-Tf-bound iron (NTBI) is mediated by DMT-1 protein ubiquitously present on the surface of cells (11, 12). In addition, the voltage-gated calcium channels of cardiomyocytes can also be iron transporters for NTBI under iron overload conditions (13). After absorption, iron enters the redox-active LIP, where it is utilized for storage in ferritin, or incorporation into iron-require proteins, or trafficking to mitochondria for the synthesis of heme and iron-sulfur (Fe-S) clusters (14) (**Figure 1**).

Cellular iron homeostasis is post-transcriptionally regulated by the iron regulatory proteins (IRP1 and IRP2) interacting with iron-responsive elements (IREs) (11). IREs are highly conserved hairpin structures of mRNAs present in 5′ or 3′ untranslated regions (UTRs) of iron metabolism genes. IRPs inhibit the initiation of translation by binding to the single 5′-UTR IREs of ferritin and FPN, whereas their binding to the multiple IRE motifs within the 3′-UTR of TFR1 and DMT1 prevents mRNAs degradation (15). The capability of IRPs binding to IRE depends on intracellular iron concentration. In iron-replete cells, IRP1 ligates the Fe-S cluster and functions as a cytosolic aconitase, which precludes IRP-IRE interaction to increase ferritin and TfR1 proteins. In the low intracellular iron environment, IRPs stabilize the mRNA of TfR1 and DMT-1 to enhance iron uptake and inhibit iron excretion by suppressing the translation of FPN (16, 17).

The hepcidin-FPN1 axis also plays a pivotal role in controlling cellular iron flux, particularly in cardiomyocytes. Distinct from systemic iron regulation, hepcidin can be produced locally in the heart, and functions as an autocrine protein to regulate iron levels in cardiomyocytes (18). Interestingly, cardiac hepcidin is upregulated in response to hypoxia to retain cellular iron, while systemic hepcidin is downregulated (19). Such regulation may be an adaptive mechanism to maintain cardiac function.

IRON AND FERROPTOSIS

Iron Metabolism and Ferroptosis

Ferroptosis is a novel form of regulated cell death driven by iron-dependent lipid peroxidation. It is distinct from other types of regulated cell death (**Table 1**), which can be suppressed by iron chelators (e.g., deferoxamine) (20). In the process of ferroptosis, reactive oxygen species (ROS) are overproduced by accumulated intracellular iron, and extensive oxidation of polyunsaturated fatty acid is triggered, resulting in the damage of cellular membrane structure and cell death (20). Thus, modulation of iron metabolism-related genes may regulate ferroptosis by affecting cellular iron homeostasis. The nuclear receptor coactivator 4 (NCOA4) is a selective cargo receptor for the autophagic degradation of ferritin, namely, ferritinophagy, which can increase intracellular iron and induce ferroptotic cell death (21). Overexpression of ferritin heavy chain 1 impaired ferritinophagy and inhibited ferroptosis in PC-12 cells (22). Senescent cells with impaired ferritinophagy were more resistant to ferroptosis (23). Moreover, blockade of cellular iron export *via* genetically deleting FPN has been reported to develop morphological and molecular features of ferroptosis in hippocampus neurons (24). The overexpression of FPN in neurons could alleviate neuronal apoptosis and ferroptosis after

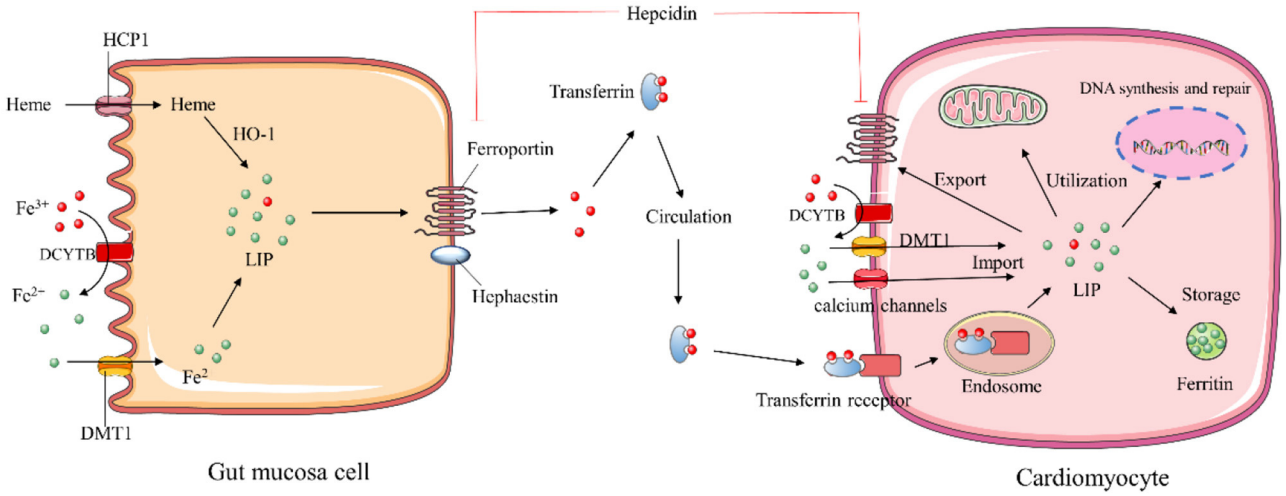

FIGURE 1 | Schematic description of iron homeostasis. HCP1, heme carrier protein 1; HO-1, heme oxygenase 1; DMT-1, divalent metal transporter protein 1; DCYTB, cytochrome b reductase; LIP, labile iron pool.

intracerebral hemorrhage (25). Taken together, the modulation of cellular iron metabolism might provide a novel therapeutic target for ferroptosis-associated disease.

Regulatory Pathways of Ferroptosis

Current knowledge indicates two major pathways, the glutathione peroxidase 4 (GPX4)-glutathione (GSH) and ferroptosis suppressor protein 1 (FSP1)-coenzyme Q (CoQ) pathways, involved in the regulation of ferroptosis (26, 27) (**Figure 2**).

Glutathione peroxidase 4 is a selenocysteine-containing, GSH-dependent enzyme capable of catalyzing the reduction of lipid hydroperoxides (26). Genetic manipulation studies revealed that constitutive deletion or inactive mutant of GPX4 leads to early embryonic lethality. As a result, conditional GPX4 knockout mice were generated to study the mechanisms of GPX4 deficiency-induced cell death. The inducible ablation of GPX4 causes mitochondrial damage and lipid peroxidation-mediated ferroptosis event (28). Thus, several ferroptosis inducers, such as Ras-selective lethal 3, can trigger the accumulation of lipid hydroperoxides and result in cell death by directly inhibiting GPX4 (29). Conversely, overexpression of GPX4 has been reported to protect against oxidative injury in various cell types (30). *ApoE*$^{-/-}$ mice overexpressing GPX4 showed decreased oxidized lipids and atherosclerotic lesions in the aorta compared with *ApoE*$^{-/-}$ control mice (31). Overexpression of mitochondrial GPX4 can also protect ischemia/reperfusion (I/R)-induced cardiac injury (32).

GSH is synthesized from glutamate, cysteine, and glycine in two steps under the catalysis of the cytosolic enzymes glutamate-cysteine ligase and glutathione synthetase, participating in the regulation of ferroptosis (33). Cysteine is the most limiting amino acid for GSH synthesis. Inhibiting its import through the system Xc^- is sufficient to trigger ferroptosis *in vitro* (34). System Xc^- is a cystine/glutamate antiporter that facilitates the exchange of cystine and glutamate across the plasma membrane (34). Thus, inhibition of system Xc^- can lead to deprivation of cellular GSH and impair the function of GPX4 to suppress lipid peroxidation and ferroptosis.

Recent evidence indicates that the FSP1-CoQ10 pathway co-operates with GPX4 and GSH to suppress phospholipid peroxidation and ferroptosis, as a stand-alone parallel system. FSP1, also called apoptosis-inducing factor mitochondrial 2 (AIFM2), was predicted to induce apoptosis by a caspase-1 independent pathway, due to its biochemical similarities to AIFM1 (35). However, FSP1 is recruited to the plasma membrane by myristoylation, where it functions as an oxidoreductase that catalyzes the regeneration coenzyme Q10 (CoQ10) (35), instead of inducing apoptosis. Ubiquinol is the reduced form of CoQ10 generated by the mevalonate pathway. It can act as a lipophilic radical-trapping antioxidant to regulate ferroptosis by halting the propagation of lipid peroxides (35).

Ferroptosis and Cardiomyopathy

Although the physiological action of ferroptosis remains elusive, it has been studied in several cardiomyopathies, namely, diabetic cardiomyopathy and doxorubicin (DOX)-induced cardiotoxicity.

Diabetic cardiomyopathy, characterized by cardiac hypertrophy, diastolic dysfunction, and intracellular lipid accumulation, is the common complication of diabetes (36). It has been reported that GPX4 expression was reduced in both high glucose-treated cardiomyocytes and the left ventricular myocardial tissues of *db/db* mice (37). A recent study found that inhibition of cardiac autophagy could activate nuclear factor E2-related factor 2 (Nrf2)-mediated ferroptosis and lead to myocardial damage in type 1 diabetic mice (38).

DOX is a second-generation anthracycline chemotherapeutic drug used in many malignancies. It often causes cardiotoxicity (39). In a mouse model of DOX-induced cardiomyopathy,

TABLE 1 | Hallmarks of major types of regulated cell death.

Type	Morphological changes	Cellular events	Major regulators	Trigger signals
Ferroptosis	Mitochondrial shrinkage and increased mitochondrial membrane density	Iron accumulation; lipid peroxidation; ROS accumulation	Positive: TFRC, LOXs, ACSL4, LPCAT3, ALOX15, GLS2, NCOA4, VDAC2/3, RAS, NOX, TfR1, TP53, GLS2s, BECN1 Negative: GPX4, FSP1, HSPB1/5, SLC7A11, NFS1	Iron overload, GSH depletion
Apoptosis	Cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation, formation of apoptotic bodies	Phosphatidylserine exposure; DNA fragment; Caspase activation; mitochondria transmembrane potential dissipation	Positive: initiator caspase (CASP2/8/910); effector caspase (CASP3/6/7); pro-apoptotic BCL2 family; TP53 Negative: anti-apoptotic BCL2 family	Death receptor activation
Autophagy	Double-membraned autolysosomes formation	LC3-I to LC3-II conversion; increased autophagic flux and lysosomal activity	Positive:ATG5/7, BECN1 and AMPK Negative: mTOR	Impaired organelles, oxidative stress
Pyroptosis	Lack of cell swelling, rupture of plasma membrane and unaffected mitochondrial integrity	Activation of CASP1 and GSDMD; GSDMDN–induced pore formation; IL-1β release	Positive: CASP1, CASP11, and GSDMD Negative: GPX4, ESCRT-III, PKA	Pathogenic microorganism infection, external stimuli
Necroptosis	Plasma membrane rupture, moderate chromatin condensation and cell swelling	RIPK1, RIPK3 and MLKL phosphorylation; DAMPs release	Positive: RIPK1/3, MLKL	Activation of TNF superfamily receptors

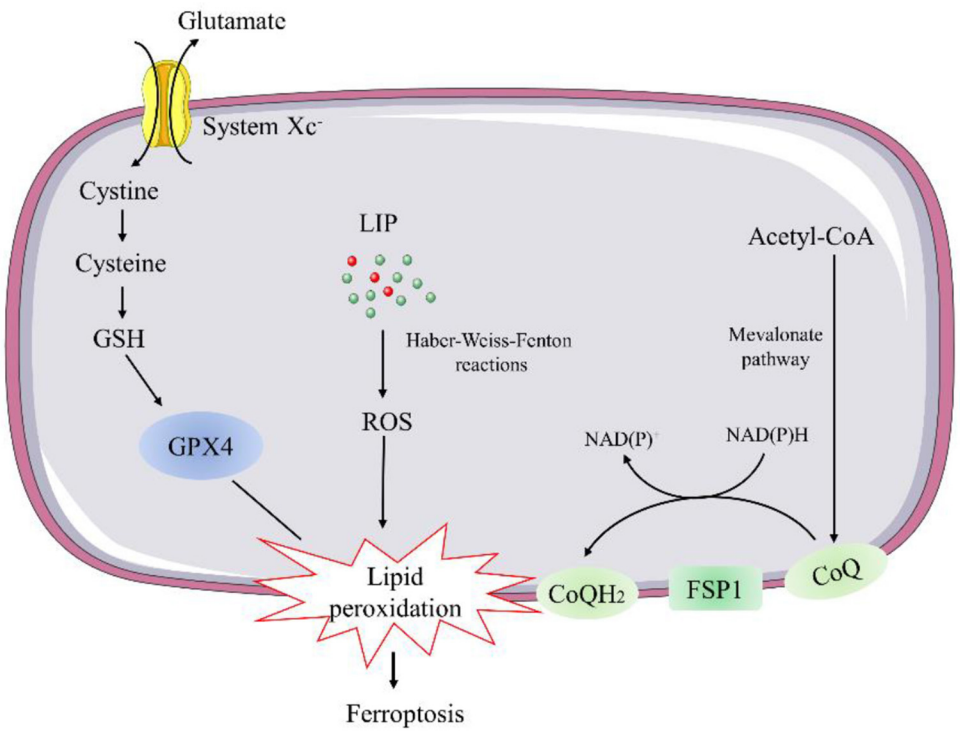

FIGURE 2 | Schematic description of regulatory pathways in ferroptosis. GSH, glutathione; GPX4, glutathione peroxidase 4; LIP, labile iron pool; FSP1, ferroptosis suppressor protein 1; CoQ, coenzyme Q.

inhibition of ferroptosis significantly improved cardiac function and reduced mortality, which was associated with the release of free cellular iron caused by HO-1 upregulation (40). DOX treatment could downregulate GPX4 and induce ferroptosis predominantly triggered in the mitochondria (41). Another study also proved that ferroptosis was involved in DOX-treated murine hearts, and acyl-CoA thioesterase 1, an important enzyme in fatty acid metabolism, might exert antiferroptosis effect in

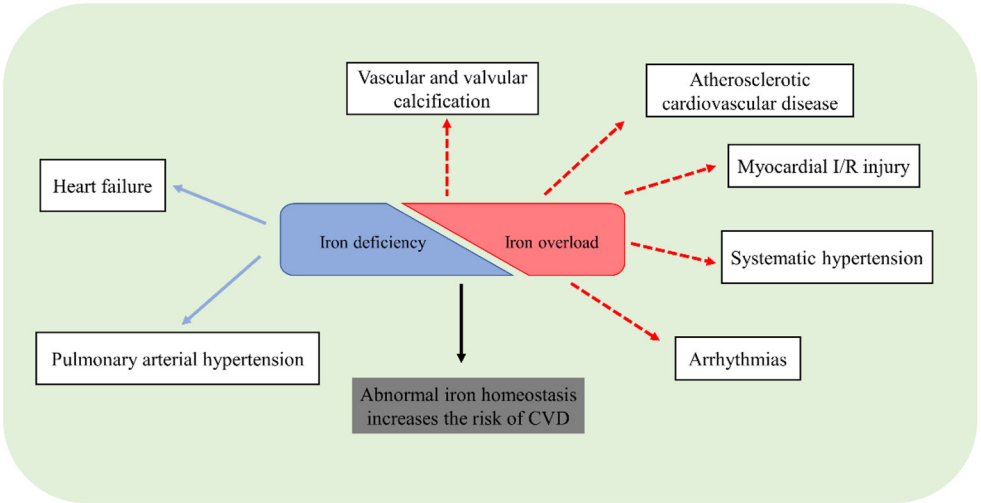

FIGURE 3 | Potential association of CVD and abnormal iron homeostasis.

DOX-induced cardiotoxicity (42). These studies highlight that ferroptosis played a crucial role in cardiomyopathy and might be a therapeutic target.

IRON AND CARDIOVASCULAR DISEASE

Role of Iron in Atherosclerotic Cardiovascular Disease

In 1981, Jerome Sullivan first proposed a hypothesis that the increased incidence of heart disease in men and post-menopausal women compared with premenopausal women could be explained by higher levels of body iron stores (5). Based on this hypothesis, numerous epidemiologic studies have investigated the role of iron in the pathogenesis of ASCVD.

The Kuopio Ischemic Heart Disease Risk Factor Study (KIHD) carried in eastern Finnish men demonstrated firstly that a high level of stored iron, assessed by increased serum ferritin, is a risk factor for myocardial infarction (MI) (43). Iron deposition was detected in coronary plaques associated with an increase of cholesterol levels in patients with atherosclerotic lesions (44). Plaques of symptomatic patients also showed higher iron concentrations and risk of cap rupture compared with plaques of asymptomatic patients (45). Several clinical studies have revealed that iron chelation therapy is beneficial to patients with CAD (46, 47). However, a systematic review and meta-analysis containing 17 prospective studies showed that there was no significant association between serum ferritin, total iron-binding capacity, serum iron, and CAD/MI, while a significant negative association was identified between transferrin saturation and CAD/MI (48). This contradicted the hypothesis that higher body iron stores represented a possible risk factor in heart disease. It may attribute to the inconsistency of the evaluated markers of serum iron in those clinical studies, and the systematic and local iron levels cannot be accurately distinguished.

The mechanism whereby iron may stimulate atherogenesis has been intensively investigated. Plenty of studies have shown an association between iron overload and atherosclerosis (49). The pathological roles of iron in atherogenesis may largely rely on its catalytically active form to generate ROS and induce lipid-peroxidation (49). Within the atherosclerotic lesions, iron overload is presented in monocytes/macrophages, endothelial cells, vascular smooth muscle cells, and platelets that all participate in the process of atherosclerosis (50). Iron overload drives endothelial dysfunction through its pro-oxidant and proinflammatory effects in endothelial cells; and promotes proliferation, apoptosis, ROS production, and phenotypic switch in vascular smooth muscle cells (51, 52). By catalyzing the atherogenic modification of low-density lipoprotein, excess iron facilitates the conversion of macrophages into foam cells (52). A recent study has reported that iron overload also enhances glycolysis and inflammation in macrophages and exacerbates the severity of atherosclerosis (53).

During the development of atherosclerosis, lipid oxidative modification and iron deposition are well-observed in plaques. It is reasonable to speculate that ferroptosis may happen in this process. A study revealed the downregulation of GPX4 in heart tissue of MI mice using quantitative proteomic analysis (54). The exosome from human umbilical cord blood-derived mesenchymal stem cells has been reported to have cardioprotective effects on mouse models of MI by inhibiting ferroptosis through suppressing DMT1 expression (55). Iron chelation therapy using desferrioxamine (DFO) has been shown to inhibit atherosclerotic lesion development (56), suggesting that ferroptosis might participate in the process of myocardial ischemia. Therefore, targeting ferroptosis might be a precise therapy of ASCVD.

Role of Iron in Myocardial Ischemia/Reperfusion Injury

Myocardial ischemia/reperfusion (I/R) injury is an important complication of percutaneous coronary intervention or thrombolysis for acute MI (57). The recanalization of an obstructive coronary artery is effective to restore blood flow and rescue the ischemic zone, but paradoxically, reperfusion can also cause cardiac damage and necrosis due to the massive production of ROS (58). Accumulating evidence suggests that iron overload is implicated in the pathology of myocardial I/R injury (59, 60). Early studies supported that high levels of iron were mobilized into the coronary flow following prolonged ischemia, and cardiac cytosolic iron level augmented in rat hearts subjected to I/R (61, 62). A hereditary hemochromatosis model of *HFE* gene knockout mice subjected to I/R injury showed increased iron deposition, cardiomyocyte apoptosis, and ROS production compared with wild-type mice (63). Furthermore, elevated mitochondrial iron was observed in myocardial I/R injury mice and human cardiac tissue samples with ischemic cardiomyopathy, while pharmacological reduction of mitochondrial iron *in vivo* protects against I/R damage (64).

Myocardial I/R can activate hypoxia-inducible factor-1 signaling and increase TfR1 expression to facilitate iron uptake (65); upregulation of TfR1 expression in I/R-treated rat hearts was along with increased iron content (66). These illustrate that I/R can induce iron overload. Recent investigations have provided evidence that ferroptosis is involved in I/R-induced cardiomyocyte damage, and targeting ferroptosis might be beneficial for I/R conditions (40). Mitochondria-specific overexpression of GPX4 alleviates cardiac dysfunction following I/R (32). Inhibition of glutaminolysis, a component of the GSH generation pathway, can also attenuate I/R-associated heart injury by blocking ferroptosis (67). Cyanidin-3-glucoside, a subgroup of flavonoids, exhibits a protective effect in the rat model of myocardial I/R injuries *via* inhibiting USP19/Beclin1-mediated ferroptosis (68). Thus, targeting ferroptosis can serve as a potential strategy to prevent I/R-induced myocardial injury. Considerable efforts have been performed to ascertain whether iron depletion by using iron chelators could exert cardiac protection effects. In some animal models, iron chelation therapy improves contractile function, increases cell viability, attenuates cardiac remodeling, and reduces the size of infarction after I/R injury (40, 59). However, these results were not reproduced in some experimental animals (69, 70). A potential reason for the discrepancy may be species specificity. Further studies are needed to test the potential clinical implications of this therapeutic strategy.

Role of Iron in Heart Failure

The role of iron deficiency is highly pronounced and deeply investigated in HF patients (71). Iron deficiency occurs in about 50% of chronic HF patients and is independently associated with increased morbidity and mortality (72). There are several mechanisms to explain HF-associated iron deficiency like dietary nutritional deficiency, reduced absorption caused by gut edema or proton pump inhibitors use, and gastrointestinal blooding due to the use of antiplatelets and anticoagulant agents (72).

Iron content was significantly decreased in left ventricular tissues of human failing hearts compared to HF-free organ donors, independent of anemia (73). Moreover, cardiac iron deficiency in HF was accompanied by reduced activity of aconitase and citrate synthase and reduced expression of ROS-protective enzymes (catalase, glutathione peroxidase, and superoxide dismutase 2), indicating that myocardial iron deficiency may contribute to the exacerbation of mitochondrial dysfunction that exists in HF (73). These findings are consistent with the recent research of Hoes *et al.*, who demonstrated that energy production and contractile function are reduced in iron-deficient human cardiomyocytes (73). Cardiomyocytes with genetic deletion of TfR1 developed mitochondrial dysfunction, interrupted mitophagy, and promoted the metabolic switch to the fetal-like pattern (74). Thus, iron deficiency-induced mitochondrial dysfunction may reciprocally impair cellular energy supplement and cardiac function.

Because iron deficiency is associated with the pathophysiology of HF, iron repletion seems to be a therapeutic strategy for HF patients. Three main clinical trials (FAIR-HF, EFFECT-HF, and CONFIRM-HF) have proven that intravenous iron supplementation improves the quality of life, exercise tolerance, and reduces hospitalization risk for aggravated HF (75–77). Other smaller trials strengthened this evidence (78, 79). However, there is no significant clinical benefit from oral iron preparations in the IRONOUT-HF trial (80). This may be explained by the impaired iron absorption in HF (81). Although intravenous ferric carboxymaltose for symptomatic HFrEF patients with iron deficiency has been recommended in guidelines, there are several unsolved issues about iron repletion in this field. The long-term safety of intravenous iron supplementation in HF patients remains to be determined. The methods of iron administration and the potential side effects of iron should be fully considered due to iron overload-related oxidative damage.

Role of Iron in Calcific Vascular and Valvular Disease

Vascular and valvular calcification refers to ectopic mineralization in vessel walls and heart valve leaflets. It is an important risk factor for adverse cardiovascular events (82). Although there are differences in the morphology and structure of heart valves and vasculature, the biological characteristics of vascular and valve calcification are similar. An osteoblast-like phenotypic transition of VSMCs and valvular interstitial cells (VICs) contributes directly to ectopic calcium deposition, respectively (83, 84). Both the vascular and valvular calcifications are clinically associated with the presence of diabetes, smoking, hypertension, and dyslipidemia (84). Experimental evidence demonstrates that oxidative stress has been verified to participate in pathological vascular and valvular calcification for a long time (85–87). In this context, iron-triggering oxidative stress is rational and speculated to be involved in vascular and valvular calcification.

Valve calcification mainly occurs in aortic valves. VICs differentiate into the pathological myofibroblasts and osteoblast-like cells which promote inappropriate extracellular matrix remodeling and calcification (88). Previous studies have observed that intraleaflet hemorrhage is associated with the progression of valve calcification, and iron deposition is observed within calcific valves. Interestingly, iron deposition can also be detected in non-calcified valves, suggesting that iron deposition occurs before calcium deposition at the sites of valve calcification (89). Valvular iron accumulation was observed in human calcific aortic valves, positively correlating with the degree of calcification (90). Furthermore, differentiated VICs induced by tumor necrosis factor-α and transforming growth factor-β showed significantly decreased expression of iron-exporter FPN, and in VICs isolated from stenotic aortic valves. In the presence of ferrous sulfate, VICs expressed increased ferritin subunits and exhibited proliferation capacity (90).

Ectopic vascular calcification is generally located either in atherosclerotic intima or non-atherosclerotic tunica media (91). Intimal calcification is related to arterial obstruction and atherosclerotic plaque rupture. Medial calcification leads to vascular stiffness and elevated blood pressure and pulse pressure (92). Although vascular calcification has been noted as a degenerative aging process for decades, calcification in both intima and media layers is recognized as an actively regulated process driven partly by VSMCs (91, 93). It was reported that holo-transferrin iron could promote human aorta VSMCs calcification *via* upregulating interleukin-24 (94). Some circumstantial evidence supported the relationship between iron accumulation and atherosclerosis progression (95, 96). However, there were conflicting results that iron citrate could reduce high phosphate-induced calcium deposition in VSMCs by preventing apoptosis and inducing autophagy (97), and inhibit the osteochondrogenic shift in VSMCs (98). Moreover, ferritin heavy chain exerted inhibitory effects on vascular calcification due to ferroxidase activity and antioxidant properties (99).

Role of Iron in Pulmonary Arterial Hypertension and Systematic Hypertension

PAH is an abnormal hemodynamic state characterized by a sustained increase in pulmonary artery pressure (≥25 mmHg) and normal pulmonary capillary wedge pressure (≤15 mmHg) in the absence of other causes of precapillary pulmonary hypertension (100). PAH is classified into idiopathic, heritable, drugs and toxins-induced, and other origins (e.g., congenital heart disease, connective tissue disease, and chronic hemolytic anemias) (101). Clinical evidence supports that iron deficiency is prevalent and correlates with reduced exercise capacity and poor outcomes in both idiopathic and heritable PAH patients (101). Intravenous iron supplementation could improve quality of life and exercise endurance capacity in PAH patients with iron deficiency in two placebo-controlled studies (102, 103). To confirm the long-term effects of iron repletion, 117 PAH patients were recruited and received placebo or intravenous iron supplementation. Eighteen-month treatment with intravenous iron supplementation brought long-term clinical benefits, namely, improved risk status and reduced PAH-associated hospitalization (104). On the other hand, oral iron appeared ineffective because of impaired gastrointestinal iron absorption caused by upregulated hepcidin (105).

The underlying pathological mechanisms of iron deficiency to the PAH may involve hypoxia, inflammation, and functional alterations of pulmonary vascular cells. Hypoxia exposure can induce vasoconstriction in pulmonary arteries, resulting in increased pulmonary artery systolic pressure (PASP). Furthermore, hypoxia-induced vasoconstriction and PASP could be augmented by iron chelation with DFO in healthy adults (106). The pulmonary hypertensive response caused by altitude-induced hypoxia could be reversed by iron infusion, reducing PASP by 6 mmHg in the sea-level residents, whereas patients with chronic mountain sickness undergoing progressive iron discharge by venesection resulted in a 25% increase in PASP (107). It is reasonable to speculate that iron deficiency, analogous to hypoxia, can increase PASP, which may partly account for the pathogenesis of PAH.

It is well-known that the pathologic hallmarks of PAH contain sustained vasoconstriction, vascular remodeling, and perivascular inflammation. Since the VSMCs in pulmonary arteries are pivotal in controlling vasoconstriction, intensive attention has been paid to deciphering the role of pulmonary arterial smooth muscle cells (PASMCs) in pulmonary vascular remodeling and hypertension. Chelation of iron *in vitro* increased the metabolic activity and proliferation of human PASMCs, while iron supplementation inhibited this process. The rats fed with an iron-deficient diet developed pulmonary vascular remodeling and hemodynamic changes similar to PAH patients, which can be reversed by iron supplementation (108). Systemic iron homeostasis is controlled by FPN and its antagonist peptide hepcidin. Hepcidin treatment caused cellular iron accumulation by internalizing FPN in human PASMCs (109). In the mice expressing hepcidin-resistant isoform fpnC326Y, specific iron deficiency of PASMCs is sufficient to develop pulmonary hypertension, which was associated with markedly increased endothelin-1 (110). These results highlight the importance of intracellular iron deficiency, other than systematic iron deficiency, in the pathogenesis of PAH.

Different from pulmonary pressure, systemic blood pressure seems to be positively associated with iron markers. Two cross-sectional studies in Korea reported that serum ferritin was positively associated with the prevalence of hypertension (111, 112). Moreover, in a large-scale longitudinal study of the Chinese population, hemoglobin and transferrin levels were positively correlated with the risk of blood pressure and incident hypertension (113). Hypertensive patients with iron overload were accompanied by sympathetic overactivation but not the parasympathetic component of cardiovascular autonomic function (114). In experimental animals, dietary iron restriction attenuated cardiovascular hypertrophy, fibrosis, and inflammation in hypertensive Dahl salt-sensitive rats (115). The dietary iron restriction also prevented the development of hypertension and renal fibrosis in aldosterone/salt-induced hypertensive mice (116). These data suggested that dysregulation of iron metabolism may be an important independent risk factor

for hypertension. However, detailed mechanistic information is lacking for the role of iron in systemic hypertension.

Role of Iron in Arrhythmogenesis

Iron overload in the heart can lead to a gradual deterioration in both cardiac mechanical function and electrical activity. Chronic iron overload has been demonstrated to induce prolonged PR-interval, heart block, and atrial fibrillation in mice (117). Abnormal electrocardiograms including prolonged PR-intervals and QRS-intervals were also observed in isolated hearts of iron-treated gerbils (118). Long-term effects of iron overload resulted in frequent arrhythmias in gerbils *in vivo*, including premature ventricular contractions and supraventricular/ventricular tachycardia (119). However, arrhythmias did not occur in gerbils and guinea pigs receiving iron overload treatment despite significantly increased cardiac and hepatic iron concentrations (120, 121). The molecular mechanisms of iron-induced arrhythmias remain elusive. Many *in vitro* studies performed in isolated cardiomyocytes have verified that free iron can directly interact and interfere with a variety of ion channels of cardiomyocytes including the L-type calcium channel, the ryanodine-sensitive calcium channel, voltage-gated sodium channel, and delayed rectifier potassium channel (13, 122). Furthermore, excessive ROS production induced by iron overload could trigger the mitochondrial inner membrane anion channel opening, resulting in mitochondrial depolarization for the cytoplasmic anion efflux, which may be one of the reasons for arrhythmias (123, 124).

As for clinical studies, the incidence of arrhythmias associated with iron overload has been well-described in β-thalassemia and hereditary hemochromatosis (125). Moreover, patients with severe thalassemia and hemochromatosis could develop HF simultaneously. Iron toxicity may contribute to cardiac structural remodeling, which disturbs cardiac electrophysiological conduction. Thus, the arrhythmias occurrence induced by iron overload is confounded by the presence of HF and may not reflect the single effect of iron overload on arrhythmias. Despite limited information regarding arrhythmias occurrence with iron overload before the development of HF, a study has demonstrated that arrhythmias were significantly increased as myocardial iron deposition in patients with β-thalassemia and preserved left ventricular systolic function (126), which suggested the independent arrhythmogenic effect of iron toxicity to some extent. In addition, cardiac arrhythmias have been reported to be ameliorated by chelation in patients with iron load, which highlights iron toxicity is associated with cardiac arrhythmias.

CONCLUSION AND FUTURE DIRECTIONS

Iron is an indispensable micronutrient for basic biological processes. Dysregulation of iron homeostasis, inappropriate iron overload or deficiency, is harmful to a living organism. Although the understanding of the role of iron in the cardiovascular system has been advanced considerably in recent years, there remains unclear in some issues. Traditional methods cannot exactly reflect iron distribution and metabolism, especially in different tissues. Application of novel instruments or methods to measure iron, like T2 star (T2*) cardiac magnetic resonance imaging, is important to identify the pathophysiological roles of iron. Although iron repletion has been employed for the treatment of HF or PAH patients with iron deficiency, future studies are still necessary to pay more attention to the clinical significance of iron status and figure out the exact association between iron homeostasis and CVD (**Figure 3**). Ferroptosis is closely associated with the pathogenesis of CVD including cardiomyopathy, ASCVD, and myocardial I/R injury. However, the mechanisms of ferroptosis in the heart and vasculature remain elusive. The safety and efficacy of iron chelation to treat ferroptosis-related CVD require further verification.

AUTHOR CONTRIBUTIONS

XZ and SL: writing and editing. XZ: funding acquisition. Both authors contributed to the article and approved the submitted version.

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Vitamin C May Improve Left Ventricular Ejection Fraction: A Meta-Analysis

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Background: Vitamin C deprivation can lead to fatigue, dyspnea, oedema and chest pain, which are also symptoms of heart failure (HF). In animal studies vitamin C has improved contractility and mechanical efficiency of the heart. Compared with healthy people, patients with HF have lower vitamin C levels, which are not explained by differences in dietary intake levels, and more severe HF seems to be associated with lower plasma vitamin C levels. This meta-analysis looks at the effect of vitamin C on left ventricular ejection fraction (LVEF).

Methods: We searched for trials reporting the effects of vitamin C on LVEF. We assessed the quality of the trials, and pooled selected trials using the inverse variance, fixed effect options. We used meta-regression to examine the association between the effect of vitamin C on LVEF level and the baseline LVEF level.

Results: We identified 15 trials, three of which were excluded from our meta-analysis. In six cardiac trials with 246 patients, vitamin C increased LVEF on average by 12.0% (95% CI 8.1–15.9%; $P < 0.001$). In six non-cardiac trials including 177 participants, vitamin C increased LVEF on average by 5.3% (95% CI 2.0–8.5%; $P = 0.001$). In meta-regression analysis we found that the effect of vitamin C was larger in trials with the lowest baseline LVEF levels with $P = 0.001$ for the test of slope. The meta-regression line crossed the null effect level at a baseline LVEF level close to 70%, with progressively greater benefit from vitamin C with lower LVEF levels. Some of the included trials had methodological limitations. In a sensitivity analysis including only the four most methodologically sound cardiac trials, the effect of vitamin C was not substantially changed.

Conclusions: In this meta-analysis, vitamin C increased LVEF in both cardiac and non-cardiac patients, with a strong negative association between the size of the vitamin C effect and the baseline LVEF. Further research on vitamin C and HF should be carried out, particularly in patients who have low LVEF together with low vitamin C intake or low plasma levels. Different dosages and different routes of administration should be compared.

Keywords: antioxidant, coronary artery bypass graft surgery (CABG), heart failure, left ventricular function, oxidative stress, percutaneous coronary intervention (PCI), randomized trials, systematic review

INTRODUCTION

In the 18th century, James Lind described extreme intolerance for exercise as a characteristic of scorbutic patients (1). In addition, other signs compatible with heart failure (HF) such as shortness of breath, lethargy, and swelling of legs were described as symptoms of vitamin C deficiency in the major monographs on scurvy (1, 2). Autopsies of patients who died of scurvy revealed cardiac hypertrophy and congestion of the lungs (2). Experimental vitamin C deprivation in healthy volunteers led to fatigue, dyspnea, oedema, chest pain and reduced autonomic reflexes (3–8).

Case reports of patients with severe vitamin C deficiency have reported fatigue, dyspnea, cardiac enlargement, oedema and orthostatic hypotension, which often disappeared quite rapidly after vitamin C administration (9–15). A few animal models found that vitamin C can improve contractility and mechanical efficiency of the heart, including effects on left ventricular ejection fraction (LVEF) (16–21). Given the overlap of the symptoms of vitamin C deficiency with the symptoms of HF, and the findings from the animal studies, it seems appropriate to investigate whether administration of vitamin C may be beneficial for some HF patients.

Vitamin C exerts a multitude of biochemical effects influencing several cardiovascular processes relevant for HF. It participates in the synthesis of norepinephrine, carnitine, nitric oxide, and in the terminal amidation of dozens of neuropeptides such as vasopressin (22–25). Vitamin C hydroxylates specific proline residues in hypoxia-inducible factor-I, which regulates hundreds of genes, and it also participates in the demethylation of DNA and histones and thereby influences the epigenome (23–26).

In randomized trials, vitamin C has affected the cardiovascular system in various ways. Meta-analyses have indicated that in some contexts vitamin C may reduce blood pressure and risk of atrial fibrillation (27, 28). It has also improved endothelial functions and baroreflex sensitivity (29–36). In patients with ischemia/reperfusion injury, vitamin C may reduce reperfusion damage with amelioration of myocardial stunning (37). Through these types of mechanisms, vitamin C may impact HF.

The goal of this meta-analysis was to analyze the findings of trials that have reported on the effect of vitamin C on LVEF as a measure of the mechanical function of the heart.

RESULTS

Description of the Included Trials

We identified 16 publications that reported 15 separate trials (**Figure 1**, **Table 1**, **Supplementary Table S1**). Seven of them were parallel group trials and five were cross-over trials (**Table 1**). Three further trials were before-after trials: LVEF was first measured at baseline and then a second LVEF measurement was carried out following vitamin C administration of 1 month (49) or 6 months (38). The Basili et al. trial was published in two reports (43, 44) and the Fernhall et al. trial in three reports (45–47). Sabri et al. (49) and Scalzo et al. (52) reported two separate trials in one paper. The total number of patients in the 15 trials was 469, with 246 participants in six cardiac trials, and 223 participants in nine non-cardiac trials, six of which were included in the meta-analysis.

In the six cardiac trials, the mean age ranged from 56 to 69 years, and the baseline LVEF ranged from 35 to 62%. Five of the cardiac trials investigated the effect of a single intravenous dose of vitamin C before or during cardiac surgery with the dose ranging from 1 to 10 g (39, 40, 43, 51, 53). A trial with HF patients used a cross-over design to investigate the effect of 4-week oral administration of vitamin C at 4 g/day compared with placebo (41).

The nine non-cardiac LVEF trials form a heterogeneous group of studies. The mean age of participants ranged from 11 to 52

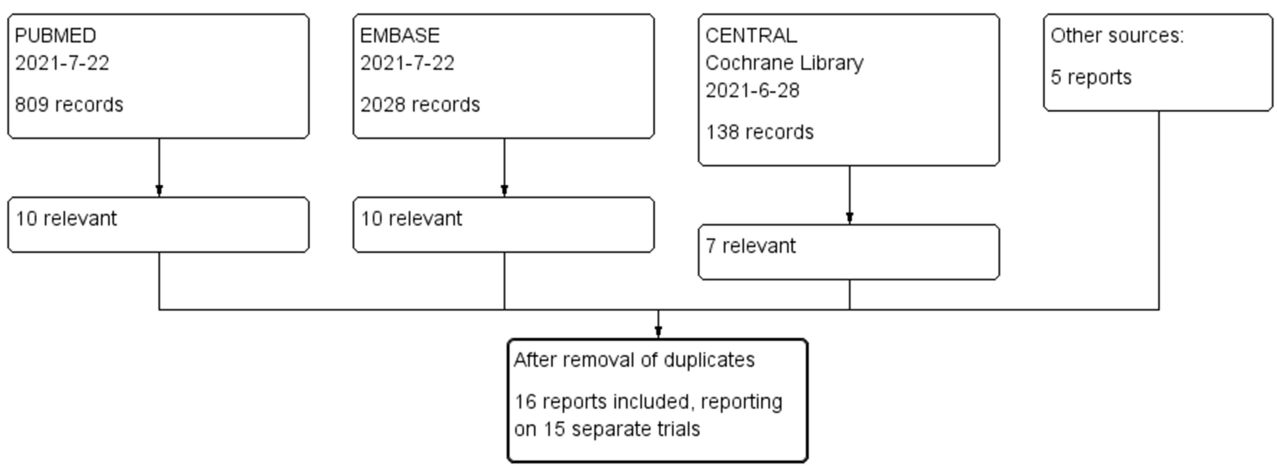

FIGURE 1 | Flow diagram of the searches, which identified 16 publications reporting 15 trials on the effect of vitamin C on LVEF (38–53). The search terms are listed in **Supplementary File 1**.

TABLE 1 | Characteristics of included trials.

Trial [ref]	N (vit C/ Control)	Age (y, mean)	Country	Context	LVEF at baseline[a]	Vit C route	Vit C dose (g/day)	Vit C days
Cardiac trials								
Guan et al. (39)	10/11	65	Japan	PCI	50%	iv	6	1
Oktar et al. (40)	12/12	56	Turkey	CABG	61.5%	iv	4	1
Ho (41)	37[b]	69	Taiwan	HF	35%	po	4	28
Basili et al. (43) Pignatelli et al. (44)	28/28	67	Italy	PCI	53%	iv	1	1
Safaei et al. (51)	29/29	57	Iran	CABG	49%	iv	2	1
Emadi et al. (53)	25/25	62	Iran	CABG	56%	iv	10	1
Non-cardiac trials								
Jensen et al. (38)	9[c]	52	Denmark	Iron-loaded adults	56%	po	0.2	180
Glavas et al. (42)	8[b]	37	Croatia	Diving	66%	po	1	1
Fernhall et al. (45) Fahs et al. (46) Fernhall et al. (47)	34/35	28	USA	Fire fighters	59%	po	2	1
Gao et al. (48)	8[d]	26	USA	Hyperoxia	59%	iv	3	1
Sabri et al. (49)	19[c]	13	Iran	Healthy	66%	po	0.25	30
Sabri et al. (49)	18[c]	11	Iran	T1D	66%	po	0.25	30
Sabri et al. (50)	20/20	13	Iran	T1D	60%	po	0.25	180
Scalzo et al. (52)	21[b]	45	USA	Exercise; Healthy	66%	iv	7.5	1
Scalzo et al. (52)	31[b]	46	USA	Exercise; T2D	64%	iv	7.5	1

[a] *Baseline LVEF is the mean of vitamin C and control groups.*
[b] *Cross-over; random order.*
[c] *Before-after study.*
[d] *Cross-over; first was the control test and thereafter the vitamin C test.*
CABG, coronary artery bypass graft surgery; iv, intravenous; LVEF, left ventricular ejection fraction; PCI, percutaneous coronary intervention; po, per oral; T1D, type 1 diabetes; T2D, type 2 diabetes.

years. The baseline LVEF was rather high in all these trials, ranging from 56 to 66%. In children with diabetes, Sabri et al. administered 0.25 g/day of vitamin C orally for 6 months (50). In two cross-over trials published in one trial report, Scalzo et al. administered 7.5 g of vitamin C intravenously before an exercise test for type 2 diabetes (T2D) patients and matched healthy participants (52). In a cross-over trial, Gao et al. studied the effect of 3 g/day intravenous vitamin C on cardiac effects of hyperoxia (48). In one before-after trial, Jensen et al. administered 0.2 g/day of vitamin C orally for 12 months to adults who suffered from transfusion-induced iron overload (38), and in two before-after trials, Sabri et al. gave vitamin C to healthy participants and to type 1 diabetes mellitus (T1D) patients (49).

The risk of bias assessment for the 15 included trials is shown in **Figure 2**. Eleven trials were randomized. Oktar et al. did not describe the method of allocation in their CABG trial (40). In three self-controlled trials, a baseline LVEF measurement was taken, followed by a period of vitamin C treatment, and then a second LVEF measurement was taken (38, 49). The reported baseline variables for the treatment groups in the parallel-group trials were balanced (**Supplementary Table S1**). Eight trials used an explicit placebo (41–43, 45, 50, 52, 53), whereas four trials reported "no treatment" in the control groups (39, 40, 48, 51). The trials by Gao et al. (48), Jensen et al. (38), Oktar et al. (40), Safaei et al. (51), and Sabri et al. (49) had the most methodological concerns (**Figure 2**). The Jensen et al. trial (38) and the both Sabri et al. (49) trials were excluded from our meta-analysis as they did not have an explicit control group or control treatment period.

In 11 trials LVEF was measured by transthoracic echocardiography (40–50, 52, 53). Biplane Simpson's rule was used to calculate LVEF in 3 trials (41, 43, 48), 2-D guided M-mode measurement of the cross-sectional axis of the LV at the papillary muscle tip level in 1 trial (42) and in the other 7 trials this was not specified (40, 45–47, 49, 50, 52, 53). Jensen et al. (38) estimated LVEF by resting multigated acquisition (MUGA) scans, Oktar et al. (40) by both MUGA scan and echocardiography and Guan et al. by left ventriculography (39). Safaei et al. did not describe the method of measurement of LVEF in their CABG trial (51).

Results of the Included Trials

Vitamin C and LVEF

For our quantitative study, we excluded three before-after trials (38, 49), restricting the meta-analysis to seven parallel group trials and five cross-over trials – 12 trials in total.

We calculated the difference in LVEF changes between the parallel vitamin C and control groups, and between the vitamin C

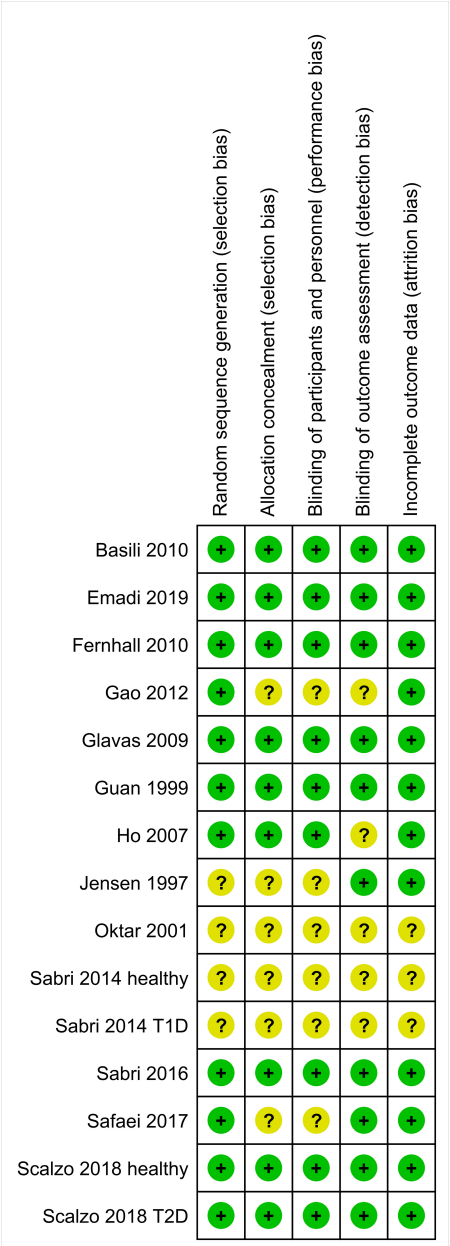

FIGURE 2 | Risk of bias summary of the trials that reported on the effect of vitamin C on LVEF. Review authors' judgments are shown for each risk of bias item for each included trial. A green plus sign (+) indicates that there is no substantial concern for bias in the particular quality item. A question mark (?) indicates that conclusions are unable to be drawn regarding potential bias. The reference numbers to the trials are shown in **Table 1**. Justifications for the quality assessments are described in **Supplementary Table S1**.

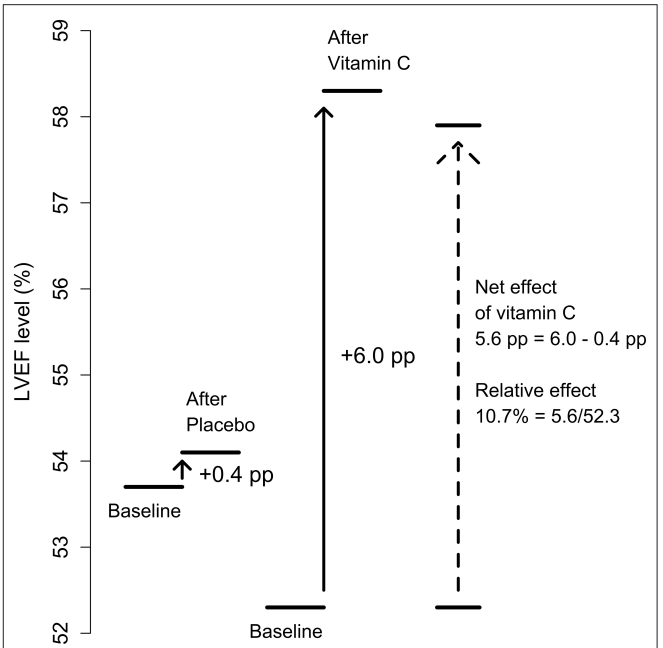

FIGURE 3 | Calculation of the effect of vitamin C on LVEF. The calculation is illustrated with figures from the Basili et al. trial during PCI (percutaneous coronary intervention) (43). In the placebo group, LVEF was increased from a baseline level of 53.7 to 54.1% after the intervention, while in the vitamin C group from 52.3 to 58.3%. These correspond to 0.4 and 6.0 percentage point (pp) increases in LVEF, respectively. Thus, subtracting the placebo group change from the vitamin C group change gives the net effect of vitamin C as 5.6 pp. On the relative scale this corresponds to a 10.7% (= 5.6/52.3) increase in LVEF due to vitamin C from the baseline level.

and control periods in the cross-over trials. We used the relative scale which has been shown to be superior in the analysis of continuous outcomes (54–56). See **Figure 3** for an illustration of the calculation for the effect of vitamin C on LVEF. A similar approach was previously used in the analysis of changes in FEV_1 levels in β_2-agonist trials (54). We analyzed the findings of cardiac and non-cardiac trials separately (**Figure 4**, **Table 1**).

In six cardiac trials with 246 patients, vitamin C increased LVEF on average by 12.0% (95% CI 8.1–15.9%; $P = 10^{-9}$) (**Figure 4**). In four trials, the benefit was statistically significant. There was no evidence of heterogeneity between the six cardiac trials ($P = 0.4$). There are methodological concerns with the Oktar and Safaei trials (**Figure 2**). Nevertheless, if these two trials are excluded in a sensitivity analysis, the estimate of vitamin C effect changes only slightly to 11.3% (95% CI 6.7–15.8%; $P = 10^{-6}$). The methodologically sound Basili trial has the greatest weight in the cardiac meta-analysis, with an estimate of effect close to the pooled effect in the cardiac group (**Figure 4**). Thus, there is no indication that methodologically inferior trials exaggerate the estimate of effect.

In the meta-analysis of six non-cardiac trials including 177 participants, vitamin C increased LVEF on average by 5.3% (95% CI 2.0–8.5%; $P = 0.0013$) (**Figure 4**). A statistically significant benefit was found in two of the individual trials. There was no evidence of heterogeneity ($P = 0.5$).

In explorative meta-regression analyses, we investigated a few relevant variables over the 12 trials as follows. There was no evidence of a difference in estimates between four trials with oral administration and eight trials with intravenous administration

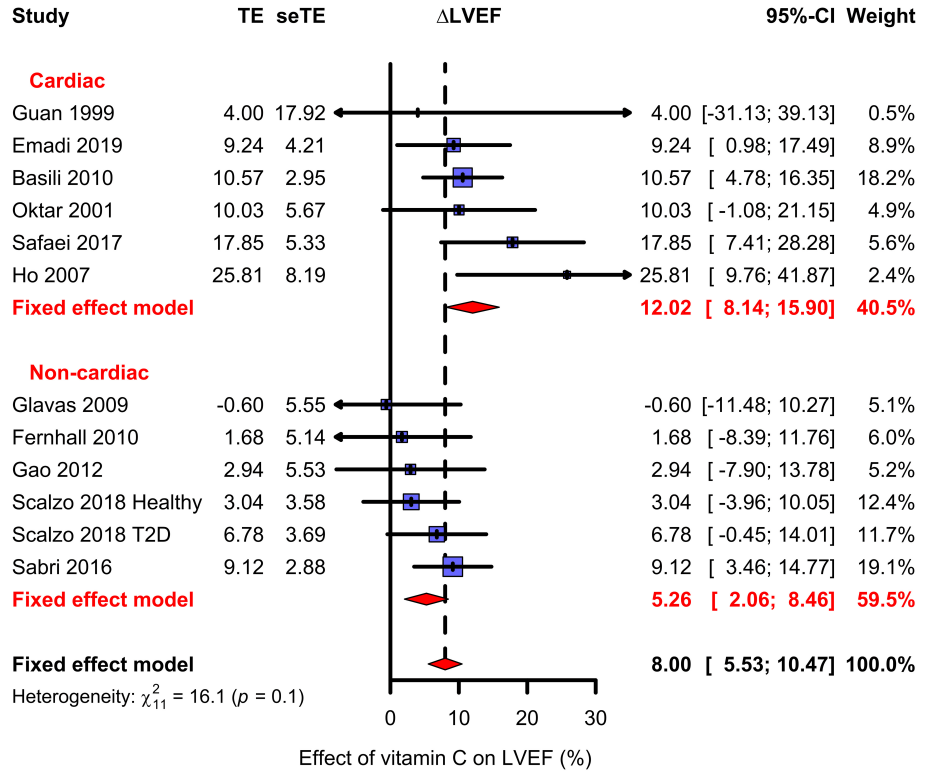

FIGURE 4 | Effect of vitamin C on LVEF. The upper subgroup shows the cardiac trials and the lower subgroup shows the non-cardiac trials. The effect of vitamin C is shown as the percentage difference between the vitamin C and placebo groups from the baseline LVEF; see **Figure 3**. The horizontal lines indicate the 95% CI for the vitamin C effect and the blue squares in the middle of the horizontal lines indicate the point estimate of the effect in the particular trial. The size of the blue square reflects the weight of the trial in the meta-analysis. The red diamond shape indicates the pooled effect and 95% CI for the two subgroups and for all 12 trials. See **Supplementary Files 1**, **2** for the description of the trials and the calculations.

($P = 0.8$). In meta-regression, the dose of vitamin C was not associated with the size of the effect ($P = 0.7$). For example, Basili (43) administered 1 g and Emadi (53) administered 10 g on one single day, but the reported effects were quite similar (**Figure 4**). See **Supplementary File 1** for the analyses.

In the 12 trials included in our meta-analysis, baseline LVEF varied from 35 to 66%, but only the Ho trial with HF patients (41) had a baseline LVEF below 48%. The relationship between the effect of vitamin C and the baseline LVEF is plotted in **Figure 5** with the cardiac trials indicated by filled circles and the non-cardiac trials by open circles. The evidence for modification of the vitamin C effect by the baseline LVEF is very strong ($P = 0.0008$). There is no evidence of residual heterogeneity around the regression line, which indicates that the meta-regression fully captures the findings of the 12 trials. The slope for all 12 trials crosses the null-effect line at a baseline LVEF of close to 70%. Given that the Ho trial (41) had by far the lowest baseline LVEF level of 35%, and is thereby particularly influential in defining the slope, it was removed in a sensitivity analysis, however, the slope did not change substantially and remained significant ($P = 0.011$). It is worth noting that most of the non-cardiac trials had participants with high baseline LVEF levels, which explains their rather low pooled estimate of effect in **Figure 4**.

Three before-after trials were excluded from our meta-analysis. The Jensen trial (38) was a study of adult patients with transfusional iron overload. During 12-month iron chelation therapy with desferrioxamine, the LVEF level of the patients significantly decreased. Thereafter, 0.2 g/day of vitamin C was administered in order to increase the efficacy of iron chelation. After 6 months of vitamin C administration, there was a mean increase in LVEF of 7.0% ($P < 0.01$) in 9 patients compared with a LVEF of about 56% before vitamin C; see **Supplementary File 2** for the calculation. Because of the continuous gradual decline in LVEF before vitamin C administration, the net effect of vitamin C would be even greater, if the period before vitamin C was adjusted for. In two pediatric trials with T1D patients and healthy controls, Sabri et al. (49) gave 0.25 g/day vitamin C for 1 month. No increase in LVEF was observed from the baseline LVEF of 66% (49). Given the baseline LVEF levels of these three trials, these findings are consistent with the predictions of our meta-regression in **Figure 5**.

Vitamin C and Other Measures of Cardiac Function in the Included Trials

Five of the included publications also reported other outcomes relevant to the topic of this study.

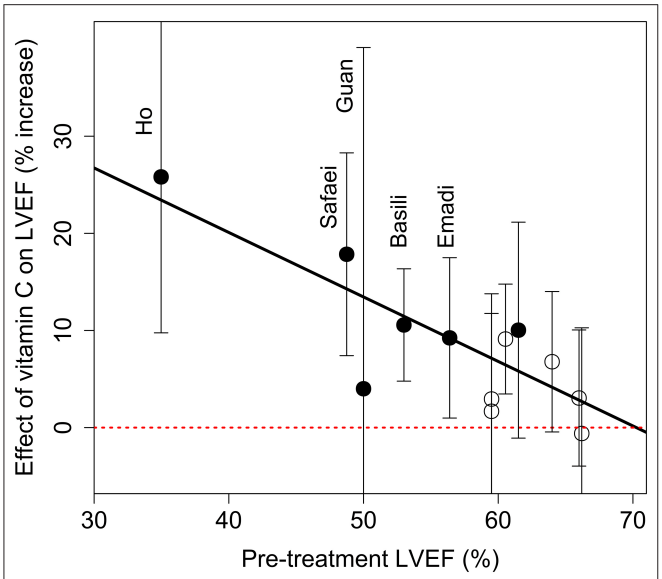

FIGURE 5 | Effect of vitamin C on LVEF by the baseline LVEF. The effect of vitamin C is shown as the percentage differences between the vitamin C and control groups from the baseline LVEF. The vertical lines indicate the 95% CI for the vitamin C effect in each trial. The cardiac trials are indicated by filled circles, and the non-cardiac trials by open circles. The red horizontal dotted line indicates the null effect. The diagonal line shows the meta-regression line with $P = 0.0008$ for the test that the slope is null. There are no indications of residual heterogeneity over the regression line, $P = 0.9$. For the calculations, see **Supplementary File 1**.

Basili et al. reported that after the percutaneous coronary intervention (PCI), the Thrombolysis in Myocardial Infarction Myocardial Perfusion Grade (TMPG, a categoric coronary flow grading system) was normal in 79% (22/28) of the participants in the vitamin C group, compared with just 39% (11/28) in the placebo group ($P = 0.01$) (43). In addition, after the PCI, the corrected Thrombolysis in Myocardial Infarction frame count (cTFC; a quantitative index for the assessment of myocardial perfusion) was lower, indicating better reperfusion in the vitamin C group (median change −41%) compared with the placebo group (median change −23%; $P < 0.0001$).

Ho found that, compared with the placebo group, the 4-week vitamin C administration increased the distance covered in a 6-min walk test by 26% in the HF patients ($P < 0.001$) and the Minnesota Living with HF questionnaire score by 53% ($P = 0.01$) (41).

Scalzo et al. found that the effect of vitamin C was not significant on left ventricular circumferential and longitudinal strain, which are measures of systolic function, whereas there was a marginally significant effect of vitamin C on the decrease in the post-peak exercise measurement of circumferential strain ($P = 0.052$) (52). In both the healthy and the T2D patients, vitamin C infusion improved diastolic function, estimated by lower values of lateral and septal E:E' ($P < 0.05$ for both) indicating enhanced relaxation. In the mitral valve deceleration time, there was interaction between the vitamin C intervention and the participant group (healthy vs. T2D) ($P = 0.018$), such that vitamin C decreased mitral valve deceleration time in participants with T2D.

Gao et al. studied the same participants before and after hyperoxia. Systolic myocardial velocity was higher during hyperoxia with a vitamin C infusion compared with the control day on which no vitamin C infusion was given ($P = 0.001$) (48). Vitamin C also led to a higher coronary blood velocity during hyperoxia compared with the control day ($P = 0.005$), and a decrease in coronary vascular resistance ($P = 0.04$).

Guan et al. reported that in patients with acute myocardial infarction undergoing direct PCI, intravenous vitamin C did not affect the cardiac index, measured by thermodilution technique at 3–4 weeks after the onset (39).

DISCUSSION

Textbooks often describe the effect that vitamin C has on wound healing, explaining the effect through the role of vitamin C in collagen metabolism. However, this is a simplistic view of the physiological functions of the vitamin. Biochemistry of vitamin C is complex, extending from several cofactor roles in diverse parts of metabolism to non-specific antioxidant effects, and further to wide-ranging epigenetic effects (22–26). The numerous biochemical effects translate to diverse changes at the clinical level. Vitamin C deficiency is associated with many symptoms characteristic of HF (1–15).

Compared with healthy people, patients with HF have lower vitamin C levels, which are not explained by differences in dietary intake levels (57–59). More severe HF seems to be associated with lower plasma vitamin C levels (57, 58). The apparent depletion of vitamin C in HF may be explained by increased metabolic consumption due to the oxidative stress associated with HF (60). In the early literature, vitamin C was suggested for treatment of HF (61–64), yet interest in the topic waned. It seems probable that the early findings were ignored because of wide-spread bias against vitamin C having effects other than treating and preventing scurvy (65).

Decreased plasma vitamin C levels have also been reported after cardiac surgery in parallel with increases in the oxidized forms of the vitamin, i.e., dehydroascorbate and ascorbate free radical (66–72). Thus, if vitamin C has an effect on cardiac function it is plausible that the decreased plasma vitamin C levels may contribute to the postoperative compromise in myocardial function after cardiac surgery.

In this systematic review, six cardiac trials were included. One of them was a 28-day oral vitamin C supplementation trial in patients with HF, whereas the others were 1-day intravenous vitamin C trials in patients who had undergone cardiac surgery or PCI. In the cardiac trials, vitamin C increased the LVEF levels on average by 12%. We also pooled six non-cardiac trials with diverse clinical contexts. In these trials vitamin C increased the LVEF levels on average by 5%. The statistically highly significant effect of vitamin C in both the cardiac and non-cardiac trials provides strong evidence that in some contexts vitamin C can influence the mechanical functions of the heart.

Three further before-after trials were consistent with our meta-regression analysis.

We did not demonstrate a dose response on the size of the effect. There was also no difference between oral and intravenous administration, which suggests that intravenous administration is not necessarily needed, despite the fact that this was the mode used in most studies (**Table 1**). However, the included trials were small and examined mostly ambulant patients, or patients undergoing elective cardiac surgery, and not patients with more severe disease with greater oxidative stress. Therefore, our comparison of oral and intravenous administration, while indicating that in some contexts oral vitamin C is effective, is not definitive and should not be generalized widely.

We found a strong negative association between the size of the vitamin C effect and baseline LVEF (**Figure 4**). However, only a single trial, which investigated HF patients (41), had baseline LVEF levels below 48% and that trial has the greatest weight in the linear regression. Nevertheless, the association remained significant even when the HF trial was removed. This modification of the vitamin C effect by baseline LVEF should be examined in further trials. Previously, the severity of disease was found to modify the effect of vitamin C on intubation time (73), the effect of vitamin C on FEV_1 decline as a result of exercise tests (74), and the effect of vitamin C on the duration of COVID-19 in outpatients (65, 75).

A few of the included trials reported effects of vitamin C on measures of cardiac physiology other than LVEF and found that vitamin C increased cardiac perfusion after PCI (43, 44), improved diastolic function in an exercise test (52), and increased systolic myocardial velocity during hyperoxia (48). In addition to the included trials, a few other trials have reported that vitamin C had an effect on the mechanical function of the heart (76–79).

Two cohort studies examined the effects of dietary vitamin C intake in HF patients who had average LVEF levels of 34% (80, 81). In both studies, higher dietary vitamin C intake was associated with a lower rate of cardiac events during follow-up. In addition, higher vitamin C intake was associated with a better health related quality of life (81). Residual confounding is a potential concern in cohort studies. However, in the randomized cross-over trial with HF patients included in our analysis, Ho found significant benefit from vitamin C administration on health-related quality of life and on the 6-min walk test (41). One of the trials included in our meta-analysis reported both a significant increase in LVEF and a significant decrease in ICU stay in the vitamin C participants (53), and another reported a significant increase in LVEF and a significant decrease in intubation time (51). It is plausible that the effect of vitamin C on the mechanical function of the heart is one of the explanations for the benefits seen in some ICU patients (65, 73, 82–84).

No effect of vitamin C on cardiovascular events was found in the Physicians' Health Study II (PHS-II) (85), in which 14,641 participants received 0.5 g/day of vitamin C for 8 years. However, the PHS-II trial recruited physicians, a group of extremely health-literate professionals who are not representative of the average population in terms of health-related lifestyles. Thus, the participants in the PHS-II trial were very different from the participants in the trials we included in our meta-analysis (**Table 1**) and so the PHS-II trial should not be considered a relevant comparison for the current meta-analysis.

It is plausible that increased intake of vitamin C may have no beneficial effect for well-nourished healthy people, but higher doses may have effects for people under heavy physiological stress, for example during cardiac surgery, or for people with HF. The possible effects of vitamin C for patients under heavy physiological stress is supported by controlled trials which found vitamin C to be beneficial for ICU patients (65, 73, 82–84), patients undergoing cardiac operations (28), patients with exercise-induced bronchoconstriction (74, 86), and patients with short-term respiratory symptoms induced by heavy physical activity (87–89).

One potential concern with meta-analyses is publication bias. Most of the included trials did not mention the findings on vitamin C and LVEF in their abstracts (39, 43, 44, 46–50, 52), and the Ho study was published only as a monograph (41). This indicates that the specific findings on LVEF were not the primary reason for publication. Furthermore, it is highly unlikely that publication bias could generate the association shown in **Figure 5**.

The method of allocation and the level of blinding during controlled trials are important issues. There was no risk of bias associated with these issues for most of the included trials (**Figure 2**). Nevertheless, even when the analysis was restricted to the four cardiac trials for which there were no methodological concerns, the evidence indicating a benefit from vitamin C was very strong.

In conclusion, in this meta-analysis, vitamin C increased LVEF in both cardiac and non-cardiac patients, with a strong negative association between the size of the vitamin C effect and the baseline LVEF. Further research on vitamin C and HF should be carried out. The most informative approach would be to investigate patients who have low LVEF together with low vitamin C intake or plasma levels, and to compare different dosages and different routes of administration. If an effect of vitamin C is demonstrated in such a setting, it would be reasonable thereafter to examine patients with higher LVEF levels or higher plasma vitamin C levels to determine the relationship between baseline characteristics and the effects of vitamin C.

METHODS

We searched for controlled trials that reported LVEF levels in parallel vitamin C and control groups, in cross-over trials (separate vitamin C and control periods for the same participants), and before-after studies. We included trials in which the administration of vitamin C was the only difference between the study groups or periods. We did not limit our search to randomized trials and did not require placebo control. We included all doses, all routes of administration and all durations of vitamin C administration.

We searched MEDLINE, EMBASE, and the Cochrane Central Register of Controlled Trials with the search phrases described in **Supplementary File 1**. Two authors (HH and AM) independently screened the titles and abstracts and identified

potentially relevant trials. Discrepancies between reviewers were resolved by discussion. We also perused the reference lists of the selected trials and relevant reviews. We identified 16 publications reporting on 15 distinct trials that satisfied our selection criteria (38–53) (**Figure 1**, **Table 1**). One author (HH) extracted study characteristics and outcomes from the trial reports and entered the data in **Supplementary Tables S1, S2** and in a spreadsheet, which are available in **Supplementary Files 1**, **2**. All authors checked the data entered against the original trial reports and all authors assessed the methodological quality of the trials (**Figure 2**, **Supplementary Table S1**). We contacted several authors to ask for details of their trials, but only three authors responded; see **Supplementary Table S1**.

The primary outcome in this analysis was the change in LVEF after the initiation of vitamin C administration. For most trials, we used variance analysis to calculate the *P*-value for the interaction between time and vitamin C treatment; see **Supplementary File 1** for the formula to calculate the F-test from the reported mean and standard deviation (SD) values of LVEF, and **Supplementary File 2** for the calculations. Thereafter we used the *P*-values to calculate comparable standard error (SE) values for the differences between the vitamin C and control observations. For the Jensen trial, we measured the LVEF changes from the 6-month time point of their figure 4A (38); see **Supplementary File 2**.

As secondary outcomes, we collected other measures of cardiac function from the included trial reports. The findings for the secondary outcomes are described in the Results section, but we did not construct forest plots for any of them because of their heterogeneity and low number.

In our analysis of the changes in LVEF, we used the relative scale, which has been shown to be superior in the analysis of continuous outcomes, because it adjusts for baseline variations and frequently leads to less heterogeneity (54–56). An illustration of the measurement of the vitamin C effect on LVEF is shown in **Figure 3** by using the Basili trial (43) as an example. Our analysis of the changes in LVEF is closely analogous to a previous analysis of changes in FEV_1 (54). In both cases the original observations are expressed as a percentage change, and the relative change caused by the treatment is calculated as a percentage effect of the percentages (**Figure 3**). We pooled the included trials with the *metagen* function of the R package *meta* (90–92), using the inverse variance, fixed effect options. For the meta-regression of the vitamin C effect on baseline LVEF, we used the *metareg* function of the *meta* package.

Although the Cochran Q test has been criticized (93), in the absence of a suitable alternative we used it to assess statistical heterogeneity among the trials in the meta-analysis, but we did not calculate the I^2 statistic. Our calculations are described in **Supplementary Files 1**, **2**.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

HH planned the study, extracted the data and entered the data into a spreadsheet, assessed the quality of the included trials, carried out the statistical analysis, and wrote the draft manuscript. EC and AM checked that the entered data were consistent with the published data, independently assessed the quality of the included trials, and participated in the critical revision of the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.789729/full#supplementary-material

Supplementary File 1 | Detailed descriptions of the included trials and statistical calculations.

Supplementary File 2 | Spreadsheet with the calculations of the results.

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Dietary α-Linolenic Acid-Rich Flaxseed Oil Ameliorates High-Fat Diet-Induced Atherosclerosis *via* Gut Microbiota-Inflammation-Artery Axis in *ApoE*$^{-/-}$ Mice

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Atherosclerosis (AS) is closely associated with abnormally chronic low-grade inflammation and gut dysbiosis. Flaxseed oil (FO) rich in omega-3 polyunsaturated fatty acids (PUFAs), which are mainly composed of alpha-linolenic acid (ALA, 18:3 omega-3), has been demonstrated to exhibit pleiotropic benefits in chronic metabolic diseases. However, the impact of dietary ALA-rich FO on AS and its associated underlying mechanisms remain poorly understood. Thus, the present study was designed as two phases to investigate the effects in atherosclerotic *Apolipoprotein E* (*ApoE*)$^{-/-}$ mice. In the initial portion, the *ApoE*$^{-/-}$ mice were randomly allocated to three groups: control group (CON), model group (MOD), and FO-fed model group (MOD/FO) and were treated for 12 weeks. The second phase used antibiotic (AB)-treated *ApoE*$^{-/-}$ mice were divided into two groups: AB-treated model group (AB/MOD) and FO-fed AB-treated model group (AB/FO). In the results, the dietary ALA-rich FO administration ameliorated atherosclerotic lesion, as well as the parameters of AS (body weights (BWs) and the total bile acids (TBA). Chronic systemic/vascular inflammatory cytokines and *in situ* macrophages (Mψs) were reduced with FO intervention. In addition, the FO improved the gut integrity and permeability by decreasing the plasma lipopolysaccharide (LPS). Moreover, gut dysbiosis and metabolites [short-chain fatty acids (SCFAs) and bile acids (BAs)] in AS were modulated after FO treatment. Intriguingly, during an AB-treated condition, a significantly weakened amelioration of FO-treated on AS proposed that the intestinal microbiota contributed to the FO effects. A correlation analysis showed close relationships among gut bacteria, metabolites, and inflammation. Collectively, these results suggested that the dietary ALA-rich FO ameliorated the AS in *ApoE*$^{-/-}$ mice *via* the gut microbiota-inflammation-artery axis.

Keywords: ALA-rich flaxseed oil, atherosclerosis, inflammation, gut microbiota, intestinal metabolites

INTRODUCTION

Atherosclerosis (AS), characterized by excessive cholesterol deposition within the artery wall, is the leading cause of cardiovascular diseases (CVD) including coronary heart disease, cerebral infarction myocardial infarction (MI), stroke, and peripheral vascular disease (1), accounting for a major cause of mortality and morbidity in the world (2, 3). Moreover, AS represents a chronic inflammatory disorder of the vascular wall involving many circulating immune cells, such as monocytes, lymphocytes, and platelets (4). Among them, the monocyte infiltration and the subsequent formation of macrophages (Mψs)-derived foam cells for the release of pro-inflammatory cytokines are crucial in the progression of AS (1, 5, 6). Monocytes are rich in cell-activating, oxidized, and low-density lipoprotein (oxLDL) in accumulating the development of lesions and forming the early plaques (known as fatty streaks) on the intima (7). The AS changes from fatty streaks to plaque rupture as well as thrombosis. Due to limited dietary management of AS, some novel strategies are urgently needed.

Chronic inflammation has been involved in the occurrence and the development of AS (8, 9). During the pathological injury of AS, a variety of pro-inflammatory cytokines, including the tumor necrosis factor (TNF)-α, the interleukin (IL)-1β, and IL-6 were increased (10). It was verified that a *Toll-like receptor* (*Tlr4*)$^{-/-}$ and/or *myeloid differentiation factor* (*Myd*)*88*$^{-/-}$ have declined the production of inflammatory cytokines in *Apolipoprotein E* (*ApoE*)$^{-/-}$ mice, thereby reducing the formation of aortic plaque (11). Furthermore, inflammation was attenuated by inhibiting the Jun N-terminal kinase (JNK) and the nuclear factor kappa-B (NF-κB) pathways in atherosclerotic *LDL*$^{-/-}$ mice (12). Consistently, clinical studies have shown a significantly increased inflammation in the AS lesion (13).

The monocytes/Mψs represent the key inflammatory cells in AS. Instability and rupture of atherosclerotic plaques occur predominantly in regions where inflammatory monocytes/Mψs adhere to the plaque shoulder areas (14). As a trigger of Mψ-mediated inflammation, lipopolysaccharide (LPS) from Gram-negative bacteria binds to TLR-4 and promotes the pro-inflammatory cytokines generation and release, which ultimately erodes the extracellular matrix and leads to the plaque rupture followed by MI or stroke (15, 16).

Gut microbiota plays a vital role in the progression of AS (17). Emerging studies have suggested that gut dysbiosis provokes the damage of intestinal mucosal barriers and enhances the intestinal permeability, thus, leading to the translocations of pathogenic bacteria and their metabolites (such as LPS) into the plasma *via* the gut-heart axis, for triggering blood vessel chronic inflammation (18). As crucial metabolites of gut microorganisms, short-chain fatty acids (SCFAs), with <6 carbon atoms (C1-C6) and mainly include acetate (C2), propionate (C3), and butyrate (C4), are essential for intestinal homeostasis (19). Importantly, a remarkable reduction of SCFAs led to a dysfunction of the gut mucosal barrier in AS (20). In addition, another microbial metabolite, the bile acids (BAs), especially the secondary BAs (SBAs), act as ligands that activated the BA-activated G protein-coupled receptor 1 (GP-BAR1) to stabilize the intestinal barrier function (21). Besides, the BAs exert various functions on intestinal lipid absorption and metabolic regulation. They were identified as the specific microbial enzymatic participants, which in turn, modulated the composition of the microbiota (22).

The flaxseed oil (FO), rich in plant-derived omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) and mainly α-linolenic acid (ALA, 18:3 ω-3) (23), has been demonstrated by researchers, including our lab to exhibit pleiotropic benefits in chronic metabolic diseases, such as alcoholic liver disease (ALD) (23), polycystic ovarian syndrome (PCOS) (24), and type 2 diabetes mellitus (T2DM) (25) and colitis (26). However, the effects of the dietary ALA-rich FO on AS and the underlying mechanisms remain elusive (27). Thus, this study was aimed to investigate the effectiveness of the dietary ALA-rich FO on the occurrence and the development of AS in *ApoE*$^{-/-}$ mice, with or without gut microbiota, which may potentially contribute to the further understanding of complicated mechanisms among AS, gut microbiota, and inflammation.

MATERIALS AND METHODS

Animals and Diets

All experiments were approved by the Ethics Committee of Ningxia Medical University (No. 2019-137). Fifty male *ApoE*$^{-/-}$ mice (8-week-old) were obtained from Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. The mice were maintained under standard, specific, and pathogen-free conditions in individual cages in a temperature-controlled room (ambient temperature 22 ± 1°C, air humidity 40–70%) with a 12 h light/dark cycle room in Laboratory Animal Center of Ningxia Medical University, Yinchuan, China. A high-fat diet (HFD) with 1.25% cholesterol (60% fat, 20% carbohydrate, and 20% protein, No. TP28520) was purchased from TROPHIC Animal Feed High-tech Co., Ltd., Nantong, China. The FO with 59.58 ± 2.47% ALA was extracted by our laboratory.

Experimental Design

As shown in **Figure 1A**, the experiment was divided into two phases. In the initial portion, after 3 weeks of acclimatization period, the male *ApoE*$^{-/-}$ mice (n = 36, 8 weeks old) were randomly divided into 3 groups (12 animals/group): (a) control group (CON), mice were fed a normal diet; (b) model group (MOD), mice were fed HFD with 1.25% cholesterol (w/w); and (c) MOD-treated with ALA-rich FO group (MOD/FO), MOD mice were fed with 10% FO (w/w) as FO intervention group. The mice in MOD groups were fed with the HFD, with an energy composition of 60% fat, 20% carbohydrate, and 20% protein. Meanwhile, the mice in the MOD/FO group received an equal amount of calories as the MOD group with the cocoa butter-derived calories that were substituted with isocaloric FO (fat). The HFD was prepared by the company in advance and was stored at 4°C. After 12 weeks of FO treatment, we fasted the mice for 12 h, and then euthanized them for further analysis. In the second phase, according to previous reports (28), after 3 weeks of antibiotic (AB) cocktail (0.5 g/L vancomycin, 1 g/L neomycin

sulfate, 1 g/L metronidazole, and 1 g/L ampicillin), the AB-treated male *ApoE*$^{-/-}$ mice ($n = 24$) were randomly divided into two groups (12 animals/group): MOD group (AB/MOD) and MOD-treated with FO group (AB/FO). After 12 weeks of HFD feeding, the mice were fasted for 12 h and then euthanized for further studies in consistence with the initial period of this study.

Histology and Morphometry Evaluations of Atherosclerotic Lesions

The pathological changes in AS were detected with *en* face oil-red O staining, HE staining, and Masson's trichrome staining ($n = 5$/group) (Details in **Supplementary Material**). Images were captured with Canon EOS 70D camera and were analyzed using Image J 1.8.0 software (National Institutes of Health, United States). The lesion area index was calculated as the percentage of aortic lumen area covered by atherosclerotic lesions. Observers were blinded to the experimental groups.

Determination of Total Bile Acids (TBA) in Serum

The levels of TBA in plasma were measured by AU400 automatic biochemical analyzer (Olympus, Japan).

Cytometric Bead Array (CBA)

The aorta tissues (100 mg) were homogenized by a glass grinder. After centrifugation at 300 × *g* for 5 min, the supernatants of homogenates were collected to determine the aortic levels of IL-1β, TNF-α, IL-6, IL-10, IL-17A, and MCP-1. These plasma and aortic inflammatory cytokines in each group were measured by LEGENDplex™ CBA mouse inflammatory cytokine kit (Biolegend, United States, No. 740150). The operation was performed three times according to the manufacturer's instructions. In brief, a series of cytokine standard dilutions were prepared. After that, 25 μl of Assay Buffer, 25 μl of each standard, 25 μl of the plasma/supernatants of aortic samples or standard dilutions of each sample, and 25 μl of mixed beads were added to all the wells in turn. All the tubes were placed on a plate shaker, shaking it at ∼500 revolutions per minute (rpm) for 2 h at room temperature. The samples were washed with 200 μl wash buffer and centrifuged at 200 × *g* for 5 min. About 25 μl of detection antibodies were added to each well after discarding the supernatant. All the tubes were placed on a plate shaker and were shook at ∼500 rpm for 1 h at room temperature. About 25 μl of Streptavidin-Phycoerythrin (SA-PE) was added to each well directly. The plate was placed on a plate shaker and shook at ∼500 rpm for 30 min at room temperature. Next, 150 μl of 1× Wash Buffer were added to each well to shake them for 1 min. Afterward, the flow cytometer was sited with cytometer setup beads and the samples were measured. The data were analyzed by using LEGENDplex software (Biolegend, United States).

Flow Cytometry Analysis

The Mψs, the significant aortic inflammatory cells (4), were digested and isolated from aortic tissues. Briefly, 1 g of aortic tissues was minced and suspended in 5 ml of Hanks balanced salt solution (HBSS) containing 0.1% (w/v) collagenase type IV (Sigma, United States) for 20 min at 37°C. Next, the specimen was washed with RPMI1640 containing 2% of fetal bovine serum (FBS) and then filtered through a 200-mesh nylon membrane. After centrifugation at 70 × *g* for 3 min at 4°C, the supernatants were discarded, and the particles were resuspended in 3 ml HBSS. After the erythrocyte lysis, samples were centrifuged for 5 min at 500 × *g*, 4°C, and then washed two times. The final concentration was adjusted to 1×10^7 cells/ml. Then, 100 μl of suspended cells were stained with KO525-conjugated anti-mouse CD45 (Biolegend, 103137, United States), FITC-conjugated anti-mouse CD11b (Biolegend, 101206, United States), and PE-conjugated anti-mouse F4/80 antibody (Biolegend, 123110, United States). The prepared samples were measured and analyzed using Accuri™ C6 flow cytometer (BD Bioscience, United States).

Plasma LPS Assay

The plasma LPS level in each group was examined using a Limulus amebocyte lysate kit (Xiamen Bioendo Technology Co., Ltd., Xiamen, China) according to the manufacturer's instructions. Briefly, the plasma was diluted with endotoxin-free water (1:4), then 50 μl of diluted plasma was put into each well in a 96-well plate. At the initial time point, 50 μl of the Limulus amebocyte lysate reagent was added to each well. The plate was incubated at 37°C for 30 min. Then, 100 μl of chromogenic substrate warmed to 37°C were added to each well, and the incubation was extended for an additional 6 min at 37°C. Finally, the reaction was stopped by adding 100 μl of 25% solution of glacial acetic acid. Optical density at 545 nm was measured with a microplate reader (Thermo Scientific, United States).

Immunofluorescence

To further determine the impacts of the dietary ALA-rich FO on inflammatory cells, the Mψs in aortic sinuses were separately detected by immunofluorescence. The frozen sections were taken out from the refrigerator, drying at room temperature for 10 min. After washing with PBS 3 times (3 min/each time), the sections were immersed in paraformaldehyde for 10 min. To minimize the scope of the subsequent incubation of the antibody and to incubate the antibody effectively, we circled the tissue with a histochemical oil pen. After soaking in PBS for 10 min, the sections were blocked with 2% albumin from bovine serum albumin (BSA) for 1 h. Then, the sections were probed with a rat anti-mouse F4/80 (1:250 dilution, Abcam, ab6640, United States) overnight at 4°C. Then samples were incubated with secondary antibody fluorescein (FITC)-conjugated goat anti-rat IgG (H+L) (1:500 dilution, Proteintech, SA00003-11, United States) for 1 h at room temperature, dropping the mounting tablets containing 4',6-diamidino-2-phenylindole (DAPI) (ZSGB-BIO, ZLI-9557, China) chromogenic agent for mounting, observing, and collecting pictures. Images were captured with an Olympus BX51 microscope (Aomori Olympus Co., Ltd., Japan). For quantification, the total numbers of positive cells of each aorta section were determined using the Image J 1.8.0 software. Observers were blinded to the experimental groups.

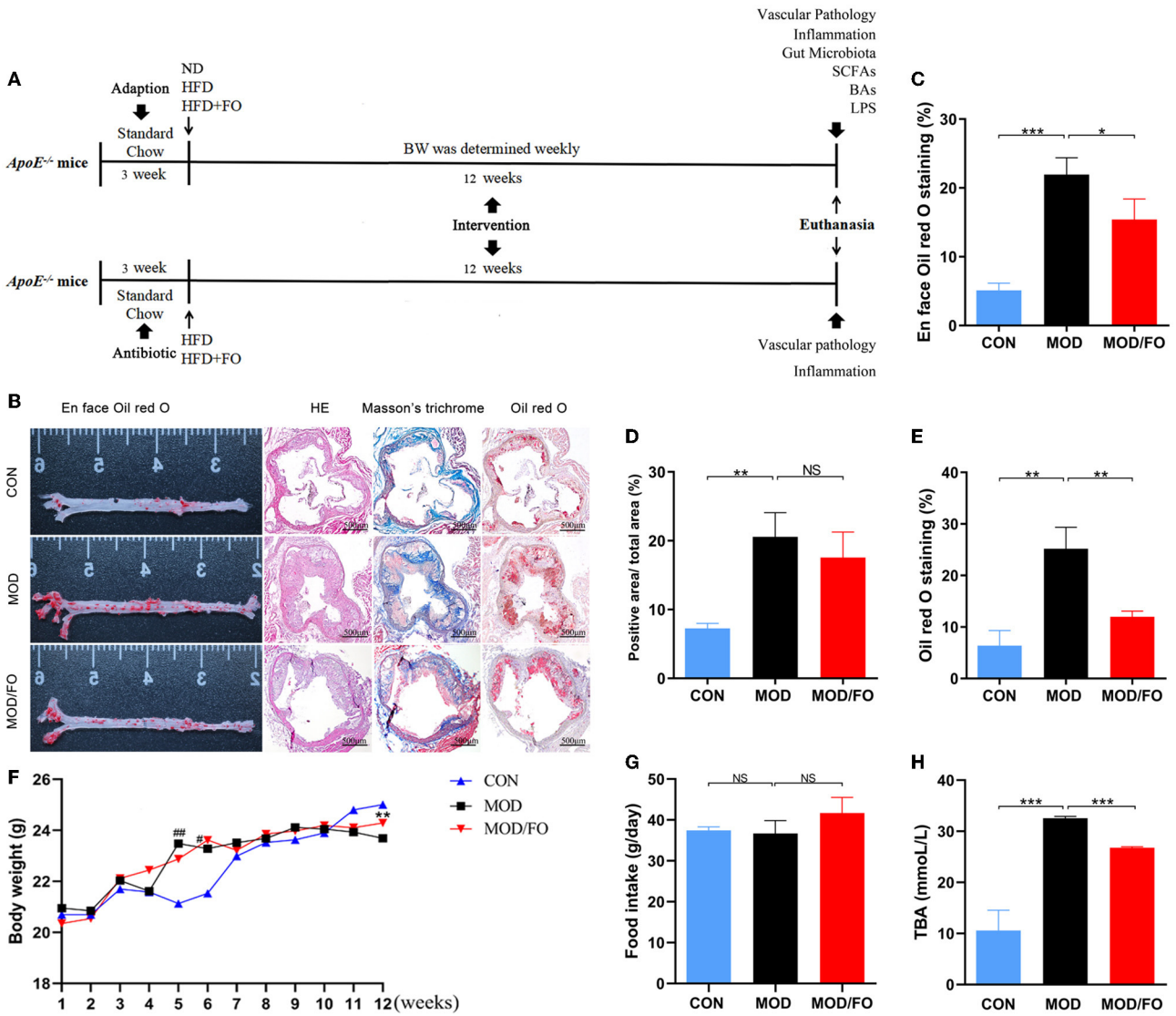

FIGURE 1 | Effects of dietary ALA-rich FO treatment on the pathological lesion and routine parameters in atherosclerotic *ApoE*$^{-/-}$ mice. **(A)** Schematic time diagram of the experimental design. **(B)** Representative stained sections of the valve area of the aorta and aortic root of the heart. Quantitative analysis as lesion area/total area (%) shown in *en* face oil-red O staining ($n = 5$/group) **(C)**, Masson's trichrome staining **(D)**, and oil-red O staining **(E)**. **(F)** BWs: Body weights. **(G)** Food intake. **(H)** TBA: total bile acid. Original magnification, ×40. The bar of 500 μm was presented in the right corner of **(B)**. Data were presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, CON group vs. MOD group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, MOD group vs. MOD/FO group. NS, no significance.

Gut Microbiota Analysis

The fecal microbial 16S rRNA gene sequencing and analysis were investigated as the previous studies (29). The mice in each group were transferred to fresh and sterilized cages after 12 weeks of feeding. The fresh feces of each group were individually collected and were immediately frozen into liquid nitrogen, then finally stored at −80°C until the DNA extraction. Cetyltrimethylammonium bromide (CTAB) method was used to extract the genomic DNA of samples, and then the purity and concentration of the DNA were detected by agarose gel electrophoresis (Details in **Supplementary Material**).

Fecal SCFAs Quantification by Gas Chromatography-Mass Spectrometer (GC-MS)

The quantification analysis of fecal SCFAs was performed using an Agilent 7890A, gas chromatography coupled with Agilent 5975C mass spectrometric detector (Agilent Technologies, United States) equipped with an HP-5MS column (0.25 × 30 mm, 0.25 μm particle size) (Suzhou Bionovogene Co., Ltd) as described previously (25). Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The initial oven temperature was held at 60°C for 5 min, ramped to 250°C at a rate of 10°C/min, and finally held at this temperature for 5 min. The

temperatures of the front inlet, transfer line, and electron impact (EI) ion source were set as 280, 250, and 230°C, respectively. Data handling was performed with an Agilent's MSD ChemStation (E.02.00.493, Agilent Technologies, Inc., United States).

Fecal BAs Quantification by Liquid Chromatograph-MS (LC-MS)

To detect the composition of BAs, 38 standard solutions were first prepared. Stock solutions were prepared using the solvent described in the instructions and the working solution was prepared using methanol through serial dilution. The standard solutions were stocked under −20°C. Then, we prepared the samples: 100 mg fecal sample was collected and was added with 300 μl methanol to precipitate the protein. Samples were oscillated for 1 min and then centrifuged at 4°C for 10 min (12,000 × *g*). The supernatant was concentrated and dried in a vacuum. The residue was dissolved with 100 μl methanol and the supernatant was ready for LC-MS analysis. The UPLC separation was performed on an Acquity UPLC system (Waters, U.K.) that is equipped with an Acquity UPLC® BEH C18 (1.7 μm, 2.1 × 100 mm, Waters) column. The temperature of the column was set at 40°C. The sample injection volume was 5 μl. The eluents consisted of 0.01% formic acid in water (eluent A) and acetonitrile (eluent B). The flow rate was set at 0.25 ml/min. A 38 min elution gradient was performed as follows: 0–4 min, 25% B; 4–9 min, 25–30% B; 9–14 min, 30–36% B; 14–18 min, 36–38% B; 18–24 min, 38–50% B; 24–32 min, 50–75% B; 32–35 min, 75–100% B; and 35–38 min, and 100–25% B. The MS analysis was performed using an AB 4,000 mass spectrometer (AB, United States) equipped with an ESI source in the negative-ion mode that is working in the multiple reaction monitoring (MRM) mode. An ion source voltage of 4.5 kV, a source temperature of 500°C, and a desolvation temperature of 380°C were used. Collision gas and the curtain gas were set at 6 and 30 psi, respectively, while both atomization gas and auxiliary gas were 50 psi.

Statistical Analysis

The data were conducted with Prism 8.01 (GraphPad Software Inc., CA, United States). The data were expressed as the mean ± SEM. Differences between multiple comparisons using 2-way ANOVA. According to the normal distribution, the differences between the two groups were analyzed by the Student's *t*-test (2-tailed). Correlation analysis was performed using the Spearman method. The $P < 0.05$ was considered statistically significant.

RESULTS

Atherosclerotic Pathological Lesion and Routine Parameters in Diverse Groups

To investigate whether dietary ALA-rich FO can improve AS, the aorta, and aortic sinus in diverse groups were pathologically stained and observed. The *en* face aorta analysis was used to reveal an atherosclerotic lesion formation with the aid of oil-red O staining. The result displayed that the high-fat diet (HFD) has significantly increased the plaque area ($P < 0.001$) (**Figure 1B**). Nevertheless, the formation of atherosclerotic plaque in the MOD/FO group was reduced compared with the MOD group ($P < 0.05$) (**Figure 1B**). The extent of atherosclerotic development at the *en* face of the aorta was quantified as a percentage of the total aortic area occupied by the oil-red O-stained lipid deposits (**Figure 1C**). Moreover, the cross-sectional analysis of atherosclerotic development at the aortic sinus revealed a lipid deposition with the aid of the oil-red O staining in atherosclerotic mice (**Figure 1B**). The component of atherosclerotic development at the aortic sinus was quantified as a percentage of aortic cross-sectional luminal area occupied by oil-red O-stained lipid deposits. There was a significant difference in the area of lipid plaque between the MOD group and CON group ($P < 0.01$) (**Figure 1E**), and FO treatment significantly inhibited the excessive lipid deposition and prevented plaque progression ($P < 0.01$) (**Figure 1E**).

Moreover, Masson's trichrome staining showed atherosclerotic plaques (red) and fibrous fatty plaques (blue) in the aortic sinus (**Figure 1B**). The component of fibrous fatty plaques at the aortic sinus was quantified as a percentage of the total aortic plaques area occupied by Masson's trichrome-stained fibrous fatty plaques (**Figure 1D**). A significant difference in fibrous fatty plaques was found between the CON group and MOD group ($P < 0.01$), but there was no significant difference after the intervention of FO ($P > 0.05$) (**Figure 1D**). Furthermore, the HE staining showed that dietary ALA-rich FO had an amelioration on atherosclerotic plaque (**Figure 1B**). Taken together, these results indicated that the treatment of dietary ALA-rich FO had the anti-atherogenic property in $ApoE^{-/-}$ mice.

We further detected alterations in basic indicators among different groups. At the initiation of the study, there was no significance in body weights (BWs) and in food intake among the 3 groups (**Figures 1F,G**). At the week 5–6 of HFD, BWs of mice in the MOD group have gained significantly higher than CON group, yet the weights began to decrease after week 10 (all $P < 0.05$), which may be due to a reduced caloric absorption capacity in the progression of the disease. At week 12, BWs in the MOD/FO group were elevated compared to the MOD group ($P < 0.01$) (**Figure 1F**). This phenomenon suggested that FO intervention has delayed the symptoms of late weight loss in AS. In terms of food intake, the average intake of mice in each group was decreased during the intervention period, but without the significant difference (**Figure 1G**), suggesting that the effects of FO on BWs were not attributed to the influence of the energy intake. Meanwhile, we detected the levels of plasma TBA. Compared to the CON group, increases in plasma TBA ($P < 0.05$) (**Figure 1H**) were observed in mice with the MOD group. Intriguingly, the ALA-rich FO intervention has reduced the abnormal TBA ($P < 0.05$) (**Figure 1H**). This result indicated that the intervention of dietary ALA-rich FO-alleviated AS might be associated with the TBA reduction.

Dietary ALA-Rich FO Altered the Levels of Plasma/Aortic Inflammatory Cytokines in AS

The anti-inflammatory effect of dietary ALA-rich FO has been demonstrated in different metabolic diseases such as polycystic ovarian syndrome (PCOS) (24) and alcoholic liver disease (ALD)

(23). Studies have shown that inflammation is closely related to AS (30). Thus, we further analyzed the influence of FO on inflammation in atherosclerotic mice (**Table 1**). The results showed that plasma levels of pro-inflammatory TNF-α, IL-1β, IL-6, and IL-17A in the MOD group were significantly increased compared to the CON group (all $P < 0.05$). After the dietary ALA-rich FO intervention, the concentrations of TNF-α, IL-1β, and IL-17A (all $P < 0.05$) in plasma were decreased compared with the MOD group. Meanwhile, the anti-inflammatory IL-10 in the MOD group was notably decreased ($P < 0.001$), but has shown no significant difference between the MOD/FO group and MOD group ($P > 0.05$). These results indicated that dietary ALA-rich FO treatment has ameliorated the systemic inflammation in $ApoE^{-/-}$ mice with AS.

In addition, we measured the levels of *in situ* aortic inflammatory TNF-α, IL-1β, IL-6 IL-17A, monocyte chemoattractant protein (MCP)-1, and IL-10 to evaluate the effects of dietary ALA-rich FO on plaque inflammation in atherosclerotic mice (**Table 1**). Similar to the above systemic inflammation, the HFD increased the levels of TNF-α, IL-1β, IL-6, and IL-17A (all $P < 0.05$) in aortic tissues, compared to the CON group. Importantly, these levels were remarkably decreased in MOD/FO group (all $P < 0.05$), except the IL-17A ($P > 0.05$). The IL-10 in the aorta in the MOD group showed a significant decrease compared with the CON group ($P < 0.05$). However, there was no difference in IL-10 between MOD/FO and MOD groups. These results suggested that dietary ALA-rich FO treatment has ameliorated the aortic inflammation in atherosclerotic $ApoE^{-/-}$ mice.

TABLE 1 | Determination of plasma and aortic inflammatory cytokine levels and plasma lipopolysaccharide (LPS) in diverse groups.

Inflammatory indicators	Groups		
	CON	MOD	MOD/FO
Aortic TNF-α (ng/mL)	10.97 ± 1.78	39.89 ± 4.35***	33.49 ± 1.96$^{\#}$
Aortic IL-1β (ng/mL)	16.27 ± 2.28	21.83 ± 1.19**	18.56 ± 1.13$^{\#\#}$
Aortic IL-10 (ng/mL)	55.19 ± 6.36	48.04 ± 1.91*	52.30 ± 4.09
Aortic IL-6 (ng/mL)	12.28 ± 1.90	15.02 ± 0.50*	13.16 ± 1.43$^{\#}$
Aortic IL-17A (ng/mL)	7.55 ± 2.14	15.77 ± 2.15***	12.60 ± 2.46
Aortic MCP-1 (ng/mL)	0.30 ± 0.03	0.35 ± 0.05	0.34 ± 0.04
Plasma TNF-α (ng/mL)	6.50 ± 1.40	10.02 ± 0.97**	8.16 ± 1.46$^{\#}$
Plasma IL-1β (ng/mL)	5.22 ± 1.13	11.09 ± 1.73***	6.92 ± 2.01$^{\#\#}$
Plasma IL-10 (ng/mL)	44.15 ± 5.74	3.16 ± 0.29***	3.25 ± 1.15
Plasma IL-6 (ng/mL)	8.76 ± 1.77	12.80 ± 2.09*	12.67 ± 1.38
Plasma IL-17A (ng/mL)	5.00 ± 1.42	9.22 ± 1.53**	7.02 ± 0.84$^{\#}$
Plasma MCP-1 (ng/mL)	0.34 ± 0.06	0.38 ± 0.03	0.38 ± 0.03
Plasma LPS (EU/mL)	0.39 ± 0.02	0.44 ± 0.04*	0.38 ± 0.03$^{\#}$

*Values are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, CON group vs. MOD group. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, MOD group vs. MOD/FO group.*

Dietary ALA-Rich FO Inhibited Inflammatory Mψs in AS

Due to the crucial role of Mψs in the inflammation of AS (31), we measured the Mψs in the orthotopic aortic tissues in diverse groups. After cell suspension from the aortic roots of atherosclerotic $ApoE^{-/-}$ mice, the Mψs stained by KO525-conjugated anti-mouse CD45, FITC-conjugated anti-mouse CD11b, and PE-conjugated anti-mouse F4/80 antibody were detected by flow cytometry. We found that Mψs in the MOD group were higher than those in the CON group ($P < 0.001$) (**Figures 2A,C**). Intriguingly, the proportions of $F4/80^{+}$ $CD11b^{+}$ cells ($P < 0.01$) (**Figure 2C**) were conversely lower in FO treatment. To further analyze the effects of FO on Mψs in the orthotopic aortic tissues, aortic $F4/80^{+}$ Mψs were measured by immunofluorescence. The aortic plaque inflammatory Mψs were increased after HFD feeding ($P < 0.01$) (**Figures 2B,D**). Importantly, the dietary ALA-rich FO intervention could dramatically attenuate these abnormal Mψs in the atherosclerotic lesion ($P < 0.01$) (**Figures 2B,D**).

Dietary ALA-Rich FO Reduced Plasma LPS Levels

The lipopolysaccharide (LPS), derived from intestinal Gram-negative bacteria, has been widely thought to contribute to the chronic inflammation in diverse metabolic diseases *via* translocation from the gut barrier and activating an inflammatory cascade in Mψ by binding the TLR-4 (18). Thus, we further tested the influence of FO intervention on plasma LPS of AS. The LPS in plasma was significantly increased in the MOD group ($P < 0.05$), compared to the CON group. This elevated LPS in plasma was significantly decreased after the FO intervention ($P < 0.05$) (**Table 1**), demonstrating that dietary ALA-rich FO possessed the ability to inhibit the LPS generation, lowering the intestinal permeability, as well as stabilizing the intestinal mucosal barrier.

Dietary ALA-Rich FO-Restored Gut Dysbiosis in AS

The above findings indicated that in AS, an elevated plasma LPS derived from intestinal pathogenic Gram-negative bacteria could be attenuated by a dietary ALA-rich FO treatment, which was closely related to gut dysbiosis. Alterations of gut microbiota have been increasingly considered to play a critical role in the pathogenesis of AS (15, 32, 33). Thus, we further investigated the changes of intestinal flora with the FO intervention. The fecal samples were analyzed by 16S rRNA high throughput sequencing, and the raw reads of gut microbiota in all groups were submitted in National Center for Biotechnology Information's Sequence Read Archive (NCBI SRA) with an accession number PRJNA624814.

As an alpha-diversity, the observed-species' index and rarefaction curve were used to analyze the abundance and diversity of the bacterial community. The observed-species index analysis showed that the abundance and diversity of gut microbiota were altered in the MOD group ($P < 0.05$) (**Figure 3A**), compared with the CON group. There was no significance after the FO intervention ($P > 0.05$) (**Figure 3A**). The rarefaction curve tended to be flat when the sequence number increased to 10,000, indicating that the amount of sequencing data was reasonable (**Figure 3B**). The overall

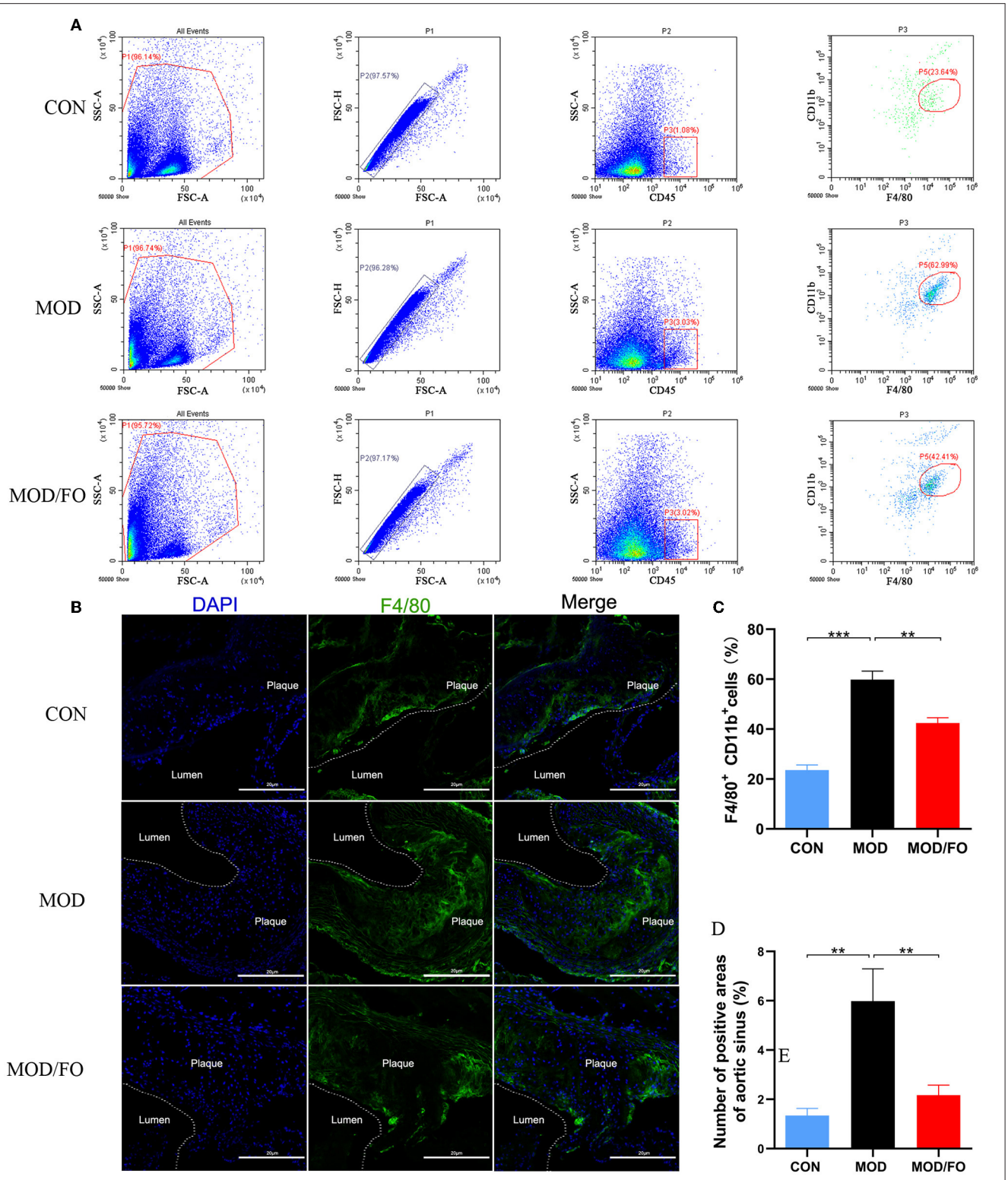

FIGURE 2 | ALA-rich FO inhibited inflammatory Mψ in atherosclerosis by flow cytometry and Immunofluorescence. After obtaining cell suspension from the aortic roots of atherosclerotic *ApoE*$^{-/-}$ mice, the Mψ stained by CD45^{+}, F4/80^{+}, and CD11b^{+} were detected by flow cytometry. **(A)** Detection of Mψ by KO525 conjugated CD45^{+}, PE conjugated F4/80^{+} antibody, and FITC conjugated CD11b^{+} antibody. **(B)** F4/80^{+} cells in immunofluorescence image. In flow cytometry, quantitative analysis as F4/80^{+} CD11b^{+} cells **(C)**. In immunofluorescence assay, quantitative analysis as F4/80^{+} cells **(D)**. Values are given as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

bacterial community structure was analyzed by the unweighted UniFrac (PCoA) (**Figure 3C**) and by the weighted distance matrices (NMDS) (**Figure 3D**). We found differential clusters in PCoA of gut microbiota between MOD and CON groups, as well as distinct clusters in PCoA after the FO supplementation compared to the MOD group (**Figure 3C**). Consistently, the NMDS analysis showed similar results (**Figure 3D**).

The differential gut bacteria of mice in diverse groups were further determined. First, the Venn diagram showed that 269 core species were observed in all groups, whereas 67, 70, or 25 species were specific in CON, MOD, or MOD/FO group (**Figure 3H**). Second, at the phylum level, *Firmicutes* and *Bacteroidetes* constituted of 2 dominant bacteria in all groups (**Figure 3E**). The proportions of *Firmicutes* and *Proteobacteria* were significantly increased and *Bacteroidetes* was decreased in the MOD group compared with those in the CON group (all $P < 0.05$) (**Figure 3F**). The ratio of *Firmicutes*/*Bacteroidetes* (F/B) was increased ($P < 0.001$) (**Figure 3G**) in the MOD group, which was reversely decreased after the intervention of dietary ALA-rich FO ($P < 0.001$) (**Figure 3G**). Thus, the ALA-rich FO had a major influence on the F/B ratio under the HFD feeding in the atherosclerotic mice.

To further evaluate the effect of FO on the genus level of the microbial community, we analyzed the top 40 species (**Figure 3I**). Compared with those in the CON group, the abundance of *Intestinimonas*, *Bilophila*, *Anaerotruncus*, *Oscillibacter*, *Negativibacillus*, *Blautia*, *Parabacteroides*, *Muribaculum*, and *Parasutterella* in the MOD group were increased ($P < 0.05$) (**Figure 3J**), whereas *Alistipes* and *Candidatus Saccharimonas* were decreased. However, after the FO administration, the abundances of *Intestinimonas*, *Bilophila*, *Anaerotruncus*, *Oscillibacter,* and *Negativibacillus* were decreased ($P < 0.05$) (**Figure 3K**). Importantly, the dietary ALA-rich FO reduced the relative abundances of *Lachnoclostridium* and *Enterorhabdus* compared with the MOD group (all $P < 0.05$) (**Figure 3K**). Collectively, our results indicate that the HFD consumption significantly altered the initial proportion of OTUs at the genus level, mainly including the increased *Intestinimonas*, *Bilophila*, *Anaerotruncus*, *Oscillibacter*, and *Negativibacillus*, as well as the decreased *Alistipes* and *Candidatus_Saccharimonas*. Conversely, the FO supplementation has restored gut dysbiosis by mainly regulating the *Intestinimonas*, *Bilophila*, *Anaerotruncus*, *Oscillibacter*, and *Negativibacillus*.

Dietary ALA-Rich FO Increased the SCFAs in Fecal Intestinal Metabolites

The intestinal metabolites' SCFAs have been reported to significantly enhance the intestinal barrier function (34). Due to the effectiveness of FO on restoring gut dysbiosis, we further examined the changes of gut microbiota's metabolites-SCFAs. As shown in the chromatogram (**Figure 4A**), each SCFA with a single peak could be clearly distinguished, indicating that the method and the data were reliable. The cluster heat map showed the difference in the contents of SCFAs among diverse groups (**Figure 4B**). The results showed that HFD decreased the amounts of acetic acid, propionic acid, and valeric acid (all $P < 0.05$) (**Figures 4C–G**) compared to the CON group; whereas the amounts of acetic acid, propionic acid, isovaleric acid, isobutyric acid, and valeric acid were increased after FO treatment, compared with the MOD group (all $P < 0.05$) (**Figures 4C–G**).

Dietary ALA-Rich FO Altered the BAs in Fecal Intestinal Metabolites

Similarly, the BAs, another type of gut microbial metabolite, have been suggested to be involved in the progression of chronic metabolic diseases (35). Thus, we further determined the influence of FO on BAs by LC-MS (**Figure 5A**). The cluster heat map showed the difference in the contents of BAs among diverse groups (**Figure 5B**). After FO intervention, allolithocholic acid (alloLCA), isolithocholic acid (isoLCA), 7-ketodeoxycholic acid (7-ketoLCA), β-ursodeoxycholic acid (β-UDCA), chenodeoxycholic acid (CDCA), and hyodeoxycholic acid (HDCA) was reduced (all $P < 0.05$) (**Figure 5C**), as well as lithocholic acid (LCA), allocholic acid (ACA), glycocholic acid (GCA), and taurocholic acid (TCA) showed an increase (all $P < 0.05$) (**Figure 5C**), revealing that dietary FO could modulate the microbial BAs metabolism in AS.

Correlation Analysis Among Gut Microbiota, Intestinal Metabolites, Inflammation

Moreover, we performed a correlation analysis among the differential bacteria, inflammation, LPS, SCFAs, and BAs in AS (**Figure 6**). The differential bacteria in the MOD group include *Intestinimonas*, *Bilophila*, *Anaerotruncus*, *Oscillibacter*, *Negativibacillus*, *Blautia*, *Parabacteroides*, *Muribaculum*, *Parasutterella*, *Alistipes,* and *Candidatus_Saccharimonas* showed close correlations with inflammation (**Figure 6A**). Interestingly, the reduced bacteria after the FO intervention involving the *Intestinimonas*, *Bilophila*, *Anaerotruncus*, *Oscillibacter*, *Negativibacillus*, *Lachnoclostridium,* and *Enterorhabdus* were positively correlated with pro-inflammatory cytokines and were negatively correlated with anti-inflammatory IL-10 (**Figure 6B**). In addition, the above downregulated bacteria after the FO intervention were negatively correlated with SCFAs, LCA, ACA, GCA, and TCA, and positively correlated with alloLCA, isoLCA, 7-ketolca, β-UDCA, CDCA, HDCA, and α-MCA (**Figure 6**). Subsequently, acetic acid, propionic acid, isovaleric acid, isobutyric acid, and valeric acid mainly showed a negative correlation with pro-inflammatory indicators and a positive association with anti-inflammatory IL-10 (**Figure 6C**). The LCA, ACA, GCA, and TCA were negatively related with inflammatory indicators, whereas other BAs metabolites alloLCA, isoLCA, 7-ketolca, β-UDCA, CDCA, HDCA, and α-MCA were positively correlated with inflammation (**Figure 6D**). Taken together, the critical differential species and SCFAs/Bas metabolites were determined in close and complicated interactions and correlations among gut bacteria, metabolites, and inflammation.

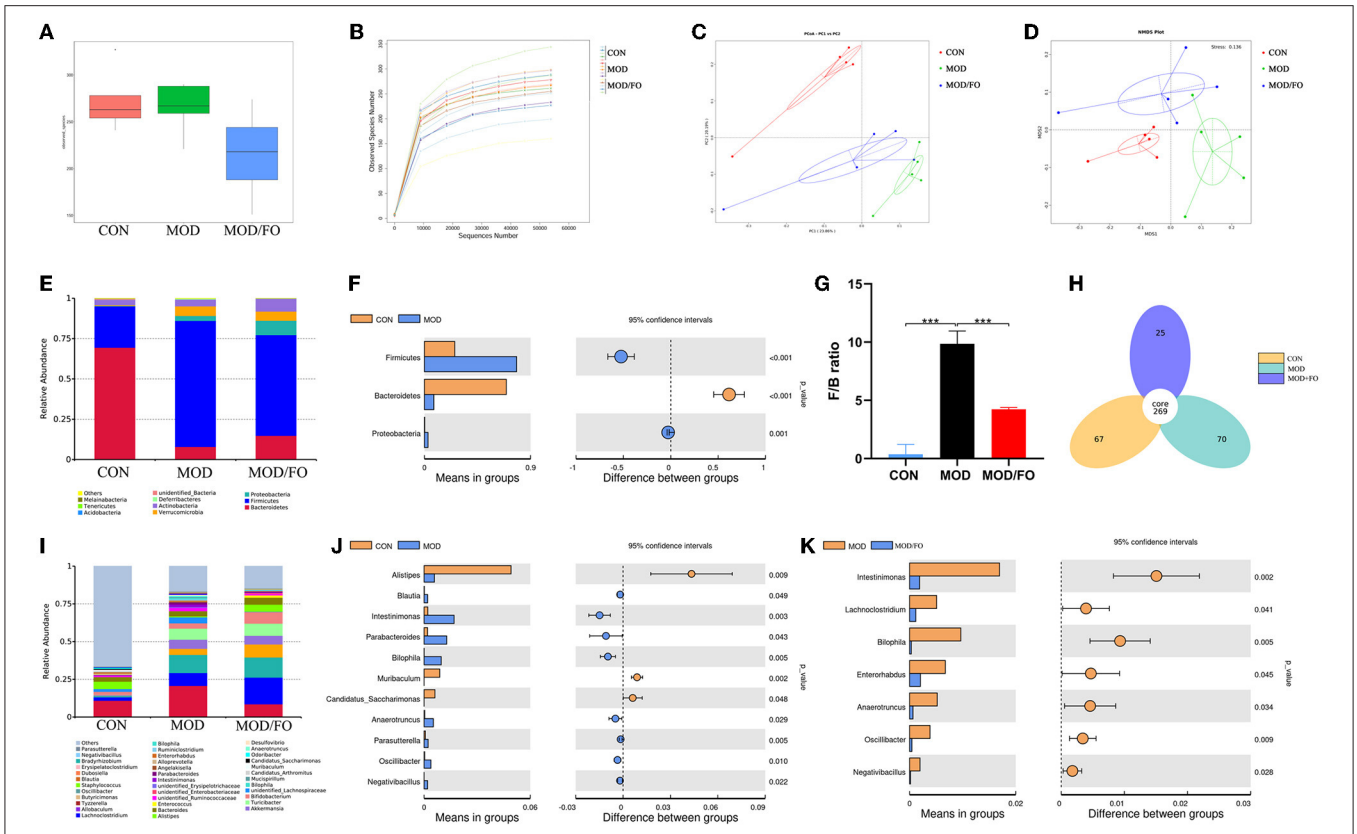

FIGURE 3 | Gut microbial community in fecal samples of different groups. **(A)** Observed-species index. **(B)** Rarefaction Curve. **(C)** PCoA analysis. **(D)** NDMS analysis. **(E–G)** The phylum level. **(H)** Venn diagram. **(I–K)** The genus level. Values are given as mean ± SEM. ***$P < 0.001$.

Determination of Gut Flora Involved in the Amelioration of Dietary ALA-Rich FO on AS by the Treatment of Antibiotic (AB) Cocktail

In the above studies, we found that the FO administration inhibited the occurrence of inflammation and LPS in AS, related to the disturbance of gut microbiota and microbial metabolites SCFAs/BAs, as well as intestinal barrier function. In this part of the experiment, to further determine whether the amelioration of FO was dependent on the improvement of gut microecology, an AB cocktail treatment was adopted to negatively demonstrate the role of gut microbiota in the FO effectiveness (**Figure 7A**). Intriguingly, we found that the plaque area in the aortic sinus was higher in AB/FO group than that in the MOD/FO group by oi- red O staining ($P < 0.05$) (**Figure 7B**), suggesting that the gut dysbiosis rectification contributed to the ALA beneficial effects on AS. Additionally, the area of atherosclerotic plaque in the AB/FO group showed milder symptoms by oil-red O staining ($P < 0.01$) (**Figures 7A,B**), compared to AB/MOD group, suggesting that ALA-rich FO treatment may partially attenuate the AS through the gut microbiota-independent pathway. The *en* face oil-red O staining and Masson's trichrome staining have shown no significant difference in AB/FO group ($P > 0.05$), compared to AB/MOD group (**Figures 7C,D**).

Finally, we measured the Mψs by PE-conjugated anti-mouse F4/80 antibody, and the FITC-conjugated anti-mouse TLR4 antibody was detected by flow cytometry in the orthotopic aortic tissues in diverse groups (**Figure 8**). We found that the aortic Mψs content within aortic lesions was higher in AB/FO group ($P < 0.05$) than that in the MOD/FO group by immunofluorescence, indicating that the modulation of gut microbiota by ALA-rich FO intervention contributed to the anti-inflammation effects on AS. In addition, the aortic Mψs content within the aortic lesions was reduced in the AB/FO group ($P < 0.01$), compared with the AB/MOD group (**Figures 8A,B**), suggesting that the suppression of the Mψs-mediated inflammation after the ALA administration in AB-treated condition was partially independent on the gut microbiota. The same result occurred in aortic Mψ as detected by flow cytometry ($P < 0.05$) (**Figures 8C,D**).

DISCUSSION

In clinical and experimental studies, the consumption of ω-3 PUFAs has exhibited pleiotropic benefits in the control of chronic diseases (36, 37). The dietary ALA-rich FO, a source of plant-derived ω-3 PUFAs, has been widely used as a systemic anti-inflammatory approach in metabolic diseases, such as diabetes and alcoholic fatty liver (23), confirming the reliability of dietary FO. This study mainly explored the protective effects of dietary FO intervention on HFD-induced AS and the underlying gut microbiota-inflammation-artery axis in this effectiveness.

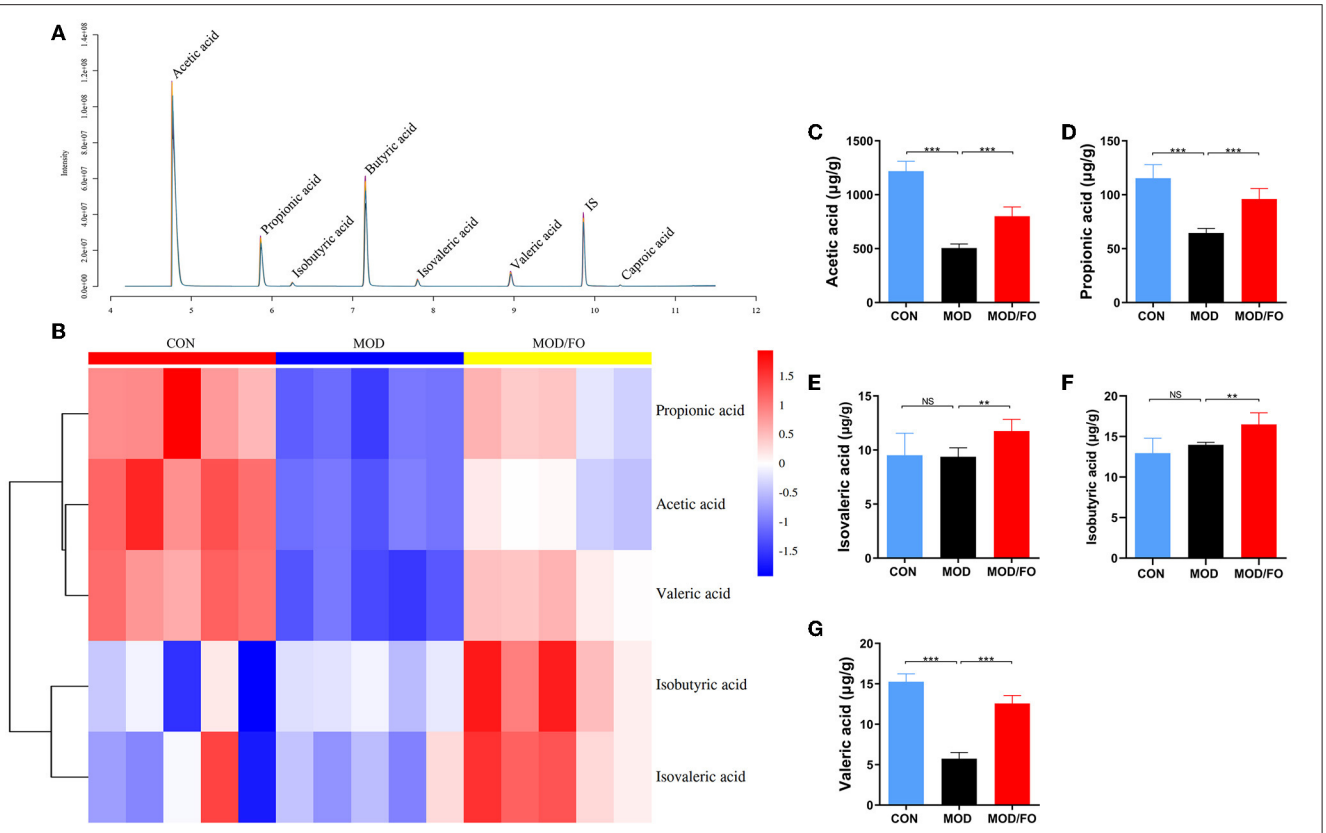

FIGURE 4 | Measurement of the contents of short-chain fatty acids (SCFAs) in fecal intestinal metabolites. **(A)** Chromatogram of mice stool. **(B)** Heat map clustering. **(C)** Determination of levels (μg/g) of Acetic acid, Propionic acid **(D)**, Isovaleric acid **(E)**, Isobutyric acid **(F)**, and Valeric acid **(G)** by gas chromatography-mass spectrometer (GC-MS). Data were presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, NS, no significance.

The mice lacking an *apolipoprotein E* are especially vulnerable to a series of complex vascular lesion symptoms under the stimulation of HFD, which were comparable to human lesions (38). In this study, we confirmed that FO could improve the AS pathological injury in atherosclerotic $ApoE^{-/-}$ mice with HFD. The FO intervention on the food intake in our study showed a limited influence, which indicated that the effectiveness of FO treatment on AS was not dependent on nutrients absorption and on the efficiency of calorie utilization in the gastrointestinal tract in AS, which was consistent with previous reports (12).

To further reveal the underlying mechanisms of dietary FO amelioration on AS, we assessed the gut microbiota alterations due to a critical role of gut dysbiosis in the pathogenesis of AS (39, 40). In this study, we found that phyla *Bacteriodetes* and *Firmicutes* were kept predominantly in diverse groups, which are paralleled with previous studies (18, 23, 32). An increase in *Firmicutes*/*Bacteriodetes* ratio is closely related to chronic metabolic diseases [(24, 25, 41)]. In this study, the increased ratio of *Firmicutes*/*Bacteriodetes* in AS was rectified by the dietary FO administration, suggesting that FO could markedly modulate the gut microbiota by decreasing the predominant *Firmicutes*/*Bacteriodetes* in phylum level (42).

Moreover, at the genus level, the reduced intestinal bacteria after the FO administration, including *Anaerotruncus*, *Enterorhabdus*, *Lachnoclostridium*, *Negativibacillus*, and *Bilophila*, were Gram-negative bacteria and anaerobic bacteria, which were closely related to the production of LPS (43, 44). In these differential bacteria, the *Anaerotruncus* that was isolated from the blood culture of a 78-year-old woman with nosocomial bacteraemia, is of clinical significance (45). Clavel (46) first isolated the *Enterorhabdus* from patients with colitis and found that it was probably associated with the inflammation of the disease (46). Intriguingly, relevant studies have shown that *Lachnoclostridium* is significantly enriched in tumors and has been identified as a novel bacterial marker for the non-invasive diagnosis of colorectal adenoma (47). Early studies have shown that a high-fat dairy diet in mice will increase bile production, leading to an increase in *Bilophila* abundance (48). Taken together, the improvement of FO intervention on AS may be due to the rectification of gut dysbiosis. In order to determine the crucial role of gut microbiota in the effectiveness of FO on the development of AS, as previously described (28), the AB cocktails were used to eliminate the effect of gut microbiota in $ApoE^{-/-}$ mice. After 3 weeks of AB cocktails, gut microflora was found with difficulty by 16S rRNA sequencing, demonstrating that mimic germ-free mice were successfully established. During the subsequent 12 weeks of treatments in diverse groups, a significantly weakened amelioration of dietary FO treatment

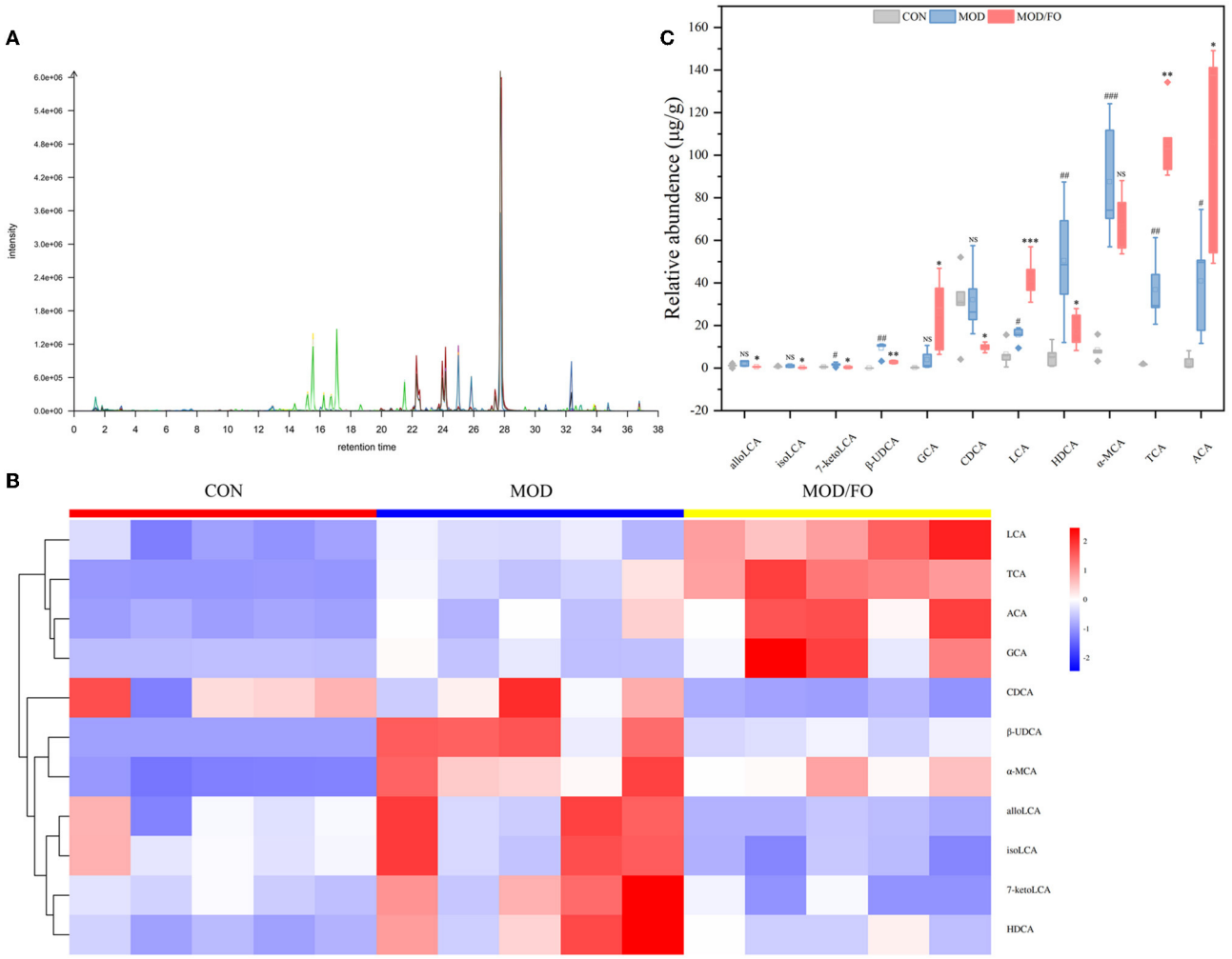

FIGURE 5 | Levels of BAs in fecal intestinal metabolites. **(A)** Chromatogram of mice stool. **(B)** Heat map clustering. **(C)** Determination of levels (μg/g) of allolithocholic acid (alloLCA), isolithocholic acid (isoLCA), 7-ketodeoxycholic acid (7-ketoLCA), β-ursodeoxycholic acid (β-UDCA), chenodeoxycholic acid (CDCA), and hyodeoxycholic acid (HDCA), lithocholic acid (LCA), allocholic acid (ACA), glycocholic acid (GCA), and taurocholic acid (TCA) by Liquid chromatography-mass spectrometer (LC-MS). Data were presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, CON group vs. MOD group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, MOD group vs. MOD/FO group. NS, no significance.

on AS suggested that intestinal microbiota contributed to the FO effectiveness.

The LPS, derived from pathogenic bacteria in gut dysbiosis, represents a causal link between gut microbiota and low-grade systemic inflammation (49). Numerous studies have demonstrated that andenterogenic endotoxemia-mediated systemic chronic inflammation aggravates the pathogenesis of AS (50). Our data revealed that the LPS in plasma were markedly decreased with FO supplementation, demonstrating that dietary FO intervention may improve the gut permeability, thus enhancing the gut barrier, to decrease the production of LPS translocation to the systemic circulation. A previous study indicated that genetic deletion of *TLR-4* and *Myd88* in *ApoE*$^{-/-}$ mice were associated with a reduced Mψ inflammatory response and aortic lesions (11). Thus, we speculated that the anti-inflammation role of dietary ALA-rich FO on AS in *ApoE*$^{-/-}$ mice might be through the LPS/TLR-4/NF-κB pathway, but the exact evidence needs to be further researched.

The above demonstrated the attenuation of FO intervention on AS through rectifying the gut dysbiosis, enhancing the gut barrier and conversing the LPS translocation. Subsequently, the impacts of dietary FO on LPS mediated-inflammation consequences were further determined. Numerous studies have solidly demonstrated that chronic inflammation is dramatically involved in the development and the rupture of atherosclerotic plaque (51, 52). The Mψs exerted a predominant inflammatory role in AS lesion formation, as well as plaque rupture (53, 54). In the present study, the proportions of F4/80^{+} cells and F4/80^{+} TLR4^{+} cells was significantly decreased with the

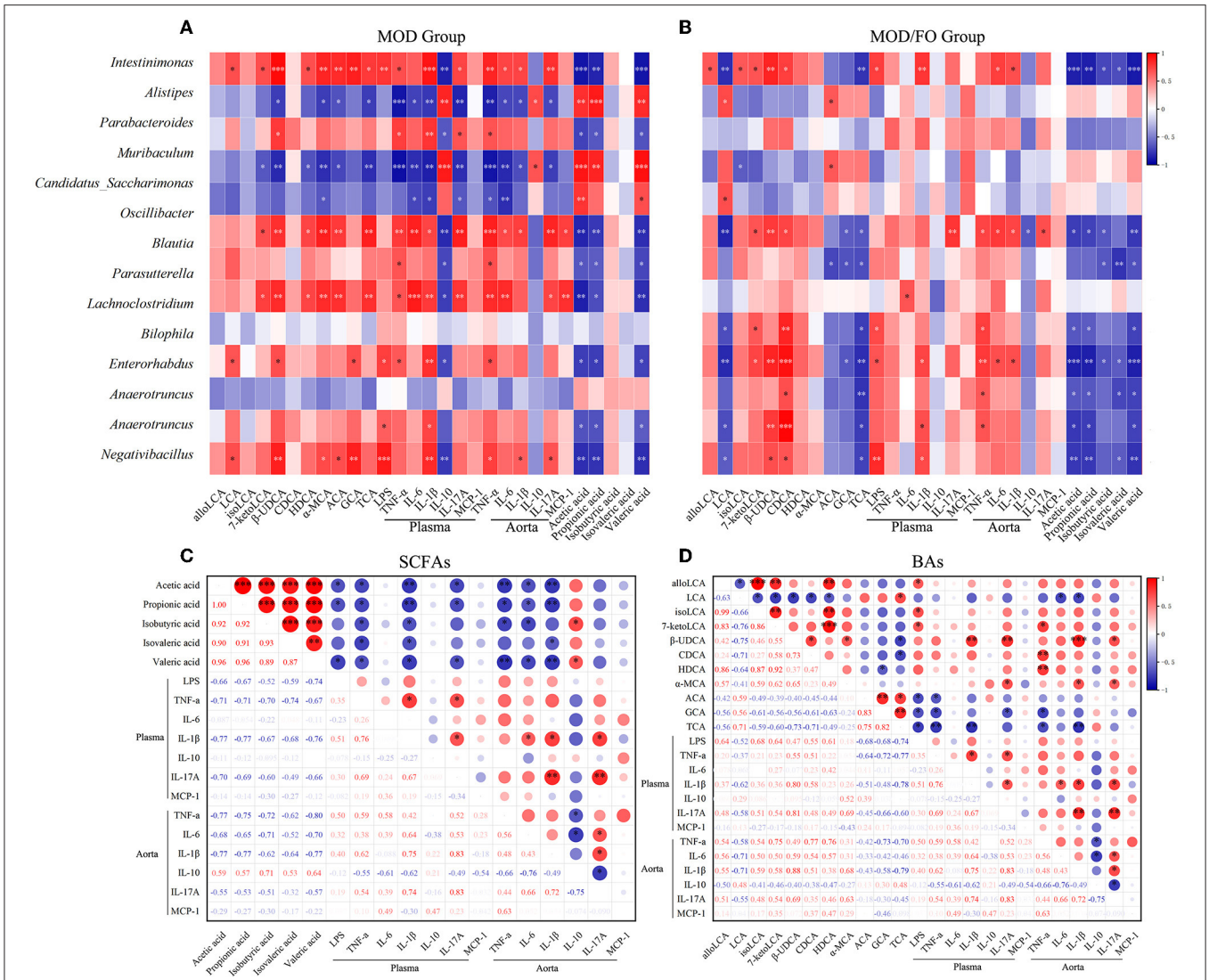

FIGURE 6 | Correlation analysis among bacteria, lipopolysaccharide (LPS), short-chain fatty acids (SCFAs), bile acids (BAs), and inflammatory cytokines. **(A)** Correlation among the differential bacteria with metabolites (SCFAs and BAs), LPS, and plasma/aortic inflammatory indicators in MOD group. **(B)** Correlation among the differential bacteria with metabolites (SCFAs and BAs), LPS, and plasma/aortic inflammatory indicators in MOD/FO group. **(C)** Correlation among SCFAs with LPS and plasma/aortic inflammatory indicators. **(D)** Correlation among BAs with LPS and plasma/aortic inflammatory indicators. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Red and blue represent the positive or negative correlation.

dietary FO administration, suggesting that the anti-inflammation effect of ALA-rich FO intervention might be mainly due to the inhibition of inflammatory Mψs. We speculated that ALA-rich FO may suppress the pro-inflammatory M1 Mψs against the exacerbated inflammation and, thus, limit the aortic injury. However, the exact molecular mechanism of FO on Mψs polarization needs to be further researched. In parallel, pro-inflammation plasma TNF-α, IL-1β, and IL-17A, as well as the aortic tissues TNF-α, IL-6, and IL-1β were suppressed after the treatment. Also, further correlation analysis discovered that pro-inflammatory cytokines and Mψs were closely related to the above differential intestinal flora, indicating that FO may regulate the gut microbiota for the contribution of inflammation suppression. Additionally, whether other inflammatory-related cells, such as regulatory T cells (Tregs), T helper cell 17 (Th17), and myeloid-derived suppressor cells (MDSCs), are involved in the improvement of FO on AS inflammation still need to be investigated in our subsequent research. Moreover, intriguingly, we found during the AB-treated condition that the FO intervention could also lower the inflammatory levels, suggesting that the anti-inflammation effect of ALA-rich FO may be partially independent of modulation of gut microbiota. Emerging evidence has demonstrated that ω-3 PUFAs, including ALA, EPA, and DHA, can serve as a novel anti-inflammation approach by directly binding these to the G protein-coupled receptor (GPR) 40, GPR120, and GPR119 (12, 27). The

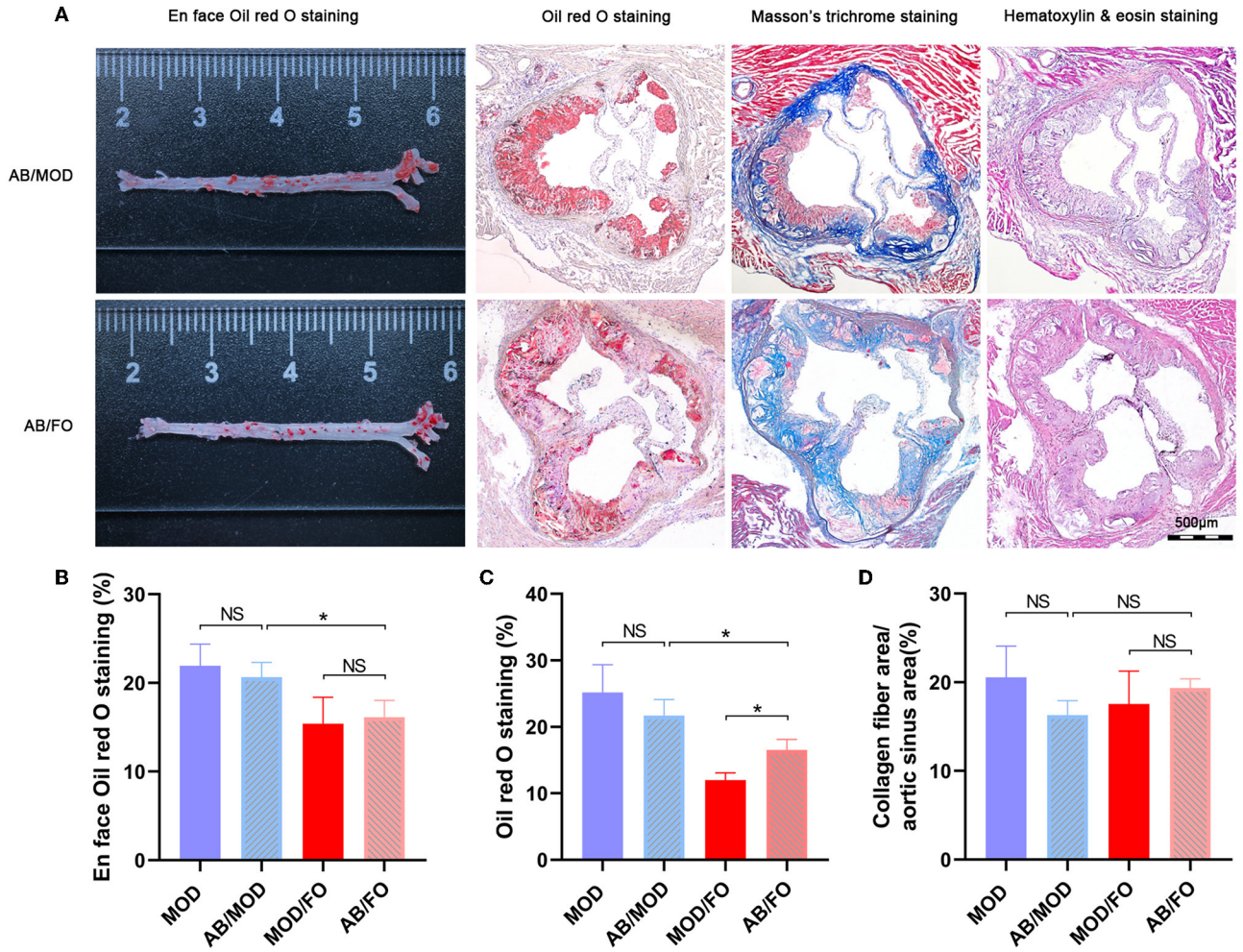

FIGURE 7 | Effects of dietary ALA-rich FO treatment on the pathological lesion in antibiotic (AB)-treated mice. **(A)** Representative sections of the valve area of the aorta and aortic root of the heart were stained with *en* face oil-red O staining, oil-red O staining, Masson's trichrome staining, and Hematoxylin and eosin staining, respectively. Quantitative analysis as lesion area/total area (%) shown in oil-red O staining **(B)**, *en* face oil-red O staining **(C)**, and Masson's trichrome staining **(D)**. *$P <$ 0.05, NS, no significance. Original magnification, ×40. The bar of 500 μm was presented in the right corner of **(A)**. All experiments were performed in triplicates.

interconversions among diverse ω-3 PUFAs and related signaling pathways are further investigated.

In the process of dietary FO administration on rectifying the gut dysbiosis in AS, gut microbiota metabolites SCFAs, tryptophan metabolism, Bas metabolism, and trimethylamine oxide (TMAO) have been reported to play a critical role in the occurrence and the development of chronic metabolic diseases (55). The SCFAs, a type of critical gut microbiota metabolites, as a link between microbiota and host homeostasis, play an important role in the regulation of inflammation and intestinal barrier function (56). However, the effect of dietary FO intervention on SCFAs in AS was only few as known previously. In this study, the increased levels of acetic acid, propionic acid, isobutyric acid, isovaleric acid, and valeric acid after dietary FO administration were negatively correlated with inflammatory indicators, indicating the beneficial effects of SCFAs in the attenuation of FO on AS. Moreover, the anti-inflammatory and immunomodulatory effects of SCFAs might be due to the activation of specific cell receptors, such as the GPR41, GPR43, and major intestinal receptors GPR109a. Additionally, the intracellular target of SCFAs *via* inhibiting the activity of histone deacetylase (HDACs) is involved in the regulation of expression of genes promoting pathogenesis in many diseases (57). Besides, the SCFAs promote the increase of intestinal TJP (tight junction protein) through intestinal mucosal receptors of monocarboxylate transporter 1 (MCT-1) and sodium-coupled monocarboxylate transporter 1 (SMCT-1), to block the LPS translocation into the blood circulation and to ultimately suppress the systemic inflammation (19). The accurate mechanisms of these different SCFAs metabolites *via* binding to multiple specific corresponding sensors in AS, with or without dietary FO treatment, still need to be separately determined.

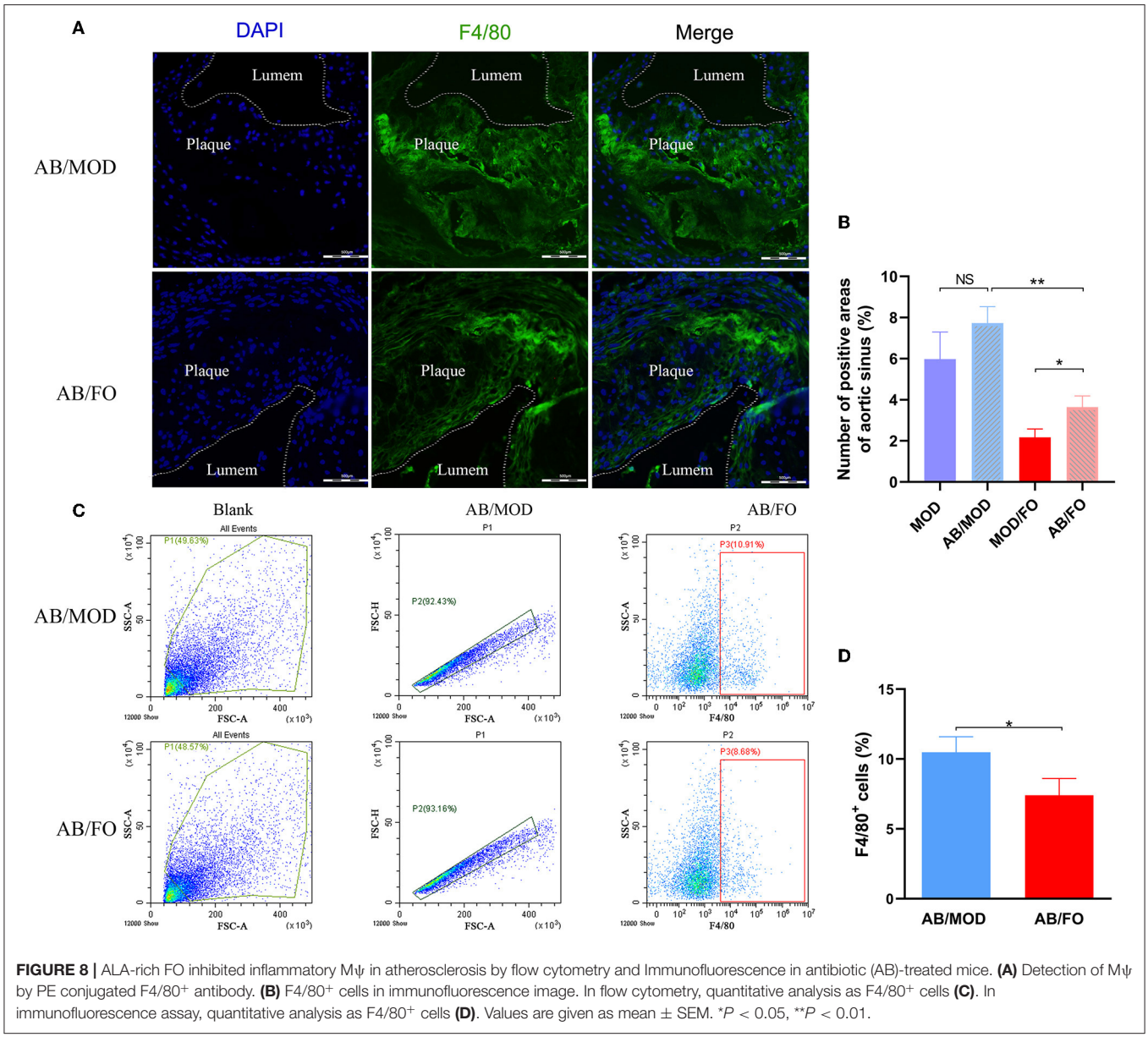

FIGURE 8 | ALA-rich FO inhibited inflammatory Mψ in atherosclerosis by flow cytometry and Immunofluorescence in antibiotic (AB)-treated mice. **(A)** Detection of Mψ by PE conjugated F4/80$^+$ antibody. **(B)** F4/80$^+$ cells in immunofluorescence image. In flow cytometry, quantitative analysis as F4/80$^+$ cells **(C)**. In immunofluorescence assay, quantitative analysis as F4/80$^+$ cells **(D)**. Values are given as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

Apart from SCFAs, the BAs' metabolites play an essential role in integrating multiple homeostatic functions in the liver and gastrointestinal tract (58). However, the impact of dietary FO intervention on BAs in AS was little known previously. Interestingly, a significant decrease of the plasma TBA indicated that the anti-AS effect of FO may be associated with the regulation of the BA biosynthesis. Furthermore, a series of differential BAs' metabolites, including decreasing alloLCA, isoLCA, 7-ketoLCA, β-UDCA, CDCA, and HDCA, as well as up-regulating LCA, ACA, GCA, and TCA, were found with the dietary FO administration, showing that the ALA-rich FO had a capacity of regulating the gut microbiota-associated BAs' metabolites for the potential protection against AS. Further correlation analysis also demonstrated that these differential BAs' metabolites were markedly related to atherosclerotic inflammation. Accumulating studies have revealed that BA-sensing receptors, including farnesoid X receptor (FXR) and G protein-coupled BA receptor 1 (TGR5) (59), have mediated the anti-inflammation effects *via* NcoR1-NF-κB/NLRP3 and cAMP-PKA-NF-κB/NLRP3 pathways, respectively (60, 61). Thus, the exploration of the underlying mechanisms of these differential BAs' metabolites *via* binding to specific receptors in AS, with or without dietary FO treatment, might contribute to the understanding of BAs' feedback regulation in AS.

CONCLUSION

This study highlighted that the dietary ALA-rich FO mainly ameliorated the HFD-induced AS *via* gut microbiota-inflammation-artery axis in *ApoE*$^{-/-}$ mice, which potentially

served as the inexpensive interventions for the prevention and treatment of the disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA624814.

ETHICS STATEMENT

This animal experiment has been approved by Ningxia Medical University Ethics Committee (No. 2019-137).

AUTHOR CONTRIBUTIONS

HW, XZ, and YiL: conceptualization and validation. YiL, ZY, YuL, TW, YaL, ZB, YR, HM, TB, HL, RW, LY, NY, XZ, and RY: methodology and investigation. YiL and ZY: software and formal analysis. HW: resources and project administration. YiL, ZY, YuL, and TW: data curation. YiL, ZY, and HW: writing—original draft preparation. HW and YiL: writing—review and editing. YiL: visualization. XZ and HW: supervision. HW, XZ, and SJ: funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.830781/full#supplementary-material

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REVIEW
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Short-Chain Fatty Acids in the Metabolism of Heart Failure – Rethinking the Fat Stigma

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Heart failure (HF) remains a disease with immense global health burden. During the development of HF, the myocardium and therefore cardiac metabolism undergoes specific changes, with decreased long-chain fatty acid oxidation and increased anaerobic glycolysis, diminishing the overall energy yield. Based on the dogma that the failing heart is oxygen-deprived and on the fact that carbohydrates are more oxygen-efficient than FA, metabolic HF drugs have so far aimed to stimulate glucose oxidation or inhibit FA oxidation. Unfortunately, these treatments have failed to provide meaningful clinical benefits. We believe it is time to rethink the concept that fat is harmful to the failing heart. In this review we discuss accumulating evidence that short-chain fatty acids (SCFAs) may be an effective fuel for the failing heart. In contrast to long-chain fatty acids, SCFAs are readily taken up and oxidized by the heart and could serve as a nutraceutical treatment strategy. In addition, we discuss how SCFAs activate pathways that increase long chain fatty acid oxidation, which could help increase the overall energy availability. Another potential beneficial effect we discuss lies within the anti-inflammatory effect of SCFAs, which has shown to inhibit cardiac fibrosis – a key pathological process in the development of HF.

Keywords: heart failure, short-chain fatty acid (SCFA), fatty acid oxidation (FAO), metabolic reprogramming, cardiac fibrosis (CF)

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INTRODUCTION

Heart failure (HF) is one of the leading causes of hospitalization globally and is associated with substantial mortality. In addition to an overwhelming prevalence of more than 37.7 million people globally, the incidence is expected to grow further due to the increasing age of the world's population. Several common diseases like coronary artery disease and hypertension can cause this clinical syndrome, which is characterized by a growing inability of the heart to meet the body's demand in oxygen supply in form of circulating blood (1). The development of new HF drugs has made an important contribution to improving the life expectancy of patients with HF. However, these patients still suffer from a distinctly diminished quality of life, have reduced exercise capacity and 50% still die within 5 years of the initial diagnosis (2).

HF is defined as the inability of the heart to pump sufficient blood to meet the bodies energy requirements and can manifest itself with a number of symptoms such as dyspnea, fatigue and congestion. All cardiac diseases can ultimately cause heart failure, making cardiac dysfunction the final common endpoint of heart disease. Irrespective of the etiology the myocardium undergoes a number of pathological changes once HF has developed, characterized by cardiomyocyte hypertrophy, microvascular dysfunction, fibrosis and alterations in metabolism, often referred to as pathological cardiac remodeling (3). The heart is the organ with the highest energy turnover in the body which is required to sustain the circulation, continually converting chemical energy into mechanical work. It can only store enough energy to sustain three heart beats, making it exquisitely sensitive to disturbances in the supply of oxygen and metabolites. This is not only apparent from the clinical consequences of coronary artery disease. The development of all forms of HF is also associated with maladaptive changes in cardiac metabolism that eventually cause an energy deficit.

Most cardiac energy (60–90%) is generated by the oxidation of (long-chain) fatty acids (FA). The remainder is thought to primarily originate from carbohydrates. The heart is, however, a metabolic omnivore that alters its substrate use depending on prevailing supply and metabolic conditions. In HF, the mitochondrial capacity to oxidize FA and carbohydrates is diminished and ATP production is reduced (4). Although FA remain the main energy source for the failing heart, a "substrate switch" from FA toward glucose oxidation is thought to occur (5). Based on the dogma that the failing heart is oxygen-deprived and on the fact that carbohydrates are more oxygen-efficient than FAs, metabolic drugs for HF have so far aimed to either stimulate glucose oxidation or inhibit FA oxidation (4). Unfortunately, decades of clinical trials testing various compounds have not produced meaningful clinical benefits. We believe that it is time to rethink the concept that fats are bad for the heart. In this review, we will first introduce the metabolism of the healthy heart, subsequently that of the diseased, failing heart, and finally the mechanisms enabling fatty acids to potentially play a role in the treatment of HF in spite of their bad reputation.

CARDIAC METABOLISM OF THE HEALTHY, PHYSIOLOGICAL HEART

As mentioned, the heart can switch between various substrates as sources of energy, and many adaptive metabolic changes occur during sustained exercise and pregnancy (6). The most dramatic change occurs during the transition from fetal to adult state in which glucose and lactate are the main cardiac energy sources, FA oxidation predominates thereafter (7). This switch toward FA oxidation is thought to be mediated by long-chain fatty acids (LCFAs) in breast milk, which activate the peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) - peroxisome proliferator-activated receptor (PPAR) -α, -β, and -δ pathways as key transcriptional regulators of the genes responsible for FAO. This has several downstream effects, such as higher expression of fatty acid transporters, increased mitochondrial biogenesis and mitochondrial metabolism, leading to the described switch in substrate preference (8).

In the physiological adult heart of model organisms, ≈65% of ATP derives from FAO, the lion's share being long-chain fatty acids and ≈30% from oxidation of pyruvate, which is either the product of lactate oxidation or glycolysis, leaving only a minimal amount of ≈5% coming from anaerobic pathways (9–11). The metabolic benefit of this energetic switch is obvious, the net energy yield of long-chain FAO is 105 ATP molecules per molecule of palmitic acid, a common long-chain fatty acid. This is substantially higher than for a glucose molecule where oxidation results in 31 ATP per molecule or two ATP molecules for anaerobic glycolysis. The process of FAO is well-described, but a short overview of the steps of FAO is necessary to understand the changes in dysfunctional myocardium that are discussed below. Fatty acids enter the body via the digestive tract. While LCFAs and medium-chain fatty acids (MCFAs) require transporters to be taken up by enterocytes, short-chain fatty acids (SCFAs) can diffuse freely into the intestinal cells (12). Within the enterocyte, LCFAs and MCFAs together with lipoproteins form chylomicrons for transport. Chylomicrons are transported via the lymphatic system and enter the bloodstream via the thoracic duct. SCFAs are transported directly to the blood via the portal vein to the liver and are then released into systemic circulation (12). From the blood, SCFAs are taken up for further metabolization and oxidation by cardiomyocytes and other cell types of the heart.

The regulation of FA oxidation by cardiac myocytes is controlled at two regulatory hubs, the passage across the cell membrane and the passage across the mitochondrial membrane, although those differ depending on the chain length of the FA. LCFAs are taken up in a transporter-dependant manner. To enter the cell LCFAs bind to fatty acid translocase (FAT/CD36), which leads to internalization of both FAT/CD36 and LCFAs (13, 14). Inside the cell LCFAs are bound to carnitine to enable transport across the inner mitochondrial membrane by the carnitine shuttle consisting of carnitine palmitoyltransferase (CPT) 1 and 2. Since this is the rate-limiting step of FAO, CPT1 is considered the pacemaker enzyme of FAO (10, 15). MCFAs enter the cell via monocarboxylate transporter 1 (MCT1) and are also transported to the mitochondrial matrix by CPT1. MCFAs with a chain length of eight carbon atoms or less, can diffuse freely across the mitochondrial membrane (16, 17). SCFAs enter the cell via MCT1, or diffuse into the mitochondria in a transporter-independent manner. Their passage into the mitochondrion is also transporter-independent. This makes the utilization of SCFAs and MCFAs less susceptible to perturbations in transporter abundance and kinetics. However, oxidation of SCFAs is largely independent of the described transporters, which might provide the vital advantage over MCFAs in terms of oxidation rates, a fact which we will discuss in more detail later (18). Once inside the mitochondrion, all fatty acids are broken down through beta-oxidation, although the process is

TABLE 1 | Overview of characteristics of different fatty acids according to chain length.

	SCFA	MCFA	LCFA
Carbon chain length	1–6	6–12	13–21
Common FAs in human context	Acetic acid (C2) Propionic acid (C3) Butyric acid (C4)	Caprylic acid (C8) Capric acid (C10) Lauric acid (C12)	Myristic acid (C14) Palmitic acid (C16) Stearic acid (C18)
Typical food containing FAs	- Dairy products - Vinegar - Fermentation of fiber*	- Dairy products - Palm oil - Coconut oil	- Dairy products - Palm oil - Olive oil - Meat

SCFA, short-chain fatty acids; MCFA, medium-chain fatty acids; LCFA, long-chain fatty acids (20–23).

initiated depending on the length of the fatty acid by very-long-chain, long-chain, medium-chain and short-chain acyl-CoA dehydrogenase (VLCAD, LCAD, MCAD, SCAD) (19). The following steps of beta-oxidation are hydration, oxidation with release of electrons which enter the respiratory chain and release of an acetyl-CoA molecule which enters the Krebs cycle. This cycle is repeated several times, each time shortening the chain of FA by one molecule of acetyl-CoA; the longer the chain, the more cycles are required to degrade the FA (19). For an overview of the characteristics of LCFA, MCFA and SCFA (see **Table 1**).

Despite its negative clinical stigma as a nutrient that leads to obesity and atherosclerosis when consumed in excess, fat is essential for proper cardiac function. However, effective FAO is only guaranteed under abundant supply of oxygen (9, 24). In response to repetitive endurance stress such as in swimming, the heart becomes physiologically hypertrophied. In addition to structural adaptations such as increased capillary density, enlargement of cardiomyocytes and a moderate increase in cardiac mass, cardiac metabolism also changes (25). Upregulation of PGC-1α -PPAR-α is responsible for an increase in mitochondrial fatty acid oxidation capacity – which leads to overall increased FA utilization and oxidation (26). Additionally, PGC-1α activation induces mitochondrial biogenesis, further contributing to the increase in oxidative phosphorylation (4, 26).

The heart is considered to be a metabolic omnivore, adapting its substrate use on the prevailing conditions and availability. In the physiological setting, however, most of its energy is derived from the oxidation of LCFA. In the next section, we will explain why in the context of HF passage across the cell membrane and transport into the mitochondria are so critical.

CARDIAC METABOLISM IN THE DISEASED, PATHOLOGICAL MYOCARDIUM

Overall oxidation of FAs has been shown to be decreased in HF. This has been demonstrated in rats with cardiac hypertrophy from pressure overload and in myocardial infarction, both of which resulted in a decrease in palmitate utilization (27, 28). Oxidation of oleate, another LCFA, is decreased in pacing-induced HF in dogs (29). The findings from animal data are consistent with human data, as patients with nondiabetic dilated cardiomyopathy (CM) and, in a second study, patients with idiopathic CM had decreased palmitate oxidation (30, 31). These changes are reflected by alterations in gene expression as well. MCAD expression was downregulated in the above-mentioned model of pacing-induced HF in dogs and an infarct rat model, so was long-chain fatty acid transporter FAT/CD36 in the infarction model (28, 29). CPT1 and 2, the crucial parts of the carnitine shuttle, are also downregulated in rodent HF models (27, 32). The reduction in fatty acid oxidation is at least partially mediated by the downregulation of PPAR-α and PGC1- α, which has been shown in a rodent HF models as well as in patients with both ischemic and non-ischemic end-stage HF (33, 34). In addition, the expression of transporters CPT1 and FAT/CD36 were found to be reduced in ischemic and dilated end-stage HF in model organisms and in humans as were the enzymes responsible for beta oxidation of very-long chain and long-chain FA, VLCAD, and LCAD. The latter mechanism is thought to be responsible for a "backlog" in FA metabolism, resulting in accumulation of toxic lipid intermediates in the heart, further aggravating the metabolic derangements in HF (32, 35, 36).

Even though the above-described studies are not consistent on all levels, there is overwhelming and consistent evidence that fatty acid oxidation is perturbed in HF. The reduction in FAO leads to an increase in anaerobic metabolism in the form of glycolysis in HF with an overall decrease in the available ATP (27, 37). This reduction in FA oxidation is considered fetal remodeling – although it must be mentioned that the metabolic differences are far less pronounced than between the adult and the fetal heart. The failing heart continues to yield 70% of its energy from FAO and it is questionable whether this should be considered a "metabolic switch" or rather a disruption in FA oxidation capacity. Of note glucose oxidation is also downregulated in advanced HF, indicating that a swich toward glucose oxidation is less likely. Nevertheless, the evidence does support the hypothesis, that the energy deficiency in the failing heart is caused by the inability to process long chain fatty acids (33, 38). The disappointing results from efforts to increase carbohydrate metabolism in heart failure, have led to the search for an alternative fuel for the failing heart. Recent work from our department and from other research groups have identified ketone bodies as a promising candidates, at least in part because they are taken up by cardiac mitochondria in a transporter-independent fashion (39, 40). Intriguingly, short-chain fatty acids (SCFAs), have similar bioenergetic properties as ketone bodies and appear to be even more efficient fuels.

SHORT-CHAIN FATTY ACIDS (SCFAS) IN A HEALTHY, PHYSIOLOGICAL SETTING

Fatty acids with a carbon chain of up to six atoms are considered SCFAs (41). The most relevant ones in human metabolism are acetate (C2), propionate (C3), and butyrate (C4). SCFAs originate

from the fermentation of indigestible dietary fiber in the colon by the gut bacteria and are taken up by non-ionic diffusion and active transport into the colonocytes for which butyrate is the main source of energy. Propionate and acetate are transported via the portal vein into the liver, where propionate is further metabolized e.g., it serves as a substrate for gluconeogenesis, whilst acetate is found in higher levels in the periphery. Some butyrate also bypasses the liver by absorption in the distal colon (42) (see **Figure 1**). The contribution of SCFAs to total energy demand in healthy adults is around 10% (44).

It is well-known that a diet high in fiber is beneficial for health, people consuming high amounts of vegetables and fruits are less likely to develop obesity, diabetes mellitus and cardiovascular disease. However, the health promoting benefits spread much wider and also include protective effects for oncological diseases such as carcinomas of the colon, breast and liver (45). The majority of the beneficial effects of high-fiber diets could be linked to the production of SCFAs by the gut microbiome. In addition to serving as a fuel, SCFAs inhibit histone deacetylases (HDACs) which are known to promote inflammation and tumourigenesis (46, 47). The exact pathway of HDAC inhibition remains unclear. As of yet, direct inhibition of enzymatic activity and binding to G-protein-coupled receptors (GPCRs) are the two main proposed pathways. The main GPCRs to which SCFAs bind are GPR41, GPR43 and GPR109A (48, 49). SCFAs also protect against food allergies and colitis, and are even considered a potential drug for Alzheimer's disease and arthritis (50–53).

In rodent studies dietary supplementation with sodium butyrate prevented the usual development of insulin resistance and obesity in a high-fat diet – while food intake remained unchanged. This was thought to be the result of an increase in PGC-1α expression, which coincided with increased energy expenditure and oxygen consumption (54). A similar effect was observed with oral application of acetate, with improved insulin tolerance in mice with diabetes type II. It also lowered expression of lipogenic genes and abdominal fat mass. The authors attributed this effect to the increased phosphorylation of AMP-activated protein kinase (AMPK), which activates the PGC-1α signal pathway and thus inhibits fatty acid synthesis while increasing FAO (55). Butyrate as well-has been proven to increase phosphorylation of AMPK in adipocytes and has a stimulating effects on mRNA expression of PGC-1 and CPT1 in skeletal muscle (54, 56, 57). Acetate also influences apetite. Acute food intake in rats was lower following acetate injection by activation of hypothalamic neurons responsible for satiety (58). Oral intake of inulin-propionate ester promoted postprandial levels of satiating hormones peptide YY and glucagon-like peptide 1 and in the long term had anti-diabetic effects such as reduced weight gain, lower intra-abdominal adipose tissue mass and higher insulin sensitivity in a population of overweight humans (59). Distal colonic acetate infusion in obese men led to higher FAO rates, a fact the authors related partially to higher levels of satieting hormone peptide YY (PYY), but suggested might also be caused by an increase in AMPK like in the rodent setting (60).

SHORT-CHAIN FATTY ACIDS IN THE DISEASED, PATHOLOGICAL MYOCARDIUM

Regarding cardiovascular health, it is of note, that patients with cardiovascular diseases have an altered intestinal microbiome compared to healthy individuals. In the gut microbiome of patients with hypertension butyrate-producing bacteria are diminished and serum butyrate levels decreased (61). Also, the degree of hypertension negatively correlates with the amount of butyrate and acetate producing bacteria. This suggests that with increasing severity of hypertension the population of acetate and butyrate producers is further reduced. Furthermore, the microbiome of HF patients shows a decreased diversity, with diminished populations of butyrate-producing strains. Since the majority of SCFAs is derived from microbial fermentation, this implies that patients with HF and cardiovascular disease have a lower overall capacity to produce SCFAs (62). If that is the case, therapies aiming to restore the balance of the gut microbiome would improve the capacity to produce SCFAs. This could increase circulating serum levels of SCFAs, thereby increase the availability of nutrients for the failing heart.

Murashige et al. analyzed which nutrients the failing human heart uses to fulfill its energy demands. They found ketone, lactate and acetate metabolism to be increased. Even though the overall contribution of SCFAs to cardiac ATP production was relatively low, acetate extraction was increased by ≈20% in the HF cohort. Acetate uptake by the heart was also proportional to the circulating levels, suggesting that there is a large cardiac spare capacity for acetate oxidation that may be utilized for therapeutic purposes. In other words, increasing systemic SCFAs could be beneficial for energy production in HF (63). The increase in ketone as well as SCFA oxidation, is likely due to the fact, that their uptake into cardiac mitochondria is independent of CD36 and CPT1, enzymes that are downreglated in HF (18, 56, 64) (see **Figure 2**).

In an *ex-vivo* langendorff perfused heart model, Carley et al. (65) compared the oxidation rates of butyrate and the ketone body 3-OHB. Ketone body metabolism is increased in HF and substitution of ketones has been successfully tested as a strategy to improve myocardial function in HF mouse and rat models (39). Surprisingly, ATP yield from butyrate was significantly higher than from ketone bodies, both in healthy and failing hearts. They did not observe differences in metabolic regulation between substrates, suggesting that SCFAs are more efficient fuels than ketone bodies (65).

The same authors also evaluated further metabolic changes in human and rat HF. As expected, enzymes responsible for LCFA oxidation were reduced in failing hearts. Interestingly, however, they discovered a higher expression of acyl-CoA synthetase medium-chain family member 3 (ACSM3), an enzyme involved in butyrate oxidation (56, 66). ACSM3 is specific for fatty acids with carbon chains lenghts between 4 and 14 – this would make butyrate an ideal fuel candidate since it is a C4 fatty acid. Acetate and propionate would not be increasingly oxidized by this upregulation as they are no target substrates with C2 and

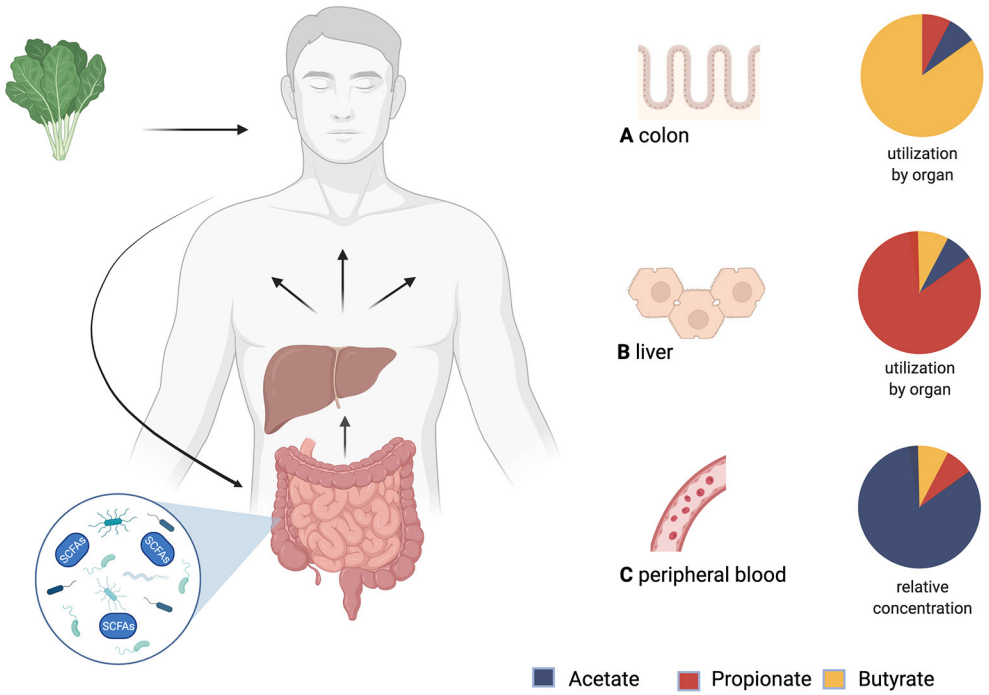

FIGURE 1 | Production, distribution and yield of short-chain fatty acids (SCFAs) in different tissues. After consumption, undigested fiber reaches the colon and is fermented by microbiota. The product of this process are SCFAs. Via the portal vein SCFAs are transported to the liver and then systemically distributed. **(A)** Utilization of SCFAs in the colon: Butyrate is an important energy source for colonocytes. **(B)** Utilization of SCFAs in the liver: Propionate is mostly metabolized in the liver. **(C)** Relative concentrations of SCFAs in peripheral blood: Acetate has the highest peripheral concentrations. This is followed by propionate, while butyrate shows the lowest peripheral concentrations (43). Created with BioRender.com.

C3 length. Considering that systemic acetate levels are higher than those of butyrate, further studies are necessary to clarify if one SCFA is more favorable as an energy source, or if even a combination of SCFAs might be beneficial.

As to our knowledge there are no reports of SCFAs activating AMPK in the myocardium. However, SCFAs have not been long investigated in the context of cardiac health, but effect similar to those described in other tissues (liver, skeletal muscle, brown adipose tissue) are conceivable (49, 54, 55). It would be interesting to evaluate whether AMPK activation by SCFAs takes place in the heart and if the extent differs between the individual SCFAs. This may have therapeutic potential, as AMPK is likely to be the major pathway of SCFA activation of PGC-1. As mentioned before, PGC-1 is a key regulatory pathway for FAO. By activating the PGC-1 pathway SCFAs might be able to at least partially restore the FAO capacities of the failing heart thus increasing its total ATP levels. Also there is indirect evidence suggesting SCFAs activate the PPAR-α -PGC-1 pathway in the myocardium. Crawford et al. (67) investigated the effect of fasting in germ-free mice and mice that had received a gut microbiome transplant. The physiological response to fasting would be an increase in PPAR-α expression leading to increased FAO and ketone body formation to maintain a constant energy supply. However, the germ-free mice had a markedly dimished expression of PPAR-α compared to those with a healthy gut microbiome. Considering the fact that SCFAs are known to increase PPAR-α in other tissues, it seems likely that the decreased PPAR-α expression in germ-free mice is caused by their lack of a SCFAs-producing microbiome.

The anti-inflammatory effect of SCFAs also plays a role in pathological cardiac remodeling. Tang et al. (68) altered the gut flora of mice by treatment with antibiotics to investigate the influence of gut microbiota on the postinfarction myocardial repair. They observed a significant increase in mortality in the antibiotic-treated mice, concomitant with a decrease in SCFA levels. The higher mortality was mitigated by 50% by giving SCFAs immediately prior to the infarction event. They attributed this effect mainly to an altered immune response taking into consideration the changes in a certain population of monocyte infiltration in the periinfarct zone. In terms of function, treatment with a probiotic cocktail containing Lactobacillus -a genus of bacteria known to produce SCFAs- was able to improve ejection fraction after the infarct event, although circulating SCFA levels remained unchanged. In a hypertension mouse model, Bartolomaeus et al. (69) demonstrated that oral treatment with propionate reduces development of atherosclerosis, but also reduces pathological cardiac hypertrophy and fibrosis. As the cardioprotective effects of propionate were less pronounced when given to a population of regulatory T cell-depleted

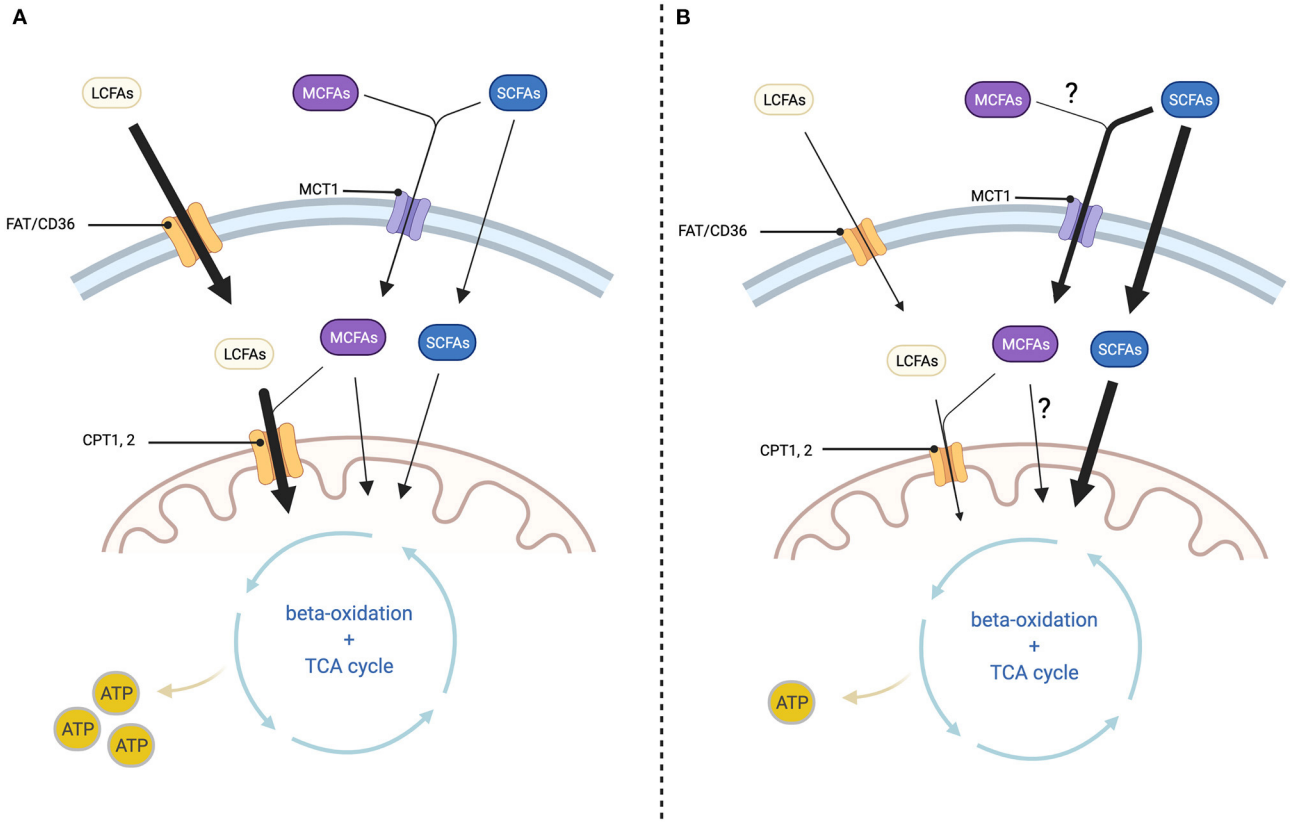

FIGURE 2 | Cellular fatty acid transport depending on chain length and changes in HF. **(A)** In healthy myocardium: Long-chain fatty acids (LCFAs) enter the cell via fatty acid translocase (FAT/CD36) and the mitochondrion via the carnitine shuttle (CPT1, 2). Medium-chain fatty acids (MCFAs) enter the cell via monocarboxylate transporter 1 (MCT1) and the mitochondrion via CPT1, 2 or transporter independent. Short-chain fatty acids (SCFAs) enter the cell via monocarboxylate transporter 1 (MCT1) or through transporter-independent mechanisms. Passage across the mitochondrial membrane is as well-transporter-independent. Inside the mitochondrion both LCFAs, MCFAs and SCFAs are processed through beta-oxidation and the TCA cycle for ATP-generation. **(B)** In diseased myocardium: LCFA uptake into the cell and mitochondrion is decreased at least in part because the expression of FAT/CD36 and CPT1, 2 are diminished. On the other hand, SCFA uptake into the cell as well as the mitochondrion is increased, but this is not sufficient to prevent a reduction in ATP production. Changes in MCFA oxidation are not well-described. Created with BioRender.com.

mice, they suggested that propionate acts via a T cell-mediated pathway.

For a brief overview of studies that investigated SCFAs in the context of cardiovascular disease (see **Table 2**).

DISCUSSION AND FUTURE PERSPECTIVES

The fact that a high-fiber diet is health-promoting is well-described and this is at least partly due to the products of its fermentation, SCFAs. Long time they have only been recognized in the context of gut and bowel physiology, but their effect reaches farther than just the intestine. They have protective properties against the development of diabetes and oncological diseases and show antiinflammatory effects with possible application in the treatment of Alzheimer's disease and arthritis (52–54, 59).

But, most interesting to us, SCFAs also have a considerable potential as metabolic therapy in HF.

The abovementioned studies display SCFAs as a potent fuel since their oxidation is not dependent on the transporters of LCFAO, which are downregulated in HF (27, 65, 70). Thus, we suggest increasing systemic levels of SCFA might be an efficient way to improve cardiac function. A first step to evaluate the capability of SCFAs as energy source, would be an *in vivo* HF animal model on a diet (consisting of resistant starch/arabinoxylan oligosaccharides etc.) that leads to elevated systemic levels of SCFAs with cardiac function studies such as echocardiography, cardiac output and left ventricular pressure measurements. Analyzation of respiratory oxidation rates and determination of total ATP level in the heart would give insight on changes in mitochondrial function. A diet-based approach to increase the amount of circulating SCFAs has been established in humans in non-HF settings and would be to our knowledge safe to transfer to a human HF cohort (52, 71). However, for now, it is unclear if certain types of HF show different alterations in fatty acid metabolism and therefore deeper metabolomic insights are first necessary to identify the population most suitable for a human SCFAs trial.

TABLE 2 | Studies investigating the role of SCFAs in cardiovascular diseases.

References	Title	Model	Findings
Carley et al. (65)	Short chain fatty acids outpace ketone oxidation in the failing heart	- Rat - 14d after transverse-aortic constriction	SCFAs oxidation is increased in HF. SCFA are preferred over ketones as a fuel.
Lewandowski et al. (70)	Mitochondrial preference for short chain fatty acid oxidation during coronary artery constriction	- Pig - During coronary artery constriction	In hypoperfused myocardium, SCFA oxidation is increased.
Tang et al. (68)	Loss of Gut Microbiota Alters Immune System Composition and Cripples Postinfarction Cardiac Repair	- Mouse - Coronary artery ligation after 7d antibiotic treatment	SCFAs regulate myocardial repair after infarction.
Murashige et al. (63)	Comprehensive quantification of fuel use by the failing and nonfailing human heart.	- Human - HF with reduced EF and healthy hearts	Both HF and normal hearts rely in major parts on FA for energy. SCFA metabolism is increased in HF.
Bartolomaeus et al. (69)	Short-Chain Fatty Acid Propionate Protects from Hypertensive Cardiovascular Damage	- Apolipoprotein E knockout and WT mice - Angiotensin II infusion (14d and 28d) - oral propionate	Propionate treatment reduces cardiac hypertrophy and fibrosis, hypertension and atherosclerosis.

In addition to the described fuel function, SCFAs might bear the potential to reverse the metabolic reprogramming in HF (72). Determination of expression levels of PGC-1 and related genes would help to clarify if the FAO enhancing effects of SCFAs are restricted to liver, skeletal muscle and fat tissue, or also take place in the heart.

The anti-inflammatory effect could be assessed by gene analysis of profibrotic pathways such as alpha-smooth muscle actin and connective tissue growth factor, but also by histological stainings (68, 73).

The derangements in fatty acid oxidation occurring in the failing heart may well-be of use in terms of diagnostics and prognostics. In the long term, SCFAs might be of use as biomarkers in HF, reflecting the metabolical state of the heart.

So far SCFAs show promising results and have the characteristics necessary to act as a metabolic therapy for the failing heart in several ways. Further research is needed in order to investigate their specific pathophysiological role and whether they could be a new therapeutic approach for HF.

AUTHOR CONTRIBUTIONS

CP, KN, BB, and BW were responsible for the article drafting and revising. All authors approved the final version of the manuscript, met the criteria for authorship, and all who qualify for authorship are listed.

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The Association Between Exposure to Acrylamide and Mortalities of Cardiovascular Disease and All-Cause Among People With Hyperglycemia

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Background: Acrylamide is a common environmental volatile organic compound that humans are frequently exposed to in their daily lives. However, whether exposure to acrylamide is associated with long-term survival in patients with hyperglycemia remains largely unknown.

Methods and Results: A total of 3,601 hyperglycemic people were recruited in this study, including 1,247 people with diabetes and 2,354 people with pre-diabetes, who enrolled in the National Health and Nutrition Examination survey (2003–2004, 2005–2006, and 2013–2014). The acrylamide exposure was measured by the serum hemoglobin adduct of acrylamide (HbAA) and glycidamide (HbGA), and the ratio of HbAA and HbGA (HbAA/HbGA) was calculated, which were all categorized into quintiles. The National Death Index was used to identify the participants' death information until 2015. Cox proportional hazards (CPHs) regression models were performed to examine the survival relationship between these biomarkers and mortality. During the 28,652 person-year follow-up, 268 deaths due to the cardiovascular disease (CVD) were documented. After adjustment for multiple confounders, compared with participants in the lowest quintile of HbAA/HbGA, the participants in the highest quintile were more likely to die due to CVD (hazard ratio [*HR*] = 1.61, 95% *CI*: 1.09–2.39) and all-cause (*HR* = 1.59, 95% *CI*: 1.25–2.01). Moreover, subgroup analysis showed that the highest quintile of HbAA/HbGA in the people with diabetes or pre-diabetes was related to mortalities risk of CVD ($HR_{\text{diabetes}} = 1.92$, 95% *CI*: 1.11–3.31; $HR_{\text{pre-diabetes}} = 1.78$, 95% *CI*: 1.01–3.14) and all-cause mortality ($HR_{\text{diabetes}} = 1.81$, 95% *CI*: 1.27–2.58; $HR_{\text{pre-diabetes}} = 1.59$, 95% *CI*: 1.14–2.20). Additionally, no significant association between the levels of HbAA or HbGA and CVD mortality was observed among people with diabetes or pre-diabetes.

Conclusion: Higher levels of HbAA/HbGA are associated with greater mortalities of CVD and all-cause among hyperglycemic people.

Keywords: CVD mortality, all-cause mortality, hyperglycemia, NHANES, acrylamide

INTRODUCTION

Acrylamide is an environmentally volatile organic compound, and human beings are frequently exposed to it in their everyday life (1). Acrylamide can be found in many aspects of daily life, such as daily cosmetics, textiles and plastic, cigarettes, water purification, and dietary intakes including various heated foods, beverages, potato crisps, and coffee (2–4). In addition to the neurotoxicity and carcinogenic toxicity of high concentrations of acrylamide, a few *in vivo* and *in vitro* studies have found that long-term exposure to low concentrations of acrylamide could induce hepatic oxidative stress and disturb lipid metabolism in rats (5, 6), and could also stimulate the inflammatory response and cell proliferation in HepG2 cells (7). Moreover, recent population-based studies found that serum biomarkers of acrylamide exposure were associated with hyperglycemia and dyslipidemia, prompting the conclusion that daily exposure to acrylamide probably influences the daily glucose control among people with hyperglycemia (8, 9). In addition, an association between acrylamide exposure and a higher risk of cardiovascular disease (CVD) was demonstrated in a population-based study (10). However, it is still largely unknown whether exposure to acrylamide is associated with long-term survival among people with hyperglycemia.

Evidence from animal studies has demonstrated that rats exposed to acrylamide could disrupt glucose homeostasis, induce β-cell dysfunction, and reduce the area of the aortic vessel and acetylcholine to endothelial-dependent arterial diastolic reactions (11, 12). Moreover, *in vitro* experiments revealed that chronic exposure to acrylamide could induce accelerated endothelial aging, even at low concentrations (13). These mechanisms are highly overlapping with the effect of hyperglycemia on the development of CVD. Based on this evidence, we, therefore, hypothesize that exposure to acrylamide is associated with an increased risk of CVD mortality among people with hyperglycemia. To examine this hypothesis, we examined the association of serum biomarkers of acrylamide with mortalities of CVD and all-cause in the participants with hyperglycemia using the national representative sample from the National Health and Nutrition Examination Survey (NHANES).

MATERIALS AND METHODS

Study Population

The NHANES is a stratified, multilevel study using a national population sample in the United States, and its detailed information has been provided elsewhere (14). The present study included 3,601 hyperglycemic participants, consisting of 1,247 diabetes and 2,354 pre-diabetes, who were over 18 years old and measured the serum biomarkers of acrylamide. The participants who had missing information on serum acrylamide metabolites, mortality of all-cause, and CVD, and questionnaire were excluded. The NHANES program was approved by the National Health Statistics Research Ethics Committee, and all informed consent was provided by participants.

Definition of Diabetes and Pre-diabetes

According to the American Diabetes Association, diabetes was defined as conforming to at least one of the following criteria: fasting blood glucose (FBG) > 7.0 mmol/L; 2-h postprandial blood glucose > 11.1 mmol/L; HbA1c > 6.5%; or a random plasma glucose > 11.1 mmol/L. Pre-diabetes was determined as meeting at least one of the criteria created by: FBG > 5.6 and < 7.0 mmol/L; 2-h postprandial blood glucose > 7.8 and < 11.1 mmol/L; or HbA1c > 5.7% and < 6.5%.

Measurements of Serum Biomarkers of Acrylamide

The N-terminal hemoglobin adducts of acrylamide (HbAA) and hemoglobin glycidamide (HbGA) in 300 μl of human whole blood or erythrocytes were quantified using high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) based on a modified Edman reaction (15). The well-established procedure was briefly illustrated as follows: using pentafluorophenyl isothiocyanate as an Edman reagent, two adducts were isolated from the protein chain by modified Edman reaction (16). After the modified EDMAN degradation reaction, liquid–liquid extraction was performed, and the results were analyzed based on HPLC/MS/MS. The calibrator, reagent blank, and quality control materials were pretreated in the same way as the samples. Each sample was performed with two independent repeated measurements. The limits of the detection of HBbaA and HbGA were 3 and 4 pmol/g, respectively.

Main Exposure and Main Outcome

The main exposures in our study were HbAA and HbGA levels in whole blood and the ratio of HbAA and HbGA (HbAA/HbGA). In addition, the outcome was the status of mortality according to the National Death Index (NDI) until the year 2015. The NDI is a reliable death identification resource. The disease-specific death was defined as International Classification of Diseases (ICD)-10 and the death due to CVD was determined as ICD-10 codes (I00-I09, I11, I13, I20-I51, and I60-I69). In summary, a total of 268 deaths (132 in diabetes and 136 in pre-diabetes) due to CVD and 709 deaths (332 in diabetes and 377 in pre-diabetes) due to all-cause were documented during the 28,652 person-year follow-up.

Covariates Assessment

The covariates in our study included age (years), sex (women/men), race/ethnicity (Mexican American/non-Hispanic white/non-Hispanic black/other), smoking (yes or no), drinking status (yes or no), education levels (less than high school education and high school and above), annual household income (more than $100,000/$45,000–$75,000/$20,000-$45,000/<$20,000), occupation, body mass index (BMI, kg/m^2), energy (kcal), exercise (yes or no), mean blood pressure (mmHg), whether to use insulin hypoglycemic tablets (yes or no), whether to take medication for hypertension (yes or no), and whether to take medication for cholesterol (yes or no).

Statistical Analyses

Demographic and anthropometric characteristics were presented using the mean and standard deviation (SD) for the continuous variables and the number and percentage for categorical variables. The general linear model adjusted for age and sex was used to compare the differences in the biomarkers of acrylamide by different baseline characteristics. All statistical analyses were conducted by R 4.0.2 software, and all tests were two-sided. The value of $p < 0.05$ was considered statistically significant.

Cox Proportional Hazards (CPH) Models

Cox proportional hazards (CPHs) models were conducted to calculate hazard ratios (*HR*s) and 95% *CI* for all-cause and CVD mortalities. The time scale in the Cox model used for the follow-up time was obtained by person-months from the date of the interview to their death, or the end of 2015, which was used for the time scale this Cox model. The HbAA, HbGA, and HbAA/HbGA were respectively categorized into quintiles, and the lowest quintile is regarded as the reference group. The confounders in the CPH model included age, sex, race, smoking status, drinking status, exercise, energy, education level, occupation, annual household income, BMI, mean blood pressure, whether to take medication for diabetes, whether to take medication for hypertension, and whether to take medication for cholesterol.

RESULTS

Participant Characteristics

The differences in the whole blood HbAA, HbGA, and HbAA/HbGA levels according to the baseline characteristics are presented in **Table 1**. The results of the normality test showed that HbAA, HbGA, and HbAA/HbGA levels were non-normally distributed (**Supplementary Figure 1**). The participants with higher HbAA levels were younger, men, non-Hispanic blacks, with a lower use rate of medication for glucose and blood pressure, and higher use of medication for cholesterol, and higher smoking rate, drinking, and energy intake (all the $p < 0.05$). Similarly, the participants with higher HbGA levels were younger, Mexican American, with a lower use rate of medication for blood pressure and higher use of medication for cholesterol, and higher rates of smoking, drinking, and energy intake (all the $p < 0.05$). Additionally, the participants with obesity, diabetes, or hypertension had lower levels of HbAA, and the participants with hypertension had lower levels of HbGA, respectively (all the $p < 0.05$). Meanwhile, the participants with higher HbAA/HbGA levels were men, non-Hispanic blacks, with lower medication use for glucose and cholesterol, higher medication use for blood pressure, and higher rates of smoking, drinking, and energy intake. The ratio of HbAA/HbGA was significantly lower in obese, diabetes, and dyslipidemia participants ($p < 0.05$).

Acrylamide Exposure and Mortality

The relationships of HbAA, HbGA, and HbAA/HbGA in whole blood with CVD and all-cause mortalities among people with hyperglycemia are presented in **Table 2**. Compared with those in the lowest quintile of HbAA/HbGA, the participants in the highest quintile were more prone to die due to CVD and all-cause, with the *HR*s (95% *CI*) being 1.61 (1.09–2.39) and 1.59 (1.25–2.01), respectively, and sex was not a significant effect modifier of the above association ($p_{\text{effectmodificationwithsex}} > 0.05$). Meanwhile, compared with the participants in the lowest quintile of HbAA, the participants in the highest quintile of HbAA had a greater risk of CVD mortality (*HR*: 1.84, 95% *CI*: 1.00–3.37) and all-cause mortality (*HR*: 2.21, 95% *CI*: 1.46–3.05). Moreover, no significant association between quintiles of HbGA with CVD mortality was observed.

Further, in line with the results of the total sample, results of subgroup analysis in the people with diabetes and pre-diabetes indicated that compared with the participants in the lowest quintile of HbAA/HbGA, the participants in the highest quintile of HbAA/HbGA had a greater risk of CVD mortality (*HR*: 1.92, 95% *CI*: 1.11–3.31 for diabetes and *HR*: 1.78, 95% *CI*:1.01–3.14 for pre-diabetes) and all-cause mortality (*HR*: 1.81, 95% *CI*:1.27–2.58 for diabetes and *HR*: 1.59, 95% *CI*: 1.14–2.20 for pre-diabetes), and sex was not a significant effect modifier of the above association ($p_{\text{effectmodificationwithsex}} > 0.05$) (**Tables 3**, **4**). Meanwhile, no significant association between quintiles of HbAA or HbGA with mortality from CVD was observed among people with diabetes and pre-diabetes, respectively.

Additionally, the significant modification effects of sex on the relationship between HbAA and CVD mortality in the total sample, and the association of HbGA with mortalities of CVD and all-cause were observed among the total sample, and the samples of pre-diabetes and diabetes, respectively. We therefore examined this above association by sex, which is presented in the **Supplementary Materials**. In the total sample, the increased HbAA levels were negatively related with CVD mortality in men; whereas it was positively related with CVD mortality in women (**Supplementary Table 1**). Moreover, no significant relationship between HbGA and CVD mortality was observed in men (**Supplementary Table 2**); whereas an increase in HbGA was negatively associated with CVD or all-cause mortality in women (**Supplementary Table 3**). These findings indicated that compared with the ratio of HbAA and HbGA, the individual association of HbAA or HbGA with CVD mortality was more easily influenced by sex.

DISCUSSION

To our knowledge, the present study is the first to examine whether exposure to acrylamide is associated with long-term survival among people with hyperglycemia. In this study, we observed that higher levels of the ratio between HbAA and HbGA were related to greater CVD and all-cause mortalities among people with hyperglycemia, independent of other traditional risk factors of CVD. Moreover, this association was relatively robust, which was consistently observed among people with diabetes and pre-diabetes, respectively.

Currently, only a few cross-sectional studies and one prospective study have examined the association of the biomarkers of acrylamide with blood glucose, lipids, and CVD

TABLE 1 | The hemoglobin biomarkers of hemoglobin adduct of acrylamide (HbAA), hemoglobin adduct of glycidamide (HbGA), and HbAA/HbGA are grouped by various characteristics of the total hyperglycemia population.

Characteristics	HbAA (pmol/g Hb)		*P* fortrend	HbGA (pmol/g Hb)		*P* fortrend	HbAA/HbGA		*P* fortrend
	Medians	IQRs		Medians	IQRs		Medians	IQRs	
Age			<0.001			<0.001			0.4
< 60 years	57.4	(42.0, 102.0)		54.1	(37.8, 81.5)		1.15	(0.93, 1.45)	
≥ 60 years	47.1	(36.4, 64.6)		43.0	(31.5, 60.4)		1.15	(0.94, 1.41)	
Sex			<0.001			0.13			<0.001
Male (%)	53.9	(39.4, 88.7)		47.6	(33.2, 70.5)		1.22	(1.00, 1.49)	
Female (%)	49.5	(37.6, 72.8)		49.0	(35.3, 70.2)		1.07	(0.88, 1.31)	
Race			<0.001			<0.001			<0.001
Mexican American	53.4	(42.0, 70.7)		52.8	(39.7, 70.6)		1.04	(0.88, 1.27)	
Non-hispanic White	51.5	(38.7, 81.3)		48.9	(34.5, 72.9)		1.14	(0.92, 1.39)	
Non-hispanic Black	53.5	(37.2, 100.5)		44.2	(30.6, 66.0)		1.31	(1.06, 1.67)	
Others	45.4	(33.1, 69.2)		42.2	(33.8, 67.4)		1.10	(0.92, 1.29)	
Current smoking			<0.001			<0.001			<0.001
Never	45.0	(35.5, 57.4)		42.0	(31.5, 56.2)		1.09	(0.89, 1.30)	
Active	114.0	(74.3, 164.0)		79.9	(55.5, 115.0)		1.36	(1.12, 1.69)	
Current drinking			<0.001			<0.001			<0.001
Never	46.9	(36.6, 65.4)		46.0	(33.1, 63.5)		1.08	(0.88, 1.31)	
Current	54.5	(39.6, 89.6)		48.8	(34.3, 73.2)		1.19	(0.97, 1.47)	
College graduate or above			0.2			0.11			0.3
<High school	53.7	(39.2, 83.4)		49.7	(35.1, 72.1)		1.15	(0.93, 1.43)	
≥High school	51.0	(38.2, 77.3)		47.6	(33.6, 69.0)		1.15	(0.94, 1.42)	
Income			0.12			0.043			0.6
<10,000	52.1	(38.4, 85.1)		48.8	(34.2, 73.3)		1.16	(0.93, 1.43)	
≥10,000	51.1	(38.5, 73.1)		47.7	(33.7, 66.9)		1.15	(0.94, 1.41)	
Energy			<0.001			<0.001			<0.001
<1,829 kcal	49.7	(37.5, 72.3)		46.6	(33.2, 67.0)		1.14	(0.92, 1.38)	
≥1,829 kcal	53.9	(39.7, 90.7)		49.5	(35.1, 73.6)		1.17	(0.95, 1.46)	
Regular exercise			0.7			0.027			<0.001
Yes	51.3	(39.7, 70.9)		49.5	(36.4, 69.0)		1.10	(0.90, 1.34)	
No	52.0	(37.8, 85.7)		47.6	(33.2, 70.3)		1.18	(0.96, 1.46)	
Obesity			<0.001			0.6			<0.001
Yes	49.8	(37.7, 73.4)		47.9	(37.7, 95.6)		1.10	(0.91, 1.34)	
No	54.8	(39.8, 95.6)		49.2	(39.8, 95.6)		1.23	(1.00, 1.53)	
Diabetes			<0.001			0.060			<0.001
Yes	49.7	(37.0, 72.0)		47.2	(34.4, 68.4)		1.11	(0.89, 1.35)	
No	52.9	(39.5, 85.1)		48.4	(33.4, 73.6)		1.18	(0.96, 1.46)	
Hypertension			<0.001			<0.001			0.2
Yes	48.6	(36.7,71.7)		45.1	(32.4, 64.5)		1.15	(0.93, 1.40)	
No	55.3	(40.8,88.4)		51.6	(36.1, 75.2)		1.16	(0.94, 1.44)	
Dyslipidemia			0.5			<0.001			<0.001
Yes	52.1	(38.1, 87.1)		51.1	(35.3, 76.8)		1.11	(0.89, 1.36)	
No	51.5	(38.5, 77.3)		47.2	(33.5, 68.4)		1.17	(0.95, 1.44)	
Take medication for diabetes			<0.001			0.2			<0.001
Yes	49.3	(36.7, 69.8)		47.6	(33.0, 67.0)		1.10	(0.88, 1.33)	
No	52.4	(39.1, 84.0)		48.3	(34.3, 71.2)		1.17	(0.95, 1.45)	
Take medication for hypertension			<0.001			<0.001			0.027
Yes	47.5	(36.4, 67.7)		44.4	(32.3, 62.3)		1.15	(0.92, 1.38)	
No	55.1	(38.9, 102.0)		48.5	(32.0, 85.5)		1.14	(0.98, 1.46)	

(Continued)

TABLE 1 | Continued

Characteristics	HbAA (pmol/g Hb)		*P* fortrend	HbGA (pmol/g Hb)		*P* fortrend	HbAA/HbGA		*P* fortrend
	Medians	IQRs		Medians	IQRs		Medians	IQRs	
Take medication for cholesterol			<0.001			0.007			0.001
Yes	49.5	(37.8, 71.3)		46.6	(33.4, 67.0)		1.13	(0.91, 1.38)	
No	46.1	(35.6, 61.5)		43.9	(31.0, 60.8)		1.16	(0.88, 1.39)	

Continuous variables are presented as medians (IQRs). Categorical variables are presented as percentages. Diabetes is defined by FBG ≥ 7.0 mmol/L; 2-h PBG ≥ 11.1 mmol/L, random plasma glucose ≥ 11.1 mmol/L, or HbA1c ≥ 6.5%. Pre-diabetes is determined as meeting at least one of the criteria created by: 2-h PBG ≥ 7.8 and < 11.1 mmol/L; FBG ≥ 5.6 and < 6.9 mmol/L, or intermediate HbA1c ≥ 5.7% and < 6.4%.

TABLE 2 | Adjusted hazard ratios (*HRs*) for the associations between HbAA, HbGA, HbAA/HbGA and CVD or all-cause mortality in the total hyperglycemia population.

	CVD mortality		All-cause mortality	
	Case/*N*	HR (95%CI)	Case/*N*	HR (95%CI)
HbAA(pmoL/g Hb)				
Q1(<36.2)	79/722	1	182/722	1
Q2(36.2–46.3)	54/723	0.74(0.51–1.07)	159/723	0.95(0.76–1.19)
Q3(46.3–59.0)	47/719	0.73(0.49–1.11)	130/719	0.88(0.68–1.13)
Q4(59.0–95.7)	51/718	1.16(0.73–1.85)	127/718	1.18(0.88–1.59)
Q5(>95.7)	37/719	1.84(1.00–3.37)	111/719	2.21(1.46–3.05)
P for trend		0.002		<0.001
$P_{sex*HbAA}$		0.018		0.222
HbGA(pmoL/g Hb)				
Q1(<31.5)	67/726	1	179/726	1
Q2(31.5–42.0)	65/716	0.86(0.60–1.23)	170/716	0.84(0.68–1.05
Q3(42.0–55.2)	54/722	0.71(0.47–1.08)	132/722	0.65(0.50–0.84)
Q4(55.2–78.8)	48/718	0.70(0.44–1.13)	117/718	0.62(0.46–0.83)
Q5(>78.8)	34/719	0.60(0.33–1.07)	111/719	0.70(0.49–0.99)
P for trend		0.448		0.007
$P_{sex*HbGA}$		0.005		0.008
HbAA/HbGA				
Q1(<0.89)	55/718	1	140/718	1
Q2(0.89–1.06)	45/717	0.95(0.64–1.41)	142/717	1.13(0.89–1.43)
Q3(1.06–1.24)	61/727	1.32(0.91–1.92)	131/727	1.05(0.83–1.34)
Q4(1.24–1.50)	53/721	1.27(0.87–1.87)	141/721	1.24(0.98–1.58)
Q5(>1.50)	54/718	1.61(1.09–2.39)	155/718	1.59(1.25–2.01)
P for trend		0.062		0.001
$P_{sex*HbAA/HbGA}$		0.126		0.124

Adjustments included age, sex, race, income, education, occupation, exercise, smoking, alcohol intake, energy, BMI, mean blood pressure, take medication for diabetes, and taking medication for hypertension and cholesterol. Case/N, number of case subjects/total; Q, quintile.
*The symbol * indicates the interaction between two variables.*

mortality among the general population (8, 17, 18). However, there are some contradictions among these results. The previous study found that a higher HbAA level was associated with lower fasting insulin and glucose (19), whereas the recent study found that increased urine biomarkers of acrylamide were associated with increased fasting blood glucose, probably through lipid peroxidation (20). Moreover, it has been found that HbGA is positively associated with serum triglycerides and low-density lipoprotein cholesterol, suggesting that a higher level of HbGA is associated with dyslipidemia (17), which contradicts the recent results regarding the association between whole blood HbGA and CVD, reporting that higher levels of HbGA were associated with the lower prevalence of CVD and CVD-mortality (21). The conflicting findings of these studies suggest that both HbAA and HbGA have limitations as markers of acrylamide exposure alone, whereas the present study integrates two acrylamide metabolites, HbAA and HbGA, by using a ratio of the two as biomarkers of acrylamide exposure for a more comprehensive evaluation. Furthermore, these conflicting results warrant a study focusing on people with diabetes or pre-diabetes to elucidate

TABLE 3 | Adjusted HRs for the associations between HbAA, HbGA, HbAA/HbGA and CVD or all-cause mortality in diabetes population.

	CVD mortality		All-cause mortality	
	Case/N	**HR (95%CI)**	**Case/N**	**HR (95%CI)**
HbAA(pmoL/g Hb)				
Q1(<34.9)	40/250	1	87/250	1
Q2(34.9–43.7)	24/249	0.68(0.40–1.15)	70/249	0.94(0.67–1.31)
Q3(43.7–56.1)	23/251	0.62(0.34–1.11)	60/251	0.81(0.56–1.18)
Q4(56.1–83.8)	21/248	0.67(0.33–1.34)	58/248	0.98(0.64–1.53)
Q5(>83.8)	24/249	1.23(0.55–2.77)	57/249	1.60(0.95–2.67)
P for trend		0.075		0.030
$P_{sex*HbAA}$		0.057		0.114
HbGA(pmoL/g Hb)				
Q1(<30.7)	31/250	1	82/250	1
Q2(30.7–41.7)	32/249	0.95(0.57–1.59)	77/249	0.87(0.63–1.20)
Q3(41.7–54.2)	26/250	0.79(0.43–1.44)	66/250	0.70(0.48–1.02)
Q4(54.2–76.6)	20/249	0.74(0.36–1.53)	54/249	0.67(0.43–1.05)
Q5(>76.6)	23/249	0.81(0.37–1.80)	53/249	0.64(0.38–1.06)
P for trend		0.914		0.347
$P_{sex*HbGA}$		0.042		0.023
HbAA/HbGA				
Q1(<0.85)	25/249	1	58/249	1
Q2(0.85–1.02)	26/250	1.23(0.70–2.14)	72/250	1.40(0.99–1.99)
Q3(1.02–1.19)	25/248	1.17(0.66–2.06)	62/248	1.23(0.85–1.77)
Q4(1.19–1.45)	24/251	1.15(0.65–2.03)	64/251	1.29(0.90–1.85)
Q5(>1.45)	32/249	1.92(1.11–3.31)	76/249	1.81(1.27–2.58)
P for trend		0.153		0.021
$P_{sex*HbAA/HbGA}$		0.407		0.762

Adjustments included age, sex, race, income, education, occupation, exercise, smoking, alcohol intake, energy, BMI, mean blood pressure, take medication for diabetes, and taking medication for hypertension and cholesterol. Case/N, number of case subjects/total; Q, quintile.
*The symbol * indicates the interaction between two variables.*

the association between exposure to acrylamide and long-term survival in this specific population.

In this study, although we observed the positive association of HbAA with CVD and all-cause mortalities in the total sample, this relationship could not be consistently observed among the people with diabetes or pre-diabetes, suggesting that this significant association in the total sample was likely driven by the relatively large sample size. In contrast, the association of higher levels of the ratio of HbAA and HbGA with mortalities of CVD and all-cause could be consistently observed among people with diabetes or pre-diabetes, suggesting that the combination of HbAA and HbGA was more important than focusing on the individual effect of HbAA or HbGA on the development of CVD among people with hyperglycemia. In fact, when acrylamide enters circulation, a proportion of acrylamide reacts with hemoglobin to form HbAA (22), and the other proportion of acrylamide can be metabolized by binding to glutathione (GSH) (23), which further can be oxidized into glycidamide by cytochrome P450 (24), and the epoxidation of hydrolyzate can largely convert glycidamide into non-toxic glycine amide due to the body's self-protective mechanism (25). The ratio of HbAA and HbGA probably reflects the balance of the detoxification metabolism in the body (4), and based on the findings of this study, the balance of metabolic networks for acrylamide may play a more critical role in the development of CVD among people with hyperglycemia.

Moreover, a series of the previous *Vivo*- and *Vitro*-experiments elucidated the mechanisms underlying the relationship between exposure to acrylamide and CVD mortality (26–28). In the past few years, there has been some progress in understanding the underlying impact of acrylamide on CVD, which is that acrylamide exposure may lead to oxidative stress and disturbance of physiological function (26, 28). A human study has found that the consumption of acrylamide-containing potato chips could cause significant changes in the levels of oxidative stress and inflammation response, probably increasing the risk of atherosclerosis (27). Moreover, zebrafish research has shown that acrylamide exposure could aggravate oxidation and degradation of low-density lipoprotein with increased production of reactive oxygen species (ROS) and cause dose-dependent fat accumulation in the liver (29). In addition, another animal study indicated exposure to acrylamide could cause a significant decrease in the GSH level (30) and stimulate oxidative stress, which might strike the blood vessels and exacerbate the progression of atherosclerosis (31). Further, *vitro* experiments also revealed that chronic exposure to acrylamide

TABLE 4 | Adjusted HRs for the associations between HbAA, HbGA, HbAA/HbGA and CVD or all–cause mortality in pre-diabetes population.

	CVD mortality		All-cause mortality	
	Case/*N*	**HR (95%CI)**	**Case/*N***	**HR (95%CI)**
HbAA(pmoL/g Hb)				
Q1(<37.1)	38/471	1	91/471	1
Q2(37.1–47.3)	31/472	0.79(0.48–1.32)	89/472	0.97(0.71–1.32)
Q3(47.3–60.2)	23/470	0.81(0.45–1.46)	64/470	0.89(0.62–1.28)
Q4(60.2–104.0)	30/473	1.53(0.82–2.84)	73/473	1.38(0.93–2.04)
Q5(>104.0)	14/468	1.66(0.67–4.14)	60/468	2.38(1.44–3.94)
P for trend		0.108		<0.001
$P_{sex*HbAA}$		0.057		0.114
HbGA(pmoL/g Hb)				
Q1(<31.9)	33/474	1	92/474	1
Q2(31.9–42.2)	36/468	0.96(0.58–1.57)	98/468	0.93(0.69–1.25)
Q3(42.2–56.0)	33/472	0.91(0.52–1.60)	67/472	0.65(0.45–0.93)
Q4(56.0–79.8)	20/470	0.61(0.31–1.20)	60/470	0.61(0.41–0.91)
Q5(>79.8)	14/470	0.65(0.27–1.55)	60/470	0.85(0.55–1.39)
P for trend		0.579		0.023
$P_{sex*HbGA}$		0.042		0.023
HbAA/HbGA				
Q1(<0.91)	27/469	1	75/469	1
Q2(0.91–1.09)	25/470	1.04(0.60–1.80)	76/470	1.05(0.76–1.45)
Q3(1.09–1.27)	32/474	1.52(0.90–2.57)	63/474	0.94(0.67–1.32)
Q4(1.27–1.55)	26/472	1.16(0.67–2.01)	81/472	1.22(0.89–1.69)
Q5(>1.55)	26/469	1.78(1.01–3.14)	82/469	1.59(1.14–2.20)
P for trend		0.186		0.014
$P_{sex*HbAA/HbGA}$		0.407		0.762

Adjustments included age, sex, race, income, education, occupation, exercise, smoking, alcohol intake, energy, BMI, mean blood pressure, and taking medication for diabetes and hypertension, take medication for cholesterol. Case/N, number of case subjects/total; Q, quintile.
*The symbol * indicates the interaction between two variables.*

and glycidamide could induce accelerated endothelial aging, even at low concentrations (13). The results of our study showed that acrylamide exposure was significantly associated with CVD and all-cause mortalities, which could be partially supported by these studies above.

Further, we observed that among the people with diabetes, the participants whose ratio was ≥1.45 had greater CVD mortality compared with control; whereas among the people with pre-diabetes, the participants whose ratio was ≥1.55 had greater CVD mortality compared with control. These findings suggested that the ratio for increasing the risk of CVD mortality is lower in diabetes than in pre-diabetes, which adds to our understanding of how to develop an adequate range for the ratio among people with diabetes in the future. It has been reported that people with diabetes have higher levels of glutathione and cytochrome P450 2E1 than other people (32). Therefore, in people with diabetes, the circulating acrylamide tends to convert to HbGA, probably making the ratio of HbAA and HbGA significantly lower than in other people, and this mechanism supported the findings in this study.

This present study examined the relationship between exposure to acrylamide and the mortalities of CVD and all-cause, demonstrating the importance of the balance of metabolic networks for acrylamide among people with hyperglycemia. This association documented in our study had relatively strong robustness after adjusting for a range of important classical confounders. These findings also provided evidence for the impact of environmental exposure on the improvement of long-term survival for people with diabetes. Diabetes care professionals should be aware of the current findings from this study regarding the deleterious effect of exposure to acrylamide in daily life. This information is of importance in providing individualized treatment strategies in relation to avoiding environmental exposure for patients with diabetes. In addition, we recognized that this study had several limitations. First, we had adjusted a range of potential confounders, whereas the present study was still observational research, and some unmeasured confounding factors cannot be excluded. Second, this study was not able to distinguish between different types of diabetes. More research in the future is needed to examine this association in terms of type 1 and type 2 diabetes to provide more comprehensive evidence. Last, the measurement of exposure to acrylamide was only conducted at baseline. The studies with more frequent measurements may offer a more complicated blueprint of changes in the exposure to acrylamide and long-term survival among people with hyperglycemia.

CONCLUSION

Exposure to acrylamide, which is indicated by the higher ratio of HbAA and HbGA in the whole blood, is related to higher CVD and all-cause mortalities among people with diabetes and pre-diabetes.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: National Health and Nutrition Examination Survey https://www.cdc.gov/nchs/nhanes/index.htm.

AUTHOR CONTRIBUTIONS

CS and TH planned the work. JX, XS, HJ, and CH carried out the statistical analysis. HW wrote and reported the work. WW and WJ played critical roles in revising the manuscript. All authors made a significant contribution to this article, critically assessed and reviewed the paper, and approved the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.930135/full#supplementary-material

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Dietary magnesium and risk of cardiovascular and all-cause mortality after myocardial infarction: A prospective analysis in the Alpha Omega Cohort

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Background: An adequate intake of magnesium has been associated with lower risks of cardiovascular disease (CVD) and all-cause mortality in population-based studies. Whether an adequate magnesium intake is important for reducing long-term mortality risk after myocardial infarction (MI) is not yet clear.

Objective: We examined magnesium intake in relation to CVD, all-cause and coronary heart disease (CHD) mortality, on top of drug treatment, in patients who had experienced an MI.

Methods: We included 4,365 Dutch patients aged 60–80 y from the Alpha Omega Cohort with a history of MI <10 y before study enrollment. Dietary data over the past month were collected at baseline using a 203-item validated food frequency questionnaire from which magnesium intake was calculated. Patients were followed for cause-specific mortality through December 2018. HRs for mortality in tertiles of energy adjusted magnesium intake were obtained from multivariable Cox proportional hazard models, adjusting for age, sex, education, obesity and other lifestyle and dietary factors. Associations were also studied in relevant subgroups, including patients with diabetes and diuretics users. Restricted cubic splines were used for studying the continuous association of magnesium intake with CVD mortality.

Results: The average magnesium intake was 302 ± 78 mg/day and 28% of male and 33% of female patients had adequate intakes. Magnesium containing supplements were used by 5.4% of the cohort. During a median follow-up of 12.4 years (48,473 person-years), 2,035 patients died, of which 903 from CVD and 558 from CHD. Higher magnesium intakes (>320 g/d), compared to the reference group (<283 mg/d), were associated with a lower risk of CVD mortality (HR: 0.72; 95% CI: 0.54–0.98) and all-cause mortality (HR: 0.78; 95% CI: 0.64–0.95) in the fully adjusted model. A non-significant inverse association was found for CHD mortality. Associations for CVD mortality were slightly stronger in diuretic users (HR: 0.55; 95% CI: 0.34–0.89). Results were similar after excluding magnesium supplement users.

Conclusion: An adequate intake of magnesium may be important for lowering long-term mortality risk after MI, especially in patients treated with diuretics. The Alpha Omega Trial was registered at clinicaltrials.gov as NCT03192410.

KEYWORDS

dietary magnesium, myocardial infarction, cardiovascular disease, mortality, patients

Introduction

Population-based studies have shown inverse associations of dietary magnesium with risk of cardiovascular diseases (CVD) and risk of hypertension, metabolic syndrome and type 2 diabetes (T2D) (1–4). Bagheri et al. showed a relative risk for CVD mortality of 0.92 (95% CI: 0.87–0.97) for high vs. low dietary magnesium intake (range from 126 to 523 mg/d) after pooling of 17 studies in which adjustment was made for total energy intake (5). In a meta-analysis of 11 prospective cohort studies by Del Gobbo et al. (3), dietary magnesium (per 200 mg/d increment) was associated with a 22% lower risk (RR: 0.78; 95% CI: 0.67–0.92) of coronary heart disease (CHD) events. Magnesium plays an essential role in blood glucose control, blood pressure regulation and myocardial metabolism (6, 7). Magnesium is derived from fiber-rich foods such as whole grains, green vegetables, legumes and nuts, and also from dairy (6). Dietary reference values (adequate intakes) for magnesium intake for European adults have been set at 350 mg/d for men and 300 mg/day for women (8).

Magnesium requirements may be different in CVD patients because of alterations in the cardiovascular system, comorbidities and/or medication use. Mild magnesium depletion is relatively common in users of loop and thiazide diuretics (9), often prescribed to CVD and T2D patients. Hypomagnesemia has been associated with insulin resistance, altered lipid metabolism, impaired endothelial function and kidney function decline (10, 11). A cohort study of 3,380 Chinese CHD patients showed higher risks of all-cause (HR: 1.59, 95% CI: 1.30–1.95) and CVD mortality (HR: 1.71, 95% CI: 1.32–2.22) when comparing higher (≥0.82 mmol/L) to lower serum magnesium concentrations (12). However, only 1–2% of body magnesium is present in the extracellular fluid and serum levels are tightly regulated (13, 14), so serum magnesium may not accurately reflect actual magnesium status (14).

Little is known about magnesium intake and long-term mortality risk in CVD patients. We therefore examined dietary magnesium and risk of CVD, all-cause, and CHD mortality, on top of drug treatment, in Dutch patients aged 60–80 y who had experienced a myocardial infarction (MI).

Methods

Design and study population

The Alpha Omega Cohort (AOC; ClinicalTrials.gov Identifier NCT03192410) is an ongoing follow-up of the Alpha Omega Trial (15). During the initial trial phase, MI patients were randomized to either receiving low doses of omega-3 fatty acids or placebo for 40 months, which showed no effect on major CVD events (16). Participants include Dutch males and females aged 60–80 y with a clinically diagnosed MI ≤10 y prior to study enrolment, most of whom received state-of-the-art cardiovascular drug treatment. After the trial, patients have continuously been followed for cause-specific mortality. Patients provided written informed consent and the study was approved by a central ethics committee (Haga Hospital, The Hague, The Netherlands) and by the ethics committees of participating hospitals.

Of 4,837 patients enrolled in the AOC between 2002 and 2006, 472 patients were excluded because of missing or incomplete dietary data ($n = 453$), or implausibly high or low energy intakes (<800 or >8,000 kcal/day for males, <600 or >6,000 kcal/day for females, $n = 19$). One participant was lost to follow-up and censored after 2.9 y. A total of 4,365 patients was included in the analysis (flow-chart of selection of population for analyses in Supplementary Figure 1).

Dietary assessment

Dietary intake data were collected at baseline by a 203-item food frequency questionnaire (FFQ) developed for the Alpha Omega Cohort. The FFQ is an extended and adapted version of a reproducible and biomarker-validated FFQ (17). Patients were asked to report their habitual intake of foods and beverages consumed during the previous month. The FFQ included questions on the frequency, amount, and type of foods, as well as preparation methods. Trained dietitians checked the questionnaires upon return and obtained additional information by phone on unclear or missing items (15). Daily intakes of foods were linked with the 2006 Dutch Food Composition Database, whereafter energy, macronutrients and micronutrient, including

magnesium intake, were calculated (18). Adequate magnesium intakes were calculated based on European magnesium guidelines and set at 350 mg/d for men and 300 mg/day for women (8). Total magnesium intake was categorized into tertiles of energy adjusted magnesium intake (low; <283 mg/d, moderate; 283–322 mg/d and high >322 mg/d). The 2015 Dutch Healthy Diet index (DHD15-index) score was calculated to reflect adherence to Dutch dietary guidelines [DHD15-index; scale from 0 to maximal adherence (0–150)] (19). Dietary supplement use was assessed at baseline by means of a self-administered Lifestyle and Health questionnaire. Information was obtained on types of supplements (including magnesium containing supplements), brand names, frequency of use and daily dosages.

All-cause mortality and cause-specific mortality

The study focuses on CVD mortality as the primary endpoint, and CHD mortality and all-cause mortality as secondary endpoints. Information on vital status and cause of death was obtained from baseline through 31 December 2018. In the period 2002–2009 (Alpha Omega Trial), an independent Endpoint Adjudication Committee assigned causes of death using information from the national mortality registry [Statistics Netherlands, (CBS)], treating physicians, and close family members, as described previously (15, 16). In the period 2010–2018, research staff assigned causes of death using information from CBS and additional information by treating physicians. Mortality coding was performed according to the International Classification of Diseases, tenth revision (ICD-10) (20), combining primary and secondary causes of death. CVD mortality comprised I20–I25 (ischemic heart disease), I46 (cardiac arrest), R96 (sudden death, undefined), I50 (heart failure), and I60–I69 (stroke). CHD mortality comprised ICD-10 codes I20–I25, I46, and R96. Person-years were calculated from the date of study enrolment to date of death or end of the study (31 December 2018), whichever came first.

Other measurements

Information on demographics, anthropometrics, lifestyle factors, medical history, and medication use was collected at baseline. Physical examinations were performed by trained research nurses. Body Mass Index (BMI) was calculated as weight (kg) divided by height squared (m^2). Obesity was classified as BMI $\geq$30 kg/m^2. Systolic and diastolic blood pressures were measured twice with an automatic device, in a seated position, after a 10-min rest. Blood lipids and glucose levels were analyzed by standard kits by using an autoanalyzer (Hitachi 912; Roche Diagnostics). Information on chronic disease history, smoking habits (never, former >10 y, former $\leq$10 y or current), educational level (only elementary, low, intermediate or high) and medication use were collected by a self-administered questionnaire.

The prevalence of diabetes mellitus was defined on basis of a self-reported physician's diagnosis, use of antidiabetic medication, and/or elevated plasma glucose ($\geq$7.0 mmol/L when fasted or $\geq$11.1 mmol/L when not fasted). Kidney function was assessed on basis the estimated glomerular filtration rate (eGFR) using the CKD-EPI formula, and categorized as impaired (eGFR < 60 ml/min/1.73 m^2) or unimpaired (eGFR $\geq$ 60 ml/min/1.73 m^2). Medication use was checked by research nurses and coded according to the Anatomical Therapeutic Chemical Classification System (21) as follows: C02, C03, C07, C08, and C09 for antihypertensive drugs and C10 for lipid-modifying drugs. Diuretics were coded as C03, with C03A for thiazide diuretics and C03D + CO3E for potassium-sparing diuretics.

Sex-specific categories were used for alcohol intake, since females may have higher and more prolonged blood levels of alcohol compared to males for the same dose of alcohol per kg of body weight (22). Alcohol intake (g/d) was derived from the FFQ and categorized as "no/light drinking" (males: <10 g/d, females: <5 g/d), "moderate drinking" (males: $\geq$10–30 g/d, females: $\geq$5–15 g/d) and "heavy drinking" (males: $\geq$30 g/d, females: $\geq$15 g/d). Physical activity was assessed by the validated Physical Activity Scale for the Elderly (PASE) (23) and categorized as low physical activity [<3 Metabolic Equivalent Tasks (METs)], intermediate physical activity [>0 to <5 days per week of moderate or vigorous activity ($\geq$3 METs)], or high physical activity ($\geq$5 days per week of moderate or vigorous activity), based on the Dutch Physical Activity Guidelines (24).

Statistical analysis

Baseline characteristics in tertiles of energy adjusted magnesium intake (mg/d) are presented as mean ± standard deviations (SDs) for normally distributed variables, medians with IQRs for skewed variables, and counts (n) including percentages (%) for categorical or dichotomous variables. Missing data (assuming missing at random) were imputed with the age and sex specific median (for continuous variables) or mode (for categorical variables). Variables that contain imputed values are BMI ($n = 6$), educational level ($n = 24$), physical activity ($n = 25$) and smoking status ($n = 1$). Multicollinearity was assessed by making use of the variance inflation factor (VIF. Multicollinearity was considered as present when VIF was higher than 5 (25). Magnesium intake was adjusted for total energy using the residual method by Willett et al. (26).

Cox proportional hazard models were used to assess the associations of energy adjusted magnesium intake in tertiles,

and the risk of CVD mortality, all-cause mortality and CHD mortality. The proportional hazards assumption was checked by log-minus-log plots and was met. Survival time (in y) was defined as the period between the date of inclusion and the date of death, censoring date, or end of follow-up (31 December 2018), whichever occurred first. HRs with 95% CIs were computed in tertiles of energy adjusted magnesium intake, using the lowest tertile as the reference.

HR were adjusted for age and sex (model 1). Model 2 additionally included smoking (4 categories), alcohol intake (3 categories), physical activity (3 categories), obesity (yes/no) and education level (4 categories). Model 3 also included dietary factors, i.e., daily intake of calcium, vitamin D, sodium (only from foods), potassium, heme iron, vitamin C, beta-carotenoids, polyunsaturated fatty acids, saturated fatty acids and total energy, prescribed fiber-rich diet (yes/no) and DHD15-index (total score). Model 4 additionally included blood pressure, kidney function (2 categories) and diabetes mellitus (yes/no), which could be potentially intermediary factors when studying magnesium intake and mortality risk.

Restricted cubic splines (RCS) were used to examine the continuous associations for energy-adjusted magnesium intake and CVD mortality (full model) and to detect a potential threshold or non-linear associations. For the RCS analysis, the reference value was set at the adequate intake for males (350 g/d) and knots were placed at the 10th, 50th, and 90th percentiles. Outliers were winsorized at the 1st and 99th percentile, meaning that outliers outside these percentiles (0–1 percentile and 99–100 percentile) were replaced with the observations closest to them.

Analyses for CVD, all-cause and CHD mortality were repeated in predefined strata by sex, diabetes mellitus (yes vs. no: 883 vs. 3,482 patients), impaired kidney function (yes vs. no: 971 vs. 3,394 patients) and use of diuretics (yes vs. no: 1,050 vs. 3,315 patients), using the full model. For diuretic and non-diuretic users, RCS plots were additionally constructed to obtain further insight in potential effect modification. Since diuretics are commonly prescribed for diabetic patients, associations between dietary magnesium and CVD mortality could be affected by diabetes status. Therefore, a sensitivity analysis excluding patients with diabetes (n = 310) from the subgroup of diuretic users (n = 1,050) was performed. To get insight in the type of diuretic that could affect the association between magnesium and CVD mortality, two additional sensitivity analyses were performed excluding thiazide users (n = 165) and potassium-sparing diuretic users (n = 65). In another sensitivity analysis, patients using magnesium containing supplements (n = 235) were excluded. Finally, subgroup analyses were performed in strata of total iron intake (low vs. high based on median intake of 10.2 mg/d) and fiber intake (low vs. high based on the median intake of 21.0 g/d), because of possible correlation with magnesium in the diet.

SPSS version 25.0 (SPSS, Inc. Chicago, IL) was used for all analyses. Two-sided P-values <0.05 indicated statistical significance.

Results

Patients were on average 69 ± 5.6 y old and 21% was female. Patients had their last MI on average 4.3 ± 3.2 y ago. The mean magnesium intake was 302 ± 78 mg/day, with 960 out of 3,432 male (28%) and 306 out of 933 (33%) female patients having adequate intakes. Magnesium containing supplements were used by 235 out of 4,365 patients (5.4%). Baseline characteristics in tertiles of energy adjusted dietary magnesium are shown in Table 1. Patients with a higher magnesium intake were more often highly educated, physically active, had a lower prevalence of impaired kidney function and were less often current smokers or categorized as heavy drinkers.

During a median follow-up of 12.4 y (48,473 person-y), 2,035 patients died, of which 903 due to CVD causes and 558 due to CHD causes. HRs for CVD mortality, all-cause mortality and CHD mortality by tertiles of energy adjusted dietary magnesium are presented in Table 2, using the lowest tertile as reference (<283 mg/d). Higher magnesium intake (>322 mg/day) was associated with a lower risk of CVD mortality (HR: 0.72; 95% CI: 0.54–0.98) and all-cause mortality (HR: 0.78; 95% CI: 0.64–0.95) in the fully adjusted model. When comparing the moderate to lower tertiles, associations were non-significant for CVD (HR: 0.93; 95% CI: 0.76–1.15) and all-cause mortality (HR: 0.93; 95% CI: 0.81–1.07). Magnesium intake was not significantly associated with CHD mortality (Table 2).

Figure 1 shows the results from the RCS analyses for energy-adjusted magnesium intake and CVD mortality, using the fully adjusted model and the adequate intake as the reference. Tests for a non-linear association were not statistically significant (p = 0.27), indicating a linear association. Highest CVD mortality risks were observed for magnesium intakes below the median intake. Protective risk estimates for CVD mortality were shown for magnesium intakes above the adequate intake.

When analyzing continuously using model 4, dietary magnesium (per 100 mg/d) was associated with CVD mortality (HR: 0.62; 95% CI: 045–0.86) and all-cause mortality (HR: 0.70; 95% CI: 0.57–0.86; Table 2). Borderline significant results were shown for the association between dietary magnesium (per 100 mg/d) and CHD mortality (HR: 0.67; 95% CI: 0.45–1.01; Table 2).

Subgroup and sensitivity analyses

Subgroup analyses for dietary magnesium and CVD mortality are presented in Table 3. Protective associations were found both in men and women, comparable to the total cohort.

TABLE 1 Baseline characteristics of 4,365 post-MI patients from the Alpha Omega Cohort, by tertiles of energy adjusted magnesium intake[a].

	Tertiles of energy adjusted magnesium intake		
	<283 mg/d (*n* = 1,453)	**283–322 mg/d (*n* = 1,459)**	**>322 mg/d (*n* = 1,453)**
Age, y	69.5 (60.5–78.5)	68.9 (59.9–77.9)	68.1 (59.1–77.1)
Females	236 (16)	348 (24)	349 (24)
Dutch ethnicity[b]	1,423 (98)	1,416 (97)	1,425 (98)
BMI, kg/m[b,3]	27.2 (22.2–32.2)	27.4 (22.4–32.4)	27.2 (23.4–31.4)
Obese	321 (22)	368 (25)	343 (24)
Educational level[d]			
Only elementary	347 (24)	281 (19)	254 (18)
Low	530 (37)	529 (36)	498 (34)
Intermediate	427 (29)	473 (32)	467 (32)
High	141 (10)	168 (12)	226 (16)
Smoking status[e]			
Never	158 (11)	266 (18)	298 (21)
Former; quit >10 y ago	208 (14)	272 (19)	287 (20)
Former; quit ≤10 y ago	768 (53)	710 (49)	684 (47)
Current	319 (22)	210 (14)	184 (13)
Physical activity[f]			
Low	710 (49)	581 (40)	492 (34)
Intermediate	499 (34)	569 (39)	567 (39)
High	240 (17)	300 (21)	382 (26)
Alcohol consumption[g]			
No or light drinking	802 (55)	817 (56)	840 (58)
Moderate drinking	391 (27)	414 (28)	407 (28)
Heavy drinking	260 (18)	226 (16)	206 (14)
Time since last MI, y[h]	3.7 (0–8.7)	3.6 (0–8.6)	3.4 (0–8.4)
Diabetes mellitus[i]	264 (18)	318 (22)	301 (21)
Impaired kidney function[j]	377 (26)	322 (22)	272 (19)
Blood pressure, mmHg[k]			
Systolic	140.0 (112.0–168.0)	141.5 (111.5–171.5)	140.0 (111.0–169.0)
Diastolic	80.0 (65.0–95.0)	80.0 (66.0–94.0)	79.5 (64.5–94.5)
Serum lipids, mmol/L			
LDL cholesterol[l]	2.5 (1.5–3.5)	2.5 (1.5–3.5)	2.5 (1.5–3.5)
HDL cholesterol[m]	1.2 (1.2–1.2)	1.2 (1.2–1.2)	1.3 (1.3–1.3)
Use of cardiovascular medication			
Antihypertensive drugs	1,299 (89)	1,321 (90)	1.308 (90)
Statins	1,214 (84)	1,265 (87)	1,268 (87)
Diuretics	367 (25)	341 (23)	342 (24)
Dietary intake			
Total energy, kJ/d	1,911 (1,164–2,658)	1,788 (1,159–2,417)	1,919 (1,293–2,545)
Dietary fiber, g/d	17 (10–24)	20 (13–27)	25 (17–33)
Fiber rich diet[n]	69 (5)	158 (11)	219 (15)
Saturated fatty acids, g/d	28 (12–44)	24 (12–36)	23 (11–35)
Polyunsaturated fatty acids, g/d	16 (6–26)	14 (6–22)	13 (5–21)
Sodium, mg/d[o]	1,986 (1,106–2,866)	2,051 (1,197–2,905)	2,353 (1,458–3,248)
Potassium, mg/d	2,770 (1,801–3,739)	3,090 (2,175–4,005)	3,734 (2,723–4,745)
Total iron, mg/d	9 (6–12)	10 (7–13)	12 (6–9)
Heme iron, mg/d	1 (0–2)	1 (0–3)	1 (0–3)

(Continued)

TABLE 1 Continued

	Tertiles of energy adjusted magnesium intake		
	<283 mg/d ($n = 1,453$)	283–322 mg/d ($n = 1,459$)	>322 mg/d ($n = 1,453$)
Calcium, mg/d	718 (319–1,117)	814 (421–1,207)	1,015 (520–1,510)
Vitamin D, µg/d	5 (2–8)	4 (2–6)	4 (2–6)
Vitamin C, mg/d	68 (16–120)	84 (26–142)	110 (37–183)
Beta-carotene, µg/d	1,409 (550–2,268)	1,537 (644–2,430)	1,761 (785–2,737)
DHD15-index score[p]	79 (61–97)	80 (62–98)	79 (60–98)

eGFR, estimated glomerular filtration rate; DHD15-index, Dutch Healthy Diet 2015 index; MET, metabolic equivalent task; MI, Myocardial Infarction.
[a]Values are means ± SDs for normally distributed variables, medians (IQRs) for skewed variables, or n (%) for categorical variables, unless otherwise indicated.
[b]Missing data for 76 patients.
[c]Missing data for 6 patients; obesity defined as BMI ≥ 30 kg/m^2.
[d]Missing data for 24 patients.
[e]Missing data for 1 patient.
[f]Missing data for 25 patients; low activity defined as ≤3 METs, intermediate activity as >3 METs on > 0 to < 5 days per week and high activity as >3 METs on ≥ 5 days per week.
[g]No/light drinking defined as <10 g/d for males and <5 g/d for females, moderate drinking as ≥10–30 g/d for males and ≥5–15 g/d for females and heavy drinking as ≥30 g/d for males and ≥15 g/d for females.
[h]Missing data for 38 patients; MI based on a verified clinical diagnosis <10 y before study enrolment.
[i]Diabetes mellitus based on a self-reported physician's diagnosis, use of antidiabetic medication, and/or elevated plasma glucose (≥7.0 mmol/L when fasted or ≥11.1 mmol/L when not fasted).
[j]Based on CKD-EPI-eGFR <60 ml/min/1.73 m^2.
[k]Missing data for 6 patients.
[l]Non-fasted, missing data for 309 patients.
[m]Non-fasted, missing data for 111 patients.
[n]Fiber rich diet was self-reported.
[o]Sodium intake only from foods, since discretionary salt use was not assessed by means of the FFQ.
[p]DHD15-index for adherence to the 2015 Dutch dietary guidelines (range 0–100), with higher scores indicating a healthier diet.

TABLE 2 HRs for magnesium intake in relation to CVD, all-cause and CHD mortality in 4,365 post-MI patients from the Alpha Omega Cohort[a].

	Tertiles of energy adjusted magnesium intake			Per 100 mg/d
	<283 mg/d	283–322 mg/d	>322 mg/d	
CVD mortality				
Cases	333	307	263	903
Model 1	1.00	0.83 (0.70–1.00)	0.64 (0.53–0.78)	0.67 (0.57–0.79)
Model 2	1.00	0.92 (0.77–1.10)	0.74 (0.60–0.90)	0.75 (0.64–0.89)
Model 3	1.00	0.90 (0.73–1.11)	0.69 (0.51–0.93)	0.58 (0.42–0.80)
Model 4	1.00	0.93 (0.76–1.15)	0.72 (0.54–0.98)	0.62 (0.45–0.86)
All-cause mortality				
Cases	750	679	606	2,035
Model 1[b]	1.00	0.86 (0.76–0.96)	0.71 (0.63–0.81)	0.75 (0.67–0.83)
Model 2[c]	1.00	0.96 (0.85–1.08)	0.83 (0.73–0.94)	0.85 (0.76–0.94)
Model 3[d]	1.00	0.91 (0.79–1.04)	0.76 (0.62–0.92)	0.66 (0.54–0.82)
Model 4[e]	1.00	0.93 (0.81–1.07)	0.78 (0.64–0.95)	0.70 (0.57–0.86)
CHD mortality				
Cases	201	195	162	558
Model 1	1.00	0.94 (0.75–1.17)	0.69 (0.54–0.89)	0.70 (0.57–0.86)
Model 2	1.00	1.04 (0.83–1.31)	0.80 (0.62–1.04)	0.80 (0.64–0.98)
Model 3	1.00	1.03 (0.79–1.35)	0.78 (0.54–1.15)	0.62 (0.41–0.93)
Model 4	1.00	1.08 (0.83–1.41)	0.84 (0.58–1.24)	0.67 (0.45–1.01)

CVD, cardiovascular disease; CHD, coronary heart disease; MI, myocardial infarction.
[a]Values are HRs (95% CIs) obtained from Cox proportional hazards models, using the lowest tertile as reference.
[b]Adjusted for age and sex.
[c]As model 1, plus smoking, alcohol intake, physical activity, obesity and education level.
[d]As model 2, plus intake of total energy, calcium, vitamin D, sodium (only from foods), potassium, heme iron, vitamin C, beta-carotenoids, polyunsaturated fatty acids, saturated fatty acids, fiber-rich diet and DHD15-Index.
[e]As model 3, plus systolic blood pressure, kidney function and diabetes mellitus.

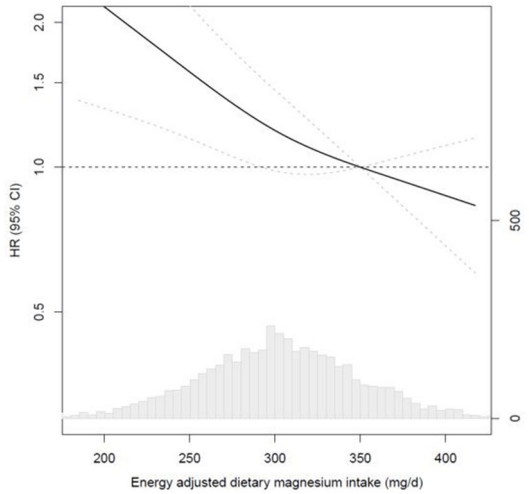

FIGURE 1
Multivariable-adjusted restricted cubic spline analyses for the continuous association of energy-adjusted magnesium intake with CVD mortality in 4,365 post-MI patients from the Alpha Omega Cohort. HRs for CVD mortality (left y-axis) are presented as a bold line for every level of magnesium intake; the reference (HR of 1.0) was set at 350 mg/d, which is the Adequate Intake for men; HRs were fully adjusted (see Table 3, model 4); knots were placed at the 10th, 50th, and 90th percentile; dotted lines indicate 95% Cis; the bottom graph shows the distribution of magnesium intakes in the Alpha Omega Cohort, with frequencies on the right y-axis. CVD, cardiovascular disease; MI, myocardial infarction.

Stronger inverse associations were found in patients using diuretics (HR: 0.55 for upper vs. lower tertile; 95% CI: 0.34–0.89), compared to non-diuretic users (HR: 0.89; 95% CI: 0.61–1.30). The presence of diabetes or impaired kidney function did not essentially modify the associations (Table 3). HRs were roughly similar across strata of total iron intake. For dietary fiber, however, stronger associations were found in patients with a relatively low fiber intake (HR: 0.54; 95% CI: 0.32–0.91). Results of subgroup analyses for dietary magnesium in relation to all-cause and CHD mortality (secondary endpoints) are presented in Supplementary Tables 1, 2. Results in subgroups were roughly similar to those for CVD mortality, expect for smaller subgroups like women or impaired kidney function where findings were inconsistent.

Sensitivity analyses were performed in the subgroup of diuretic users (Supplementary Table 3, Supplementary Figure 2). After excluding diabetic patients, the association between dietary magnesium and CVD mortality became stronger (HR: 0.47; 95% CI: 0.26–0.83). After excluding thiazide or potassium-sparing diuretic users, the associations became weaker, with HRs around 0.71 in the upper vs. lower tertiles.

Results for CVD mortality were similar after excluding a small group of patients (5.4%) who used magnesium containing supplements (HR: 0.94 in mid vs. lower tertile; 95% CI: 0.75–1.16 and HR: 0.72 in upper vs. lower tertile; 95% CI: 0.53–0.98) (data not shown in table).

Discussion

This prospective study in 4,365 Dutch patients with a history of MI showed strong inverse associations of dietary magnesium with CVD mortality and all-cause mortality. Associations with CVD mortality were mainly present in patients using diuretics and in patients with a low dietary fiber intake.

The average magnesium intake in our cohort based on FFQ data was around 300 mg/d, which is relatively low. A difference of 100 mg/d in energy adjusted magnesium intake was related to a 30–40% lower risk of CVD and all-cause mortality in (several subgroups of) our cohort. To the best of our knowledge, there are no previous studies of dietary magnesium and mortality in post-MI patients. A meta-analysis of population-based studies by Bagheri et al. showed inverse associations of dietary magnesium with CVD mortality (pooled relative risk (RR): 0.92 for highest vs. lowest comparison; 95% CI: 0.87–0.97, $n = 9$) and all-cause mortality (RR: 0.91 for highest vs. lowest comparison; 95%CI: 0.87–0.96, $n = 14$) when pooling cohort studies that adjusted for total energy intake (comparable to our study). For CHD mortality, we found a HR of 0.67 per 100 mg/d in post-MI patients, which was borderline significant. A meta-analysis by Zhao et al. (554,581 participants) of population-based studies also showed an inverse association with CHD mortality (pooled RR: 0.92 per 100 mg/d, 95% CI: 0.82–0.98) (4). Based on the totality of evidence, we conclude that magnesium intake could be more strongly related to mortality risk in CVD patients than in the general population.

Diuretics may impact magnesium status. Thiazide and potassium-sparing diuretics inhibit sodium transport in the kidney, which indirectly affects magnesium reabsorption (27). In a population-based, cross-sectional study among 9,820 participants, the use of thiazide diuretics was associated with lower serum magnesium levels and a higher risk of hypomagnesaemia (28). In the Alpha Omega Cohort, dietary magnesium tended to be more strongly associated with a lower CVD mortality risk in in diuretic users than in non-users. The prevalence of diabetes in our study was higher in diuretic users (30%) than in non-users (17%). After excluding diabetic patients from the analysis, the stronger association in diuretic users persisted. Based on sensitivity analyses by type of diuretics, we conclude that both thiazide diuretics and potassium-sparing diuretics could be involved in the relationship between magnesium intake and CVD mortality.

Dietary magnesium may interact with dietary iron, which could have an effect on CVD mortality. In our multivariable models, we could only adjust for heme iron (not for total iron)

TABLE 3 HRs for energy adjusted magnesium intake in relation to CVD mortality in subgroups of post-MI patients from the Alpha Omega Cohort[a].

	Tertiles of energy-adjusted magnesium intake*		
	<283 mg/d	283–322 mg/d	>322 mg/d
Sex			
Male ($n = 3,432/682$ cases)	1.00	0.91 (0.72–1.16)	0.73 (0.52–1.01)
Female ($n = 933$)	1.00	0.99 (0.62–1.57)	0.66 (0.32–1.34)
Diuretics[b]			
Users ($n = 1,050/364$)	1.00	0.81 (0.57–1.14)	0.55 (0.34–0.89)
Non-users ($n = 3,315/539$ cases)	1.00	1.11 (0.85–1.46)	0.89 (0.61–1.30)
Prevalent diabetes[c]			
Yes ($n = 883/222$ cases)	1.00	1.09 (0.69–1.73)	0.64 (0.33–1.22)
No ($n = 3,482/681$ cases)	1.00	0.91 (0.72–1.15)	0.76 (0.54–1.07)
Kidney function			
eGFR <60 ($n = 971/280$ cases)	1.00	0.92 (0.62–1.37)	0.75 (0.41–1.37)
eGFR ≥60 ($n = 3,394/591$ cases)	1.00	0.92 (0.71–1.18)	0.71 (0.50–1.01)
Iron intake[d]			
Low ($n = 2,182/483$ cases)	1.00	0.98 (0.74–1.30)	0.71 (0.45–1.14)
High ($n = 2,183/420$ cases)	1.00	0.92 (0.65–1.29)	0.69 (0.45–1.05)
Fiber intake[e]			
Low ($n = 2,183/489$ cases)	1.00	0.98 (0.74–1.29)	0.54 (0.32–0.91)
High ($n = 2,182/414$ cases)	1.00	1.05 (0.73–1.52)	0.90 (0.58–1.39)

CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; MI, myocardial infarction.

*Tertile cut-off values based on total cohort.

[a]Values are HRs (95% CIs) obtained from Cox proportional hazards models, using the lowest tertile as the reference. HRs are adjusted for age, sex, smoking, alcohol intake, physical activity, obesity, education level, dietary factors (see Table 2), systolic blood pressure, kidney function and diabetes mellitus (if not used as stratification factor).

[b]Diuretic use was coded according to the Anatomical Therapeutic Chemical Classification System with code C03.

[c]Diabetes mellitus based on a self-reported physician's diagnosis, use of antidiabetic medication, and/or elevated plasma glucose (≥7.0 mmol/L when fasted or ≥11.1 mmol/L when not fasted).

[d]Stratification based on median iron intake (<10.2 vs. ≥ 10.2 mg/d).

[e]Stratification based on median fiber intake (<21.0 vs. ≥ 21.0 g/d).

because of multicollinearity (VIF of total iron 7.4, results not shown). We therefore performed an additional stratified analysis by total iron intake, showing similar HRs for magnesium and CVD in patients with lower and higher iron intake. Based on our results, we conclude that the associations that we report for magnesium are (largely) independent of iron intake. It may be hypothesized that dietary fiber, rather than magnesium, contributed to the inverse associations with CVD mortality. Magnesium and fiber are partly derived from the same food sources like fruits and vegetables, cereals and legumes (29), and intakes were highly correlated in our study (VIF-5.5, results not shown). Dietary fiber may influence the absorption of minerals, including magnesium, although this is not yet fully understood (30). Protective associations of dietary fiber against CVD mortality have been found in population-based studies, as summarized in a meta-analysis of 15 cohort studies (RR: 0.91, 95% CI: 0.88–0.94 per 10 g/d increase in dietary fiber) (31). It should be noted, however, that most studies in the fiber-CVD meta-analysis (31) did not correct for magnesium and it is therefore unclear whether cardioprotective associations are attributable to dietary fiber or magnesium. In our present study of the Alpha Omega Cohort, we adjusted for prescribed fiber-rich diets in the multivariable models. Furthermore, we conducted subgroup and sensitivity analyses, in which we showed inverse associations for magnesium both in patients with low and high fiber intake and in (magnesium depleted) diuretic users. Based on the totality of findings, we think that magnesium intake independently of fiber could have contributed to the lower risk of CVD mortality in the Alpha Omega Cohort.

Our study had some limitations. Because of the observational design of the study, we should be aware of confounding. We carefully adjusted for a large number of dietary and lifestyle factors, including an overall healthy diet score. Residual confounding from unknown or imprecisely measured confounders, however, cannot fully be excluded. We used a detailed 203-item FFQ for estimating magnesium intake, but dietary assessment methods based on self-report have several shortcomings. We only assessed magnesium intake at baseline, and not during follow-up. This could have led to misclassification of patients for their true magnesium intake, and attenuation of the associations with mortality in our study. Another limitation is the

homogenous study population. Patients in the Alpha Omega Cohort were predominantly male, older, post-MI patients from the Netherlands. Results can therefore not be extrapolated to female, younger or healthy populations. Furthermore, we did not analyze blood concentrations of magnesium. However, magnesium concentrations are tightly regulated and only 1–2% of the total body magnesium is circulating (14).

To conclude, we observed a strong, linear inverse association of dietary magnesium with CVD and all-cause mortality risk after MI, which was most pronounced in patients who used diuretics. Our findings emphasize the importance of an adequate magnesium intake in CVD patients, on top of cardiovascular drug treatment.

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 Central Ethics Committee (Haga Hospital) and by an Ethics Committee in each participating hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

IE conducted the research, performed data-analysis, interpreted the data, wrote the final manuscript, and had primary responsibility for final content. EC performed data-analysis, interpreted the data, and critically reviewed the manuscript. IK performed data-analysis, drafted the first version of the manuscript, and critically reviewed the manuscript. MB supervised the data-analysis and critically reviewed the manuscript. RW critically reviewed the manuscript. JG conceived and designed the study, performed data acquisition, critically reviewed the manuscript, and had primary responsibility for final content. 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.936772/full#supplementary-material

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Dietary copper intake and risk of myocardial infarction in US adults: A propensity score-matched analysis

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Objectives: Most studies have examined the association between serum copper and myocardial infarction, but there is little evidence of the association between dietary copper intake and myocardial infarction.

Materials and methods: The study included a total of 14,876 participants from the 2011 to 2018 National Health and Nutrition Examination Survey (NHANES). Multivariate logistic regression model was used to analyze the association between dietary copper intake and the risk of myocardial infarction. To reduce selection bias, we use nearest neighbor propensity score matching (PSM) in a 1:2 ratio. Restricted cubic spline (RCS) method is used to study the non-linear relationship. Subgroup stratification was used to further investigate the association between copper intake and myocardial infarction.

Results: The median dietary copper intake was 1.0825 mg/day. A myocardial infarction had occurred in approximately 4.4% (655) of the participants. Before and after matching, multivariate logistic regression models revealed a negative correlation between dietary copper intake and the risk of myocardial infarction. The higher quartile of subjects had a noticeably lower risk of myocardial infarction in comparison to those in the first quartile of copper intake. According to RCS findings, dietary copper intake and myocardial infarction have a non-linear and dose-response relationship. According to stratified analysis, the dietary copper intake was a substantial protective element for those who were $\geq$ 50 years old, female, 25 $\leq$BMI $<$30, with history of smoking, hypertension, diabetes and ortholiposis.

Conclusion: Increased dietary copper intake was associated with a lower risk of myocardial infarction. It is especially significant in elderly-aged women, overweight individuals, smokers, hypertension, and diabetic patients.

KEYWORDS

dietary copper intake, myocardial infarction, health risk, NHANES, propensity score matching

Introduction

The major cause of mortality and morbidity worldwide is cardiovascular disease (CVD), and myocardial infarction (MI) is one of the most serious and lethal cardiovascular diseases. It is frequently fatal and worsened by cardiac failure, shock, and malignant arrhythmia (1). Because of its acute onset, high case fatality rate and poor prognosis, MI has become one of the major challenges faced by cardiovascular doctors. Therefore, it is important to detect the mechanisms and risk factors of myocardial infarction for the prevention, diagnosis and treatment. Studies have shown that the manifestation and progression of cardiovascular diseases are linked to the intake, metabolic disorders and imbalances of trace elements (2–5).

Copper, an essential micronutrient for human health and development, is involved in various biological processes in the heart muscle that are critical to cardiac metabolism and function. In one study, dietary copper supplementation is shown to replenish cardiac copper, increase VEGF, promote angiogenesis, and reverse mouse hypertrophic cardiomyopathy (6). Furthermore, copper is crucial element of superoxide dismutase 1 (SOD1) and Cytochrome c Oxidase (CcO), which are involved in antioxidant activity and mitochondrial energy metabolism (7–9). Copper restriction in the diet leads to cardiac hypertrophy, which in turn causes heart failure (10). Capillary density decreases with the loss of Cu, and a failing heart's myocardial mitochondria's structure and function are altered (11, 12). In hypertrophic hearts instigated by contraction of the upper aorta and dietary Copper deficiency, Copper supplementation enhanced the contractile function as well as structural integrity (6, 13). In fundamental investigations, several studies have shown a connection between copper depletion and myocardial ischemic infarction (14). However, in clinical studies, there is insufficient evidence to prove the relationship between copper content and myocardial infarction. Previous studies investigating the correlation between copper and CVD had shown erratic results, majority of these found the correlation of serum concentrations of these elements instead of their dietary intake (15–25). In previous studies, high serum copper was correlated with cardiovascular risk (26–30). Instead of dietary intake variations, these serum levels were linked to an increase in ceruloplasmin (and serum copper) (31, 32). The relationship between copper consumption and its serum levels is complicated by the fact that copper absorption varies with age, gender, the use of oral contraceptives as well as copper intake from different food sources (20, 26). As a result, the amount of copper in the blood cannot be used to determine total body copper status or their dietary consumption (27). Nevertheless, dietary recommendations for these elements' nutritional value cannot be influenced by the correlation between CVD and serum concentrations of these elements. As a result, dietary copper intake must be studied instead of its serum concentration to determine the risk of cardiovascular disease. However, these connections have not been addressed before. To fill the gap of this study, we collected data from the 2011 to 2018 National Health and Nutrition Examination Survey (NHANES) and conducted a comprehensive cross-sectional analysis with national representation, assessing dietary copper consumption and myocardial infarction threat to provide a theoretical basis for prevention.

Materials and methods

Study population

The NHANES study is a multi-stage, stratified, large, nationally representative study of the United States population. It is conducted by the Centers for Disease Control and Prevention's National Center for Health Statistics to assess the nutritional and physical status of Americans (33). The cross-sectional survey includes data on population demographics, diet, physical examination, and questionnaires, among others. NHANES survey data is freely available on the web to be used by data researchers and other users. For more information about NHANES, visit www.cdc.gov/nchs/nhanes/.

The study involved 39,156 participants aged 20–80 years. Among these individuals, we excluded participants who did not participate in recording copper intake (n = 10,867), participants who did not record myocardial infarction (n = 11,180), participants with missing confounding data (n = 2,055), pregnant women (n = 171), and participants with large deviations in copper intake (n = 7). A total of 14,876 participants were included in the analysis. The NCHS Ethics Review Board approved all NHANES programs, and participants or their agents provided informed consent prior to participation (34).

Variables

Copper intake was set as the dependent variable and MI as the independent variable. The dietary data was gathered from the NHANES database, and all participants gave two 24-h dietary recall interviews. The first recall interview was conducted at the NHANES Mobile Testing Center, and the second recall interview was conducted by telephone 3–10 days later. The daily total of all nutrients/food components was calculated using the USDA Food and Nutrition Diet Research database and entered into the NHANES database (35). In our study, we analyzed mean copper intake from two such 24-h recalls.

The incidence of MI was detected using the Health Status Questionnaire (MCQ). MI was said to have occurred when the participant answered "yes" to the question, "Has a doctor or other health professional ever told {you/SP}{you/s/he}. a heart

attack (also known as a myocardial infarction (my-o-car- dee-al in-fark-shun)?" (36).

Building on previous research, we included the following relevant covariates: sex, age, education level (less than 9th grade, grades 9–11, high school graduation/GED or equivalent, some college or AA degree, college graduation or above), BMI, smoking history, drinking history, hypertension, diabetes, and lipid levels. Participants were considered to have diabetes if one of the following was true: they were informed by a physician or other health professional that they had diabetes, were taking insulin, or were taking diabetes medication to lower blood sugar (37). Participants were considered to have hypertension if they met one of the following criteria: were advised by a doctor or other healthcare professional regarding their high blood pressure or were told they were taking antihypertensive medication, and had a mean systolic blood pressure (SBP) $\geq$ 140 mmHg or diastolic blood pressure (DBP) $\geq$ 90 mmHg on examination. Detailed information on the process of obtaining dietary copper intake, MI, and other covariates can be found at www.cdc.gov/nchs/nhanes/.

TABLE 1 Baseline characteristics of total participants according to copper intake quartiles.

Characteristic	Total subjects	Copper intake quartile, mg/day				
		Q1 (0.0655–0.807)	Q2 (0.807–1.082)	Q3 (1.082–1.44)	Q4 (1.44–10.6205)	*P*-value
Number	14,876	3,712	3,722	3,722	3,720	
Median intake	1.083	0.65	0.95	1.24	1.79	
Age (years old)	50.15 ± 17.45	50.38 ± 18.21	50.97 ± 17.79	50.35 ± 17.23	48.90 ± 16.46	<0.001
Sex, *n* (%)						<0.001
Male	7,318 (49.19)	1,329 (35.80)	1,686 (45.30)	1,934 (51.96)	2,369 (63.68)	
Female	7,558 (50.81)	2,383 (64.20)	2,036 (54.70)	1,788 (48.04)	1,351 (36.32)	
Level of education, *n* (%)						<0.001
Less than 9th grade	1,090 (7.33)	350 (9.43)	287 (7.71)	261 (7.01)	192 (5.16)	
9–12th grade	1,758 (11.82)	604 (16.27)	469 (12.60)	366 (9.83)	319 (8.58)	
High school graduate/GED or equivalent	3,369 (22.65)	1,037 (27.94)	914 (24.56)	783 (21.04)	635 (17.07)	
Some college or AA degree	4,730 (31.80)	1,209 (32.57)	1,192 (32.03)	1,192 (32.03)	1,137 (30.56)	
College graduate or above	3,929 (26.41)	512 (13.79)	860 (23.11)	1,120 (30.09)	1,437 (38.63)	
BMI (kg/m^2)						<0.001
<25	4,032 (27.10)	907 (24.43)	953 (25.60)	1,002 (26.92)	1,170 (31.45)	
25–30	4,775 (32.10)	1,133 (30.52)	1,164 (31.27)	1,218 (32.72)	1,260 (33.87)	
≥30	6,069 (40.80)	1,672 (45.04)	1,605 (43.12)	1,502 (40.35)	1,290 (34.68)	
Smoking history, *n* (%)						<0.001
No	8,356 (56.17)	1,937 (52.18)	2,125 (57.09)	2,149 (57.74)	2,145 (57.66)	
Yes	6,520 (43.83)	1,775 (47.82)	1,597 (42.91)	1,573 (42.26)	1,575 (42.34)	
Drinking history, *n* (%)						<0.001
No	4,945 (33.24)	1,450 (39.06)	1,281 (34.42)	1,175 (31.57)	1,039 (27.93)	
Yes	9,931 (66.76)	2,262 (60.94)	2,441 (65.58)	2,547 (68.43)	2,681 (72.07)	
Hypertension, *n* (%)						<0.001
No	8,224 (55.28)	1,911 (51.48)	1,996 (53.63)	2,083 (55.96)	2,234 (60.05)	
Yes	6,652 (44.72)	1,801 (48.52)	1,726 (46.37)	1,639 (44.04)	1,486 (39.95)	
Diabetes, *n* (%)						<0.001
No	10,671 (71.73)	2,605 (70.18)	2,590 (69.59)	2,659 (71.44)	2,817 (75.73)	
Yes	2,947 (19.81)	809 (21.79)	805 (21.63)	730 (19.61)	603 (16.21)	
IGT + IFG	1,258 (8.46)	298 (8.03)	327 (8.79)	333 (8.95)	300 (8.06)	
TC (mmol/L)	4.94 ± 1.09	4.93 ± 1.11	4.95 ± 1.11	4.95 ± 1.09	4.91 ± 1.05	0.322
TG (mmol/L)	1.72 ± 1.52	1.63 ± 1.20	1.75 ± 1.84	1.76 ± 1.56	1.74 ± 1.42	0.016
HDL (mmol/L)	1.37 ± 0.41	1.37 ± 0.43	1.37 ± 0.41	1.37 ± 0.41	1.37 ± 0.41	0.759

BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein.

Statistical analysis

The extraction and merging of NHANES data from 2011 to 2018 was done on R Studio (version 4.1.3). Baseline characteristics of continuous variables were compared using a *t*-test or non-parametric Mann-Whitney U test, and categorical variables were compared using a chi-square test or Fisher's test. Propensity score matching (PSM) uses 1:2 nearest neighbor matching algorithm. In 1983, Rubin and Rosenbaum proposed propensity score matching, which has been widely used in

TABLE 2 Characteristics of participants before and after matching by myocardial infarction.

Variables	Before matching			After matching			
	Control group (*n* = 14,221)	MI group (*n* = 655)	*P*-value	Control group (*n* = 1,300)	MI group (*n* = 654)	SMD	*P*-value
Age (years old)	49.38 ± 17.29	66.89 ± 11.36	<0.001	64.79 ± 14.05	66.87 ± 11.36	0.16 (0.07, 0.26)	0.043
Sex, *n* (%)			<0.001			0.06(-0.03, 0.16)	0.186
Male	6,892 (48.46)	426 (65.04)		805 (61.92)	425 (64.98)		
Female	7,329 (51.54)	229 (34.96)		495 (38.08)	229 (35.02)		
Level of education, *n* (%)			<0.001			0.10 (0.01, 0.20)	0.332
Less than 9th grade	1,011 (7.11)	79 (12.06)		151 (11.62)	78 (11.93)		
9–12th grade	1,660 (11.67)	98 (14.96)		205 (15.77)	98 (14.98)		
High school graduate/GED or equivalent	3,185 (22.40)	184 (28.09)		326 (25.08)	184 (28.13)		
Some college or AA degree	4,543 (31.95)	187 (28.55)		361 (27.77)	187 (28.59)		
College graduate or above	3,822 (26.88)	107 (16.34)		257 (19.77)	107 (16.36)		
BMI (kg/m^2)			<0.001			0.07(-0.02, 0.17)	0.328
<25	3,898 (27.41)	134 (20.46)		243 (18.69)	134 (20.49)		
25–30	4,569 (32.13)	206 (31.45)		451 (34.69)	206 (31.50)		
≥30	5,754 (40.46)	315 (48.09)		606 (46.62)	314 (48.01)		
Smoking history, *n* (%)			<0.001			0.10 (0.00, 0.19)	0.044
No	8,133 (57.19)	223 (34.05)		504 (38.77)	223 (34.10)		
Yes	6,088 (42.81)	432 (65.95)		796 (61.23)	431 (65.90)		
Drinking history, *n* (%)			0.537			0.05(-0.04, 0.15)	0.259
No	4,720 (33.19)	225 (34.35)		479 (36.85)	224 (34.25)		
Yes	9,501 (66.81)	430 (65.65)		821 (63.15)	430 (65.75)		
Hypertension, *n* (%)			<0.001			0.07(-0.02, 0.17)	0.128
No	8,101 (56.97)	123 (18.78)		283 (21.77)	123 (18.81)		
Yes	6,120 (43.03)	532 (81.22)		1,017 (78.23)	531 (81.19)		
Diabetes, *n* (%)			<0.001			0.19 (0.09, 0.28)	<0.001
No	10,372 (72.93)	299 (45.65)		671 (51.62)	299 (45.72)		
Yes	2,643 (18.59)	304 (46.41)		490 (37.69)	304 (46.48)		
IGT + IFG	1,206 (8.48)	52 (7.94)		139 (10.69)	51 (7.80)		
TC (mmol/L)	4.96 ± 1.08	4.44 ± 1.15	<0.001	4.58 ± 1.06	4.44 ± 1.14	0.12 (0.03, 0.22)	0.010
TG (mmol/L)	1.71 ± 1.53	1.89 ± 1.28	0.004	1.86 ± 1.48	1.89 ± 1.28	0.02(-0.07, 0.12)	0.637
HDL (mmol/L)	1.38 ± 0.41	1.27 ± 0.41	<0.001	1.29 ± 0.39	1.27 ± 0.41	0.06(-0.04, 0.15)	0.233
Copper intake quartiles (%)			<0.001			0.13 (0.04, 0.23)	0.054
Q1 (0.0655–0.807)	3,506 (24.65)	206 (31.45)		303 (23.31)	185 (28.29)		
Q2 (0.807–1.082)	3,555 (25.00)	167 (25.50)		332 (25.54)	157 (24.01)		
Q3 (1.082–1.44)	3,564 (25.06)	158 (24.12)		322 (24.77)	166 (25.38)		
Q4 (1.44–10.6205)	3,596 (25.29)	124 (18.93)		343 (26.38)	146 (22.32)		

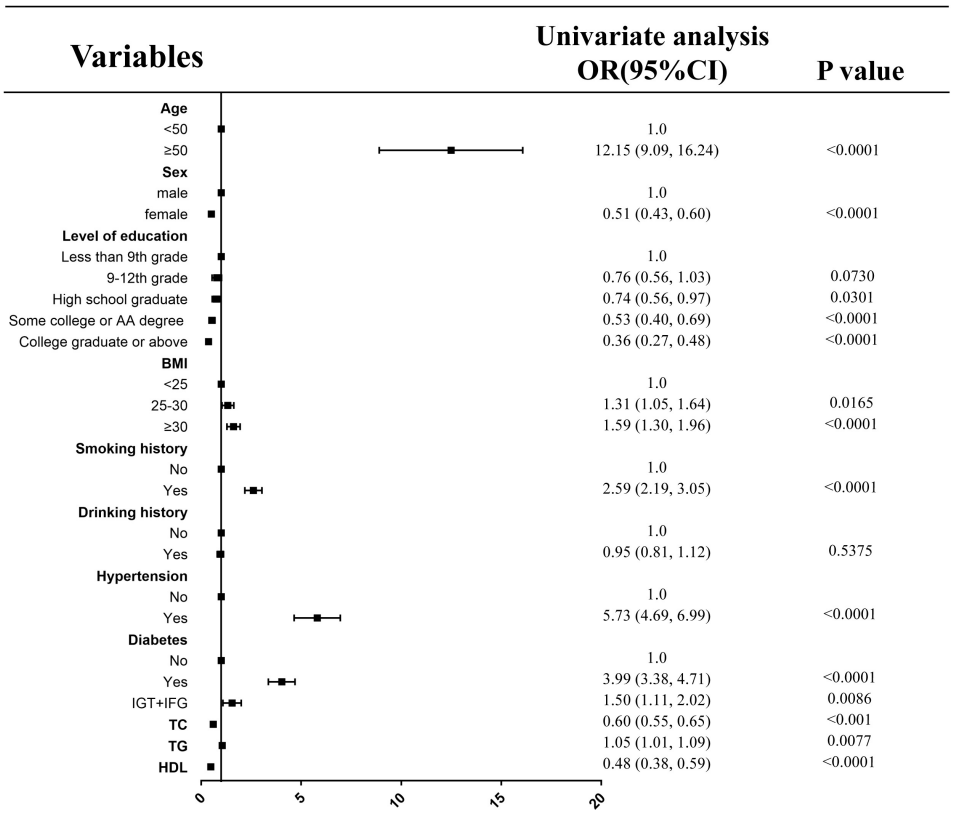

FIGURE 1
Effects of various factors on myocardial infarction by univariate analysis.

observational studies to reduce selection bias (38, 39). It is based on the concept of counterfactual and can strengthen causal arguments in observational research by reducing selection bias (40). Confounding factors such as age, sex, education level, BMI, smoking history, drinking history, hypertension, diabetes and blood lipids were selected for matching. Univariate Logistic regression was used to analyze the risk factors of myocardial infarction. Multivariate logistic regression model was used to analyze the relationship between copper intake and myocardial infarction. Possible non-linear relationships are determined by restricted cubic splines (RCS). Stratified analyses were performed according to age, sex, BMI, smoking history, hypertension, diabetes, and lipid levels. The association between copper intake and the risk of myocardial infarction was further investigated. In addition, to further test the robustness of the results, we conducted a sensitivity analysis using the inverse probability weighting of propensity scores (IPTW) method. All data analysis and graphic design were performed using R Studio (version 4.1.3), EmpowerStats,[1] GraphPad Prism (version 8.0), and Adobe Illustrator (version 2020). $P < 0.05$ was considered statistically significant.

1 http://www.empowerstats.com

Results

Baseline characteristics of total participants according to copper intake quartiles

The average age of the subjects was 50.15 ± 17.45 years old, of whom 49.19% were male (n = 7,318). The median value of Copper was 1.083 mg/day. The medians of copper in quartile group were 0.65, 0.95, 1.24, and 1.79 mg/day, respectively. There were statistically significant differences in all parameters among the four groups except TC and HDL levels (**Table 1**).

Characteristics of participants before and after matching by myocardial infarction

A total of 655 participants had MI before matching. Except for the history of drinking, the incidence of other covariates was significantly different ($P < 0.01$). Using closest neighbor propensity score matching (1:2), we established a comparable control group to further support the relationship between

dietary copper intake and MI risk(**Supplementary Figures 1 and 2**). There wasn't a significant statistical difference in the majority of baseline characteristics between the two groups after propensity score matching, which matched 1,300 participants in the control group and 654 participants in the MI group (**Table 2**). Meanwhile, the standardized mean difference (SMD) of baseline data before and after matching in the MI group was less than 0.01, which was not statistically significant (**Supplementary Table 1**).

Effects of various factors on myocardial infarction by univariate analysis

With the exception of drinking history, all other parameters were correlated with myocardial infarction. The risk of myocardial infarction (95% confidence interval) among participants aged ≥ 50 years was 12.15 (9.09, 16.24) compared with participants younger than 50 years. The odds ratio (95% confidence interval) for this correlation was 0.51 (0.43, 0.60) for female relative to male. The incidence of MI was 1.31 (1.05, 1.64) and 1.59 (1.30, 1.96) for 25 ≤ BMI < 30 Kg/m^2 and ≥ 30 Kg/m^2 compared with BMI < 25 Kg/m^2, respectively, as depicted in **Figure 1** as well as in **Table 3**.

TABLE 3 Effects of various factors on myocardial infarction by univariate analysis.

Variables	Statistics	OR (95%CI)	*P*-value
Age (years old)			
<50	7,175(48.23)	1.0	
≥50	7,701 (51.77)	12.15 (9.09, 16.24)	<0.0001
Sex, *n* (%)			
Male	7,318 (49.19)	1.0	
Female	7,558 (50.81)	0.51 (0.43, 0.60)	<0.0001
Level of education, *n* (%)			
Less than 9th grade	1,090 (7.33)	1.0	
9–12th grade	1,758 (11.82)	0.76 (0.56, 1.03)	0.0730
High school graduate/GED or equivalent	3,369 (22.65)	0.74 (0.56, 0.97)	0.0301
Some college or AA degree	4,730 (31.80)	0.53 (0.40, 0.69)	<0.0001
College graduate or above	3,929 (26.41)	0.36 (0.27, 0.48)	<0.0001
BMI (kg/m^2)			
<25	4,032 (27.10)	1.0	
25–30	4,775 (32.10)	1.31 (1.05, 1.64)	0.0165
≥30	6,069 (40.80)	1.59 (1.30, 1.96)	<0.0001
Smoking history, *n* (%)			
No	8,356 (56.17)	1.0	
Yes	6,520 (43.83)	2.59 (2.19, 3.05)	<0.0001
Drinking history, *n* (%)			
No	4,945 (33.24)	1.0	
Yes	9,931 (66.76)	0.95 (0.81, 1.12)	0.5375
Hypertension, *n* (%)			
No	8,224 (55.28)	1.0	
Yes	6,652 (44.72)	5.73 (4.69, 6.99)	<0.0001
Diabetes, *n* (%)			
No	10,671 (71.73)	1.0	
Yes	2,947 (19.81)	3.99 (3.38, 4.71)	<0.0001
IGT + IFG	1,258 (8.46)	1.50 (1.11, 2.02)	0.0086
TC (mmol/L)	4.94 ± 1.09	0.60 (0.55, 0.65)	<0.001
TG (mmol/L)	1.72 ± 1.52	1.05 (1.01, 1.09)	0.0077
HDL (mmol/L)	1.37 ± 0.41	0.48 (0.38, 0.59)	<0.0001

Correlation analysis of copper intake and myocardial infarction before and after matching

Before matching, without adjusting for any confounders, the odds ratios for the correlation of copper with myocardial infarction compared with Q1 were 0.80 (0.65, 0.99), 0.75 (0.61, 0.93), and 0.59 (0.47, 0.74) in univariate logistic regression, respectively. After adjusting for confounders (age, sex, level of education, BMI, smoking history, hypertension, diabetes, TC, TG and HDL), the differences in the correlation with MI among the other three groups were 0.78 (0.62, 0.97), 0.78 (0.62, 0.98), 0.69 (0.53, 0.88), respectively (**Table 4**).

After matching without adjusting for any confounders, in univariate logistic regression, the odds ratio for the correlation of copper with myocardial infarction was 0.70 (0.53, 0.91) in group Q4 compared with group Q1, and the remaining two groups were not statistically different. After adjusting for confounders (age, sex, level of education, BMI, smoking history, hypertension, diabetes, TC, TG and HDL), the odds ratio for the correlation between copper and myocardial infarction in the Q4 group was 0.70 (0.53, 0.93), and this correlation was not significant in the other groups (**Table 5**).

Before and after matching, we conducted RCS analysis to better understand the correlation between the risk of myocardial infarction and dietary copper intake. Before matching, we discovered an L-shaped connection between dietary copper consumption and risk of myocardial infarction in adjusted models (**Figure 2**). With increased dietary copper intake, there was a non-linear decrease in the prevalence of myocardial infarction (*P* for non-linear = 0.0109). Results from the RCS model (**Figure 3**) similarly revealed a non-linear association between dietary copper intake and the threat of myocardial infarction after matching (*P* for non-linear = 0.049).

Stratified analysis before and after matching

To determine whether the relationship between copper intake and myocardial infarction varied by age, sex, BMI,

TABLE 4 Correlation analysis between copper intake and myocardial infarction before matching.

	Model 1β (95% CI) *p* value	Model 2β (95% CI) *p* value	Model 3β (95% CI) *p* value
Copper	0.70 (0.60, 0.82) <0.0001	0.76 (0.64, 0.89) 0.0007	0.78 (0.67, 0.92) 0.0023
Copper (mg/d) quartiles			
Q1 (0.0655–0.807)	1.0	1.0	1.0
Q2 (0.807–1.082)	0.80 (0.65, 0.99) 0.0362	0.76 (0.61, 0.94) 0.0127	0.78 (0.62, 0.97) 0.0284
Q3 (1.082–1.44)	0.75 (0.61, 0.93) 0.0094	0.75 (0.60, 0.93) 0.0109	0.78 (0.62, 0.98) 0.0366
Q4 (1.44–10.6205)	0.59 (0.47, 0.74) <0.0001	0.65 (0.51, 0.83) 0.0005	0.69 (0.53, 0.88) 0.0030

Model 1: No adjustments made for confounding factors.
Model 2: Adjustments made for age, sex, level of education and BMI.
Model 3: Adjustments same as that in model 2 plus hypertension, diabetes, smoking history, TC, TG, and HDL.

TABLE 5 Correlation analysis between copper intake and myocardial infarction after matching.

	Model 1β (95% CI) *p* value	Model 2β (95% CI) *p* value	Model 3β (95% CI) *p* value
Copper	0.79 (0.67, 0.93) 0.0046	0.79 (0.67, 0.94) 0.0064	0.79 (0.67, 0.94) 0.0063
Copper (mg/d) quartiles			
Q1 (0.0655–0.807)	1.0	1.0	1.0
Q2 (0.807–1.082)	0.77 (0.60, 1.01) 0.0574	0.77 (0.59, 1.00) 0.0528	0.77 (0.59, 1.01) 0.0554
Q3 (1.082–1.44)	0.84 (0.65, 1.10) 0.2052	0.82 (0.63, 1.08) 0.1554	0.83 (0.63, 1.08) 0.1628
Q4 (1.44–10.6205)	0.70 (0.53, 0.91) 0.0079	0.70 (0.53, 0.93) 0.0124	0.70 (0.53, 0.93) 0.0125

Model 1: No adjustments made for confounding factors.
Model 2: Adjustments made for age, sex, level of education and BMI.
Model 3: Adjustments same as that in model 2 plus hypertension, diabetes, smoking history, TC, TG, and HDL.

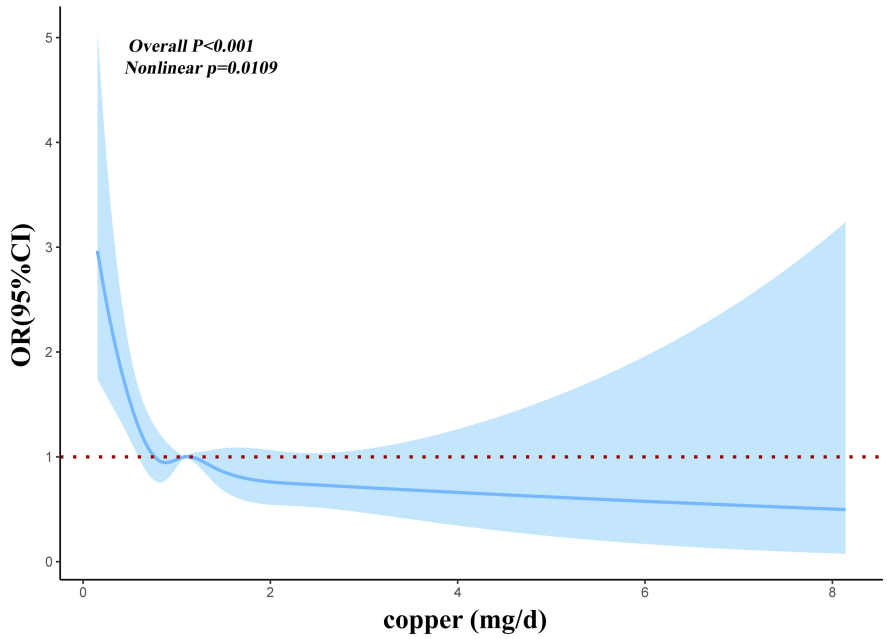

FIGURE 2
Restricted cubic spline models for the relationship between dietary copper intake and the risk of myocardial infarction before matching.

smoking status, hypertension, diabetes, and lipid profiles, stratified analyses were employed. When age was used in stratified analysis, the correlation between copper intake and myocardial infarction was more substantial in those aged ≥ 50 years, with odds ratios of 0.74 (0.62, 0.88) and 0.77 (0.65, 0.92) before and after matching, respectively. There were more significant for the correlation of copper with myocardial infarction Q4 compared with Q1, with odds

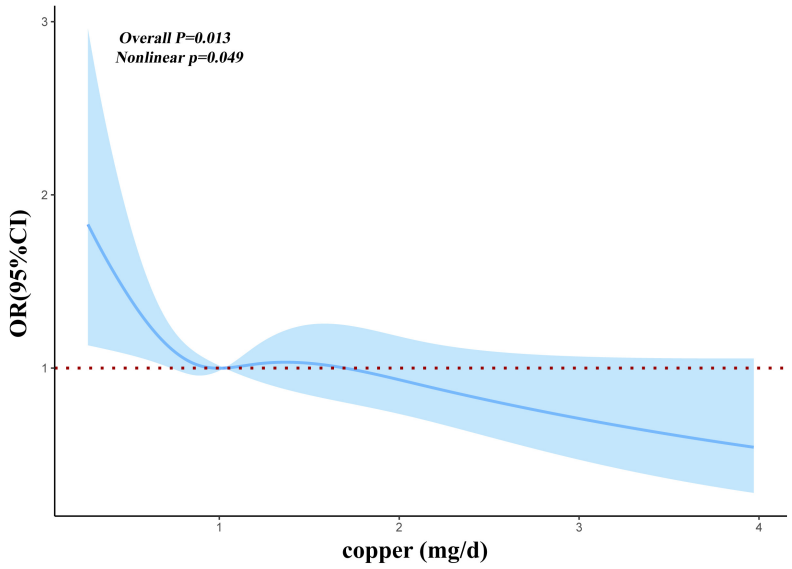

FIGURE 3
Restricted cubic spline models for the relationship between dietary copper intake and the risk of myocardial infarction after matching.

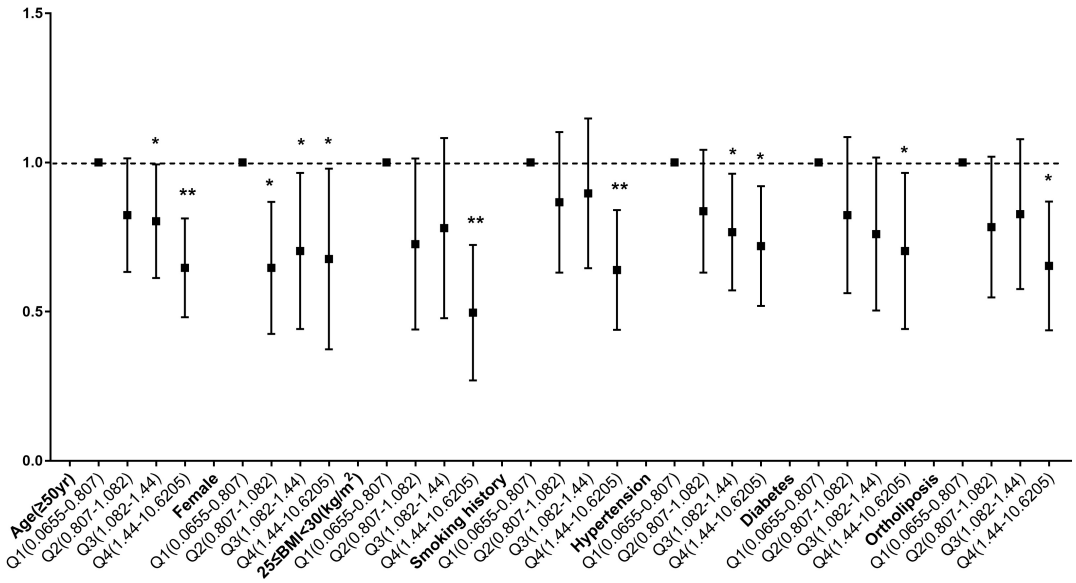

FIGURE 4
Subgroup analysis of dose-related copper intake and myocardial infarction before matching. *$p < 0.05$, **$p < 0.01$.

ratios of 0.63 (0.49, 0.82) and 0.68 (0.51, 0.91) before and after matching (**Supplementary Table 2**). Likewise, female (**Supplementary Table 3**), 25 ≤ BMI < 30 (**Supplementary Table 4**), smokers (**Supplementary Table 5**), hypertensive patients (**Supplementary Table 6**), diabetic patients (**Supplementary Table 7**) and People with normal blood lipids (**Supplementary Table 8**) had a stronger correlation between copper consumption and myocardial infarction. Before matching (**Figure 4**) and after matching (**Figure 5**), the correlation of copper intake in group Q4, with a dose-response relationship, was more significant than that in group Q1.

Sensitivity analysis

The baseline data of the two groups were weighted by inverse probability of treatment weighting (IPTW) using the propensity score for possible confounding factors, such as age,

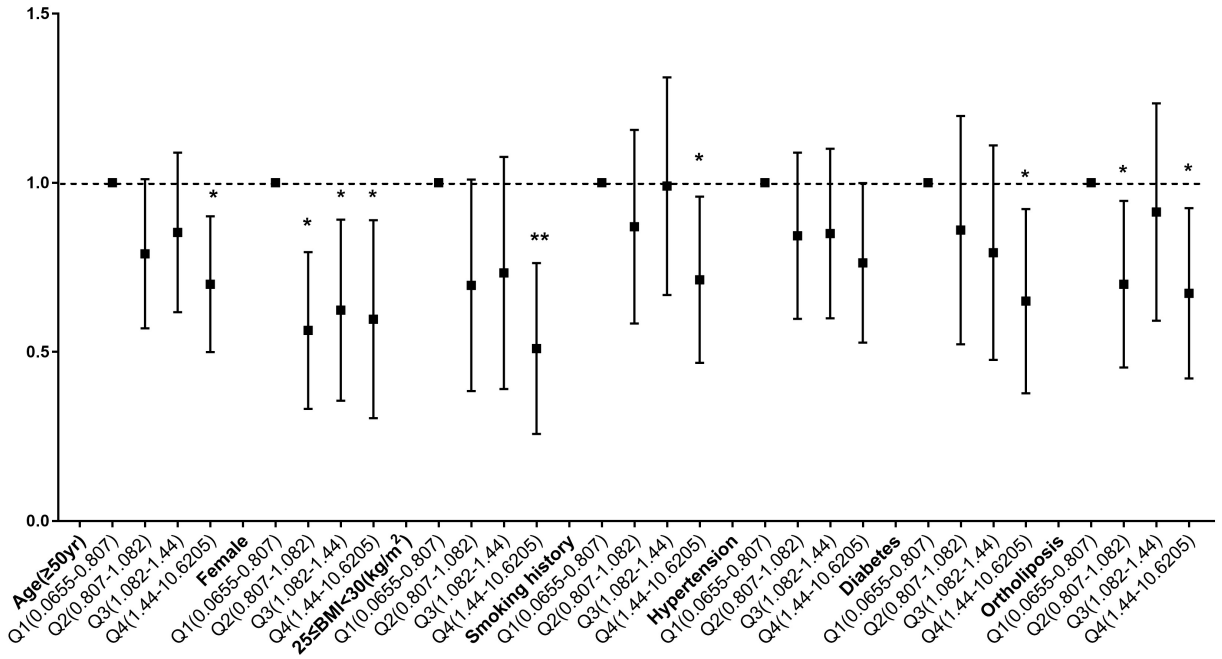

FIGURE 5
Subgroup analysis of dose-related copper intake and myocardial infarction after matching. *$p < 0.05$, **$p < 0.01$.

sex, level of education, BMI, smoking history, drinking history, hypertension, diabetes and blood lipids. After weighting, the data of the two groups were well balanced and comparable except for age (**Supplementary Table 9**). Myocardial infarction was used as the dependent variable and weighted copper intake was used as the independent variable in a multivariate logistic regression analysis. After adjusting for confounders, there was a significant negative correlation between the two groups, indicating that the above results were robust (**Supplementary Table 10**).

Discussion

The results of this study preliminarily verified the new evolutionary biological view of myocardial infarction: copper could prevent myocardial infarction (41). A few previous studies had reported the relationship between dietary copper intake and myocardial infarction, which was consistent with the results of this study. Hanish et al. (1) used Mendelian randomization to assess that copper could reduce the risk of IHD, which was consistent with the suggestion more than 40 years ago. Although this hypothesis has long existed, it has been largely ignored, and to our knowledge no large studies have evaluated the effect of copper on myocardial infarction.

Here, we analytically reviewed large population data of 14,876 cases between 2011 and 2018 to explore the probable correlation between copper consumption and the risk of myocardial infarction. The study had some major findings. First and in line with earlier studies, we found that for myocardial infarction: age, male gender, overweight and obesity, smoking, hypertension, diabetes and blood lipids level were risk factors. The present study's most significant conclusion was that the risk of myocardial infarction decreased with an increase of dietary copper intake before and after matching. It was proposed that copper intake might be a preventative measure against myocardial infarction when combined with the findings of RCS analysis. Additionally, stratified analysis further revealed that dietary copper intake was a important protective characteristic for people aged $\geq$ 50 years, female, 25 $\leq$ BMI $<$ 30, with history of smoking, hypertension, diabetes and ortholiposis.

Copper, as a powerful antioxidant, might act as preventive measure in the progress of atherosclerosis which underlined cardiovascular diseases. According to Klevay et al. (42), copper was an antioxidant, a constituent of monoamine oxidase and SOD, which benefited the blood vessel wall's fiber's by maintaining their toughness and suppleness. Copper had been shown to promote cardiac regeneration by reactivating hypoxia inducible factor 1-regulated angiogenesis, constituting another therapeutic strategy for ischemic heart disease (43). Molecular biology studies had shown that copper functioned in different organelles as a cofactor for proteins and enzymes required for cytoplasmic maturation and enzyme production (44). Copper deficiency leaded to cholesterol breakdown and disturbance of plasma lipoprotein metabolism, resulting in cholesterol deposition in damaged blood vessels and

atherosclerosis. Here, we found an inverse correlation between dietary copper intake and the threat of myocardial infarction, further emphasizing the protective effect of antioxidant minerals on cardiovascular diseases. Our findings were consistent with those of Kodali et al. (1): Copper might reduce the risk of ischemic heart disease. Yin et al. (45) showed that high levels of antioxidant micronutrients in the diet were correlated with reduced cardiovascular disease morbidity; levels of iron, zinc, and copper were inversely and non-linearly correlated with cardiovascular diseases. Therefore, trace minerals might have the ability to prevent cardiovascular disease.

This study also made a lot of subgroup analysis, which had explored the impact of copper intake on different populations. It was found that copper intake had a significant impact on myocardial infarction in elderly people (whose age $\geq$ 50 years old). This might be due to the increased risk of micronutrient deficiency in the elderly caused by pathophysiological changes (46). Although the plasma copper level was in the normal range, the availability of intracellular copper ions was reduced, thus older individuals were more prone to a range of cardiovascular diseases when they were copper deficient. Therefore, dietary copper element supplementation was appropriate for the elderly (47). By gender subgroup analysis, the effect of dietary copper intake on myocardial infarction was found to be slightly more significant in women than in men (0.80 $>$ 0.73), possibly because the effect of Cu absorption was consistently 10% higher in women than in men (27). As one of the important risk factors of myocardial infarction, hypertension was also closely related to copper ions. This study found that copper intake was more significant in hypertensive population with myocardial infarction, and there had been numerous studies on the mechanisms of copper and the development of hypertension in a series of ways, since copper was required in several enzymatic functions to maintain the integrity of the vascular system (48). A crucial enzyme in the control of blood pressure, angiotensin-converting enzyme (ACE), could be inhibited by copper at the same time (49). Copper deficiency was also correlated with impaired endothelium-dependent arterial relaxation which could lead to hypertension, possibly due to reduced activity of copper zinc superoxide dismutase (21). In addition, copper participated in the synthesis of dehydroepiandrosterone (DHEA) through oxidation of cholesterol. Therefore, the decrease of DHEA level due to copper deficiency might lead to the development of hypertension (50). Diabetes was one of the risk factors for myocardial infarction, and there were a large number of studies on Cu ions and diabetes which were consistent with the results of this study. Increased amounts of triglyceride and cholesterol production, insulin, and peroxidation damage, release may result from Cu deficiency when combined with glucose, fructose, or iron ingestion (51). Different studies had been directly or indirectly targeting Cu ions to reverse diabetic complications, and there were also results showing that classical hypoglycemic drugs had the ability to chelate Cu ions. In different cell lines, smoking could cause direct or indirect oxidative stress and copper, as a consistent antioxidant, could prevent some health issues instigated by smoking, such as lung disease, kidney failure and diabetes (52). The meta-analysis results of Wang et al. (53) were consistent with the subgroup analysis of blood lipid levels, and dietary copper had no effect on people with dyslipidemia, which may be related to the regulatory mechanism of Cu in the body.

This study also has some limitations. First of all, because it is a cross-sectional study, causal inference is not possible. Further prospective studies are required to confirm these findings. Secondly, the inclusion criteria of myocardial infarction depend on the self-reported history of myocardial infarction, and their impact on the sub-types and stages of myocardial infarction was still unclear. Despite the fact that we have incorporated numerous covariates into our analytic model, we are unable to completely rule out the influence of unidentified confounding factors. Therefore, to elucidate the correlation between dietary copper intake and risk of MI, larger clinical studies on different stages and sub-types of MI are required in the future.

Conclusion

This is a large cross-sectional study based on the NHANES database to estimate the association between dietary copper intake and the risk of MI. We found that the risk of MI decreased with increasing dietary copper intake. In addition, copper intake was found to be more protective in elderly-aged women, overweight individuals, hypertensives, smokers, and diabetic patients. It is speculated that MI may be prevented by adjusting dietary copper intake, the protective action of which, has a theoretical basis.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Health and Nutrition Examination Survey (NHANES).

Ethics statement

The NHANES study was approved by the Research Ethics Review Board of the National Center for Health Statistics, and all participants signed informed consent forms. Access to the NHANES database does not require any ethical or administrative rights. More details are available online, at www.cdc.gov/nchs/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

HW and XN proposed the strategy and implemented the extraction and collation of the data. XN, NS, and RZ validated and analyzed the data. HW and LH drafted the manuscript. QW and YL revised and finalized the manuscript. All authors 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.942000/full#supplementary-material

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Fecal microbiome transplantation and tributyrin improves early cardiac dysfunction and modifies the BCAA metabolic pathway in a diet induced pre-HFpEF mouse model

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More than 50% of patients with heart failure present with heart failure with preserved ejection fraction (HFpEF), and 80% of them are overweight or obese. In this study we developed an obesity associated pre-HFpEF mouse model and showed an improvement in both systolic and diastolic early dysfunction following fecal microbiome transplant (FMT). Our study suggests that the gut microbiome-derived short-chain fatty acid butyrate plays a significant role in this improvement. Cardiac RNAseq analysis showed butyrate to significantly upregulate *ppm1k* gene that encodes protein phosphatase 2Cm (PP2Cm) which dephosphorylates and activates branched-chain α-keto acid dehydrogenase (BCKDH) enzyme, and in turn increases the catabolism of branched chain amino acids (BCAAs). Following both FMT and butyrate treatment, the level of inactive p-BCKDH in the heart was reduced. These findings show that gut microbiome modulation can alleviate early cardiac mechanics dysfunction seen in the development of obesity associated HFpEF.

KEYWORDS

heart failure with preserved ejection fraction, gut microbiome, short chain fatty acids, branched chain amino acids (BCAAs), obesity

Introduction

Heart failure with preserved ejection fraction (HFpEF) continues to be one of the most hypercritical cardiovascular diseases in the United States that requires immediate intervention (1, 2). It accounts for more than half of heart failure patients but has only one guideline-directed treatment, the sodium-glucose cotransporter 2 (SGLT2) inhibitors (3). The pathophysiology of HFpEF is complex, and this syndrome has been increasingly characterized as heterogeneous. Better phenotyping of patients into common pathophysiological groups has been proposed as a tool to treat HFpEF better (4). Obesity is the main driver for the pathogenesis of HFpEF with more than 80% of HFpEF patients being overweight or obese, making obesity associated HFpEF a specific pathological entity (4, 5). To date, obesity associated HFpEF studies involve the induction of systemic inflammation which increases reactive oxygen species (ROS) and

oxidative stress, increases collagen deposition, limits nitric oxide (NO) bioavailability, and decreases protein kinase G (PKG) activity. This ultimately leads to cardiomyocyte hypertrophy, left ventricular (LV) stiffness, fibrosis, and the development of diastolic dysfunction and exercise intolerance (5–10). The presence of a new pathological identity termed "pre-HFpEF" has been recently identified where patients have no signs and symptoms of heart failure, they have normal ejection fraction of >50%, however, they show structural abnormalities to their hearts that resemble those found in clinical HFpEF, such as LV hypertrophy (11). It is important to understand and act on early cardiac changes observed in pre-HFpEF prior to transition to clinical HFpEF therefore our study focuses on the pre-HFpEF stage.

Among the shared risk factors between cardiovascular diseases and obesity is the accumulation of circulating branch chain amino acid (BCAA) and its decreased metabolism (12, 13). Impaired cardiac BCAA metabolism is associated with cardiac insulin resistance and the development of cardiovascular diseases (13–16). This occurs due to a decrease in the levels and activity of branched-chain alpha-keto acid dehydrogenase (BCKDH) complex that catalyzes the first irreversible step in the catabolism of branched chain amino acids (BCAAs). The increase in the levels of inactive p-BCKDH has been attributed to the downregulation of *ppm1k* gene encoding protein phosphatase 2C (PP2Cm) (17, 18). The aforementioned changes were observed once the HFpEF pathophysiology is installed, and more studies are necessary to discover the early mechanism underlying these changes.

It is well established that diet-induced obesity (DIO) mouse models are coupled with gut microbiome imbalance. Western diet (WD)-fed mice (high fat, high carbohydrate, low fiber) have significant reduction in their microbiome diversity and composition compared to control mice (19, 20), as well as a significant decrease in the short chain fatty acid (SCFA) butyrate producing bacteria *Lactobacillus* and *Lachnospiraceae* (19, 20). Interestingly, microbial metagenome and metabolomic analysis found significant reduction in butyrate producing bacteria in patients with chronic heart failure with reduced ejection fraction (HFrEF) (21). More recently, microbiome DNA sequencing analysis in HFpEF patients showed significant alterations in gut microbiome composition as well as a reduction in SCFA producing bacteria compared to control groups (22, 23). Butyrate, a microbiome-secreted SCFA, was shown to prevent cardiac hypertrophy progression in a pressure overload model of cardiac hypertrophy (24), and was found to improve cardiac function and ventricular arrhythmia in rats after myocardial injury (25).

The molecular mechanisms linking the gut microbiome imbalance, the circulating SCFAs, particularly butyrate, and the development of HFpEF are still unknown. Studies to date have not deciphered whether the microbiome imbalance observed in HFrEF and HFpEF patients is a secondary finding related to poor gut hemodynamic or the primary driver of the cardiac physiopathology. We investigated the effect of gut microbiome modulation using fecal microbiome transplantation (FMT) in the early stages of obesity associated HFpEF. We developed an obesity associated model of early cardiac dysfunction (pre-HFpEF) and focused on the early asymptomatic changes in cardiac mechanics that occur in the absence of increased intracardiac pressure.

Our study provides an insight on the potential role of gut microbiome and its metabolite butyrate in the early stage of obesity associated HFpEF and identifies the branched chain amino acids (BCAAs) metabolic pathway as a possible link between microbiome imbalance, obesity, and heart failure. These results open a new avenue not only for therapy but also for the prevention of HFpEF development and progression.

Abbreviations: HFpEF, heart failure with preserved ejection fraction; SCFA, short chain fatty acids; FMT, fecal microbiome transplantation; BCAA, branched chain amino acids; BCKA, branched chain keto acids; PP2Cm, protein phosphatase 2C mitochondria; PPM1K, protein phosphatase, Mg2 + /Mn2 + Dependent 1K; BCKDH, branched-chain alpha-keto acid dehydrogenase; p-BCKDH, phosphorylated branched-chain alpha-keto acid dehydrogenase; 4-HNE, 4-hydroxynonenal; ROS, reactive oxygen species; WD, western diet; NC, normal chow.

Results

Mice fed western diet (WD) developed early systolic and diastolic dysfunction consistent with pre-HFpEF

To study obesity associated pre-HFpEF, we developed a model of diet-induced obesity and assessed cardiac function. C57BL/6J mice were placed on WD for 14 weeks (**Figure 1A**) and compared to their littermates on normal chow (NC). We performed echocardiography measurements to investigate changes in cardiac function. As expected in a pre-HFpEF model, mice on WD had no change in their left ventricular ejection fraction (LVEF) (**Figure 1B**), and the ratio between peak velocity blood flow from left ventricular relaxation in early diastole (the E wave) to peak velocity flow in late diastole caused by atrial contraction (the A wave) (E/A ratio measurement) (**Figure 1C**) compared to NC mice. Mice fed a WD had significant decrease in global longitudinal strain (%GLS) (3.190% ± 0.9887) and longitudinal strain rate reverse peak (LSRr) ($-2.363\ s^{-1} \pm 0.6213$) indicating, respectively early signs of systolic and diastolic dysfunction (unpaired *t*-test, $P = 0.0025$, $P = 0.0005$, respectively) (**Figures 1D, E**). WD mice showed significant increase in their left ventricle posterior wall thickness during diastole (0.1368 mm ± 0.02956) (unpaired *t*-test, $P = 0.0004$) (**Figure 1F**) indicating the development of LV hypertrophy. As an early model to assess obesity associated HFpEF development, mice on WD had no significant increase in nitrosative stress or cardiac fibrosis, two players in the pathogenesis of HFpEF, indicated by no changes in *nos2* and *col1a2* (**Figures 1G, H**) expression levels. Taken together, our data show obese mice to have normal ejection fraction, early diastolic and systolic dysfunction, LV hypertrophy, with an absence of nitrosative stress and fibrosis. All of which are consistent with a diet-induced obesity pre-HFpEF phenotype.

Fecal matter transplantation (FMT) improved early systolic and diastolic dysfunction and cardiac hypertrophy in obese pre-HFpEF mice

To test whether gut microbiome alteration can reverse or delay pre-HFpEF progression, we treated obese pre-HFpEF mice with an established protocol (19) that includes an antibiotics treatment for 3 days, to deplete the gut microbiome followed by 5 days

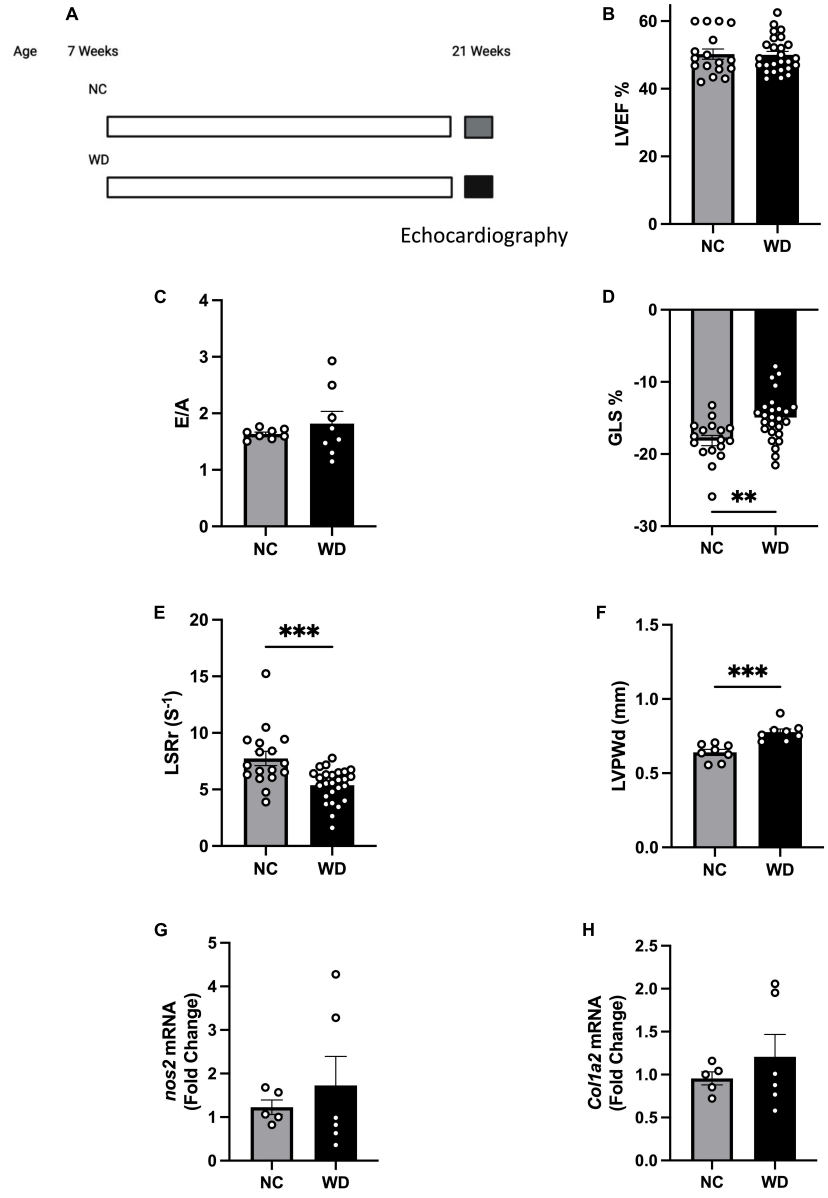

FIGURE 1
Mice fed western diet (WD) developed early systolic and diastolic dysfunction and cardiac hypertrophy. **(A)** Experimental paradigm. Normal chow group was fed NC (Teklad LM-485), while the western diet group was fed WD (TD88137, Teklad Diets; 42% kcal from fat, 34% sucrose by weight, and 0.2% cholesterol total; Envigo) for 14 weeks, starting at 7 weeks of age. Echocardiography measurements were performed at 21 weeks of age, **(B)** echocardiography measurement of left ventricle ejection fraction (LVEF), **(C)** ratio between early to atrial diastolic trans mitral flow velocity (E/A), **(D)** global longitudinal strain (%GLS), **(E)** longitudinal strain rate reverse (LSRr) (s^{-1}), **(F)** left ventricle posterior wall diameter during diastole (mm), **(G,H)** mRNA levels of *nos2* and *col1a2* in hearts of mice from different experimental groups, respectively. Statistical analysis was done using unpaired student's *t*-test. Data are mean ± S.E.M. (*$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$).

of diet switch to colonize the gut with bacteria that grows in NC conditions. Then fecal matter transplantations (FMT) were performed from either obese mice (Sham FMT) or lean mice (FMT) for 2 weeks (Figure 2A). 16S rRNA sequencing of fecal content showed FMT treated mice to have increased microbiome diversity compared to mice treated with sham FMT, indicated by higher α-diversity index (39.76 ± 12.48) (unpaired *t*-test, $P = 0.0129$) (Figure 2B). We had previously found that WD depletes *Lactobacillus* in both fecal pellets and cecal contents (19). Strikingly, FMT was able to increase butyrate-producing bacteria *Lactobacillus* abundance (0.7753% ± 0.2836) [Figure 2C (relative abundance), Supplementary Figure 1A (absolute abundance), Supplementary Datasheet 1]. Sparse Correlations for Compositional data (SparCC) network analysis identified *Lactobacillus* as a key marker of the FMT microbiome landscape as its presence was correlated with other genera (Supplementary Figure 1B) that were significantly altered between sham FMT and FMT groups (Supplementary Table 1). We measured cardiac function with echocardiography. LVEF (Figure 2D) and E/A (Figure 2E) did not change after WD nor FMT. We found that mice receiving FMT from lean mice had significant improvement in their global longitudinal strain (−2.105% ± 0.9206) (unpaired *t*-test, $P = 0.0318$) (Figure 2F). The trend in longitudinal strain rate reverse peak (LSRr) improvement did not reach statistical significance (unpaired *t*-test, $P = 0.1075$)

(**Figure 2G**). The left ventricle posterior wall thickness (LVPWd) was significantly decreased by FMT treatment (−0.1151mm ± 0.04217) (unpaired *t*-test, $P = 0.0182$) (**Figure 2H**). In addition, we found no changes in *nos2* and *col1a2* (**Figures 2I, J**) expression levels between sham FMT and FMT groups. These data indicate that modulation of gut microbiota induced by FMT from lean mice to obese mice improves early systolic and diastolic dysfunction, as well as LV hypertrophy in obese pre-HFpEF mice. Overall, these findings suggest a key role for SCFA producers *Lactobacillus* in the reversal of diet-induced obesity pre-HFpEF.

Tributyrin treatment improved early cardiac dysfunction and cardiac hypertrophy in obese pre-HFpEF mice

We identified *Lactobacillus,* a SCFA-producer, as a key marker of the FMT microbiome landscape. We also recently showed that circulating serum levels of the SCFA butyrate were significantly increased in obese mice after FMT (19). We wanted then to investigate whether cardiac function improvements in obese pre-HFpEF mice after FMT treatment can be recapitulated by the SCFA butyrate. We previously tested various routes of butyrate treatment, doses, and drug compounds and observed an increase in circulating butyrate in blood of tributyrin (three butyrate molecules with a glycerol backbone) -treated mice (19). We then treated WD-fed mice with tributyrin (5 g/kg body weight) or vehicle for 3 weeks (**Figure 3A**). We measured the circulating levels of butyrate in Tributyrin treated mice using mass spectrometry and we found it to be significantly increased compared to vehicle treated mice (228.5 μM ± 55.08) (one-way ANOVA with repeated measures followed by Tukey's multiple comparison test, $P < 0.005$) (**Figure 3B**). Next, we measured changes in cardiac function with echocardiography and similar to our findings after FMT treatment, LVEF and E/A did not change between NC, WD and WD + Tributyrin treated mice (**Figures 3C, D**). As seen in **Figure 1**, mice on WD had significant decrease in their%GLS compared to NC mice (8% ± 2.108), and this change was significantly improved with tributyrin treatment (−10.43% ± 1.928) (one-way ANOVA with repeated measures followed by Tukey's multiple comparison test, $P < 0.0001$) (**Figure 3E**). We also found Tributyrin treatment to significantly improve LSRr in WD fed mice compared to vehicle treatment (4.002 s^{-1} ± 1.574) (one-way ANOVA with repeated measures followed by Tukey's multiple comparison test, $P < 0.05$) (**Figure 3F**) indicating effectiveness of tributyrin treatment in eliminating early signs of systolic and diastolic dysfunction. Additionally, changes in LVPWd observed in WD fed mice compared to NC were eliminated after tributyrin treatment (one-way ANOVA with repeated measures followed by Tukey's multiple comparison test, $P < 0.05$) (**Figure 3G**). Severe exercise intolerance is very common in obese and HFpEF patients (9, 10); therefore, we performed an exercise exhaustion test. Mice fed WD had significantly decreased running distance compared to NC (−87.50 m ± 31.61), and this difference was eliminated by tributyrin treatment (37.73 m ± 28.91) (one-way ANOVA with repeated measures followed by Tukey's multiple comparison test, $P < 0.05$) (**Figure 3H**). These results suggest that FMT and butyrate share common mechanisms in improving early cardiac function and LV hypertrophy in obese pre-HFpEF mice.

Tributyrin treatment increased *ppm1k* transcript in the heart

To better understand the molecular mechanisms leading to improvements in early cardiac function in obese pre-HFpEF mice following Tributyrin treatment, we performed cardiac RNA sequencing analysis of WD and WD + Tributyrin treated mice. Out of 23,564 transcripts identified, using an FDR (false discovery rate) of 10%, we found 34 transcripts to be altered with tributyrin treatment (**Figure 4A** and **Supplementary Datasheet 2**). Among the significantly altered genes, is the transcript protein phosphatase Mg2 + /Mn2 + dependent 1K (*ppm1k*). Tributyrin treated mice had a significant increase in *Ppm1k* transcript levels compared to WD treated mice (795.0 read count ± 175.7) (**Figure 4B**, false-discovery rate (FDR)-adjusted $P < 0.1$). *Ppm1k* encodes protein phosphatase 2C (PP2Cm), which in turn is responsible for the dephosphorylation and activation of branched-chain alpha-keto acid dehydrogenase (BCKDH) complex that catalyzes the first irreversible step in the catabolism of branched chain amino acids (BCAAs); L-leucine, L-valine, and L-isoleucine, and in turn improves BCAA metabolism (17, 26). In addition to the increase in *ppm1k* transcript levels, we found the encoded PP2Cm protein levels to be significantly increased (1.958 A.U. ± 0.7367) in the heart of WD + Tributyrin group compared to WD (unpaired *t*-test, $P = 0.0197$) (**Figures 4C, D**). Without butyrate treatment, *Ppm1k* mRNA level had a trend toward decreasing in WD-fed mice compared to NC and a trend toward increasing in lean FMT treated mice compared to sham-FMT (**Figures 4E, F**). Similarly, PP2Cm protein levels trended lower in WD-fed mice (**Figures 4G, H**) and higher in FMT compared to sham FMT treated mice (**Figures 4I, J**).

This further implies that improvements seen with FMT treatment share common mechanism with treatment with SCFA butyrate.

Tributyrin treatment decreased the p-BCKDH inactive enzyme in the BCAA metabolism pathway

Impaired BCAA metabolism occurs due to a decrease in the levels and activity of BCKDH and an increase in the levels of inactive p-BCKDH and that has been attributed to the downregulation of *ppm1k* translated protein PP2Cm (17, 18). We found the protein levels of p-BCKDH to be significantly increased in WD fed mice compared to NC (5.477 A.U. ± 0.7178) (unpaired *t*-test, $P < 0.0001$) (**Figures 4K, L**). However, p-BCKDH was significantly blunted in the heart after FMT treatment (−0.3352 ± 0.1266) (unpaired *t*-test, $P = 0.0380$) (**Figures 4M, N**) as well as after tributyrin treatment (−0.3004 ± 0.1031) (unpaired *t*-test, $P = 0.0121$) (**Figures 4O, P**). The levels of circulating BCAAs in serum was not changed between WD and WD + Tributyrin in both male and female treated mice (**Figure 4Q**). To better assess the cardiac BCAA metabolism, we measured cardiac BCAAs concentration. We found no significant changes in cardiac BCAA between the three different groups (**Figure 4R**). BCAA catabolism defects are known to be associated with heart failure development due to increase in oxidative stress and reactive oxygen species (ROS) (3, 19–22, 27–29). Elevated ROS levels lead to lipid peroxidation, where unsaturated lipids are converted to lipid peroxides that generate highly reactive and damaging lipids such as 4-hydroxynonenal (4-HNE) (30, 31). Therefore, we investigated

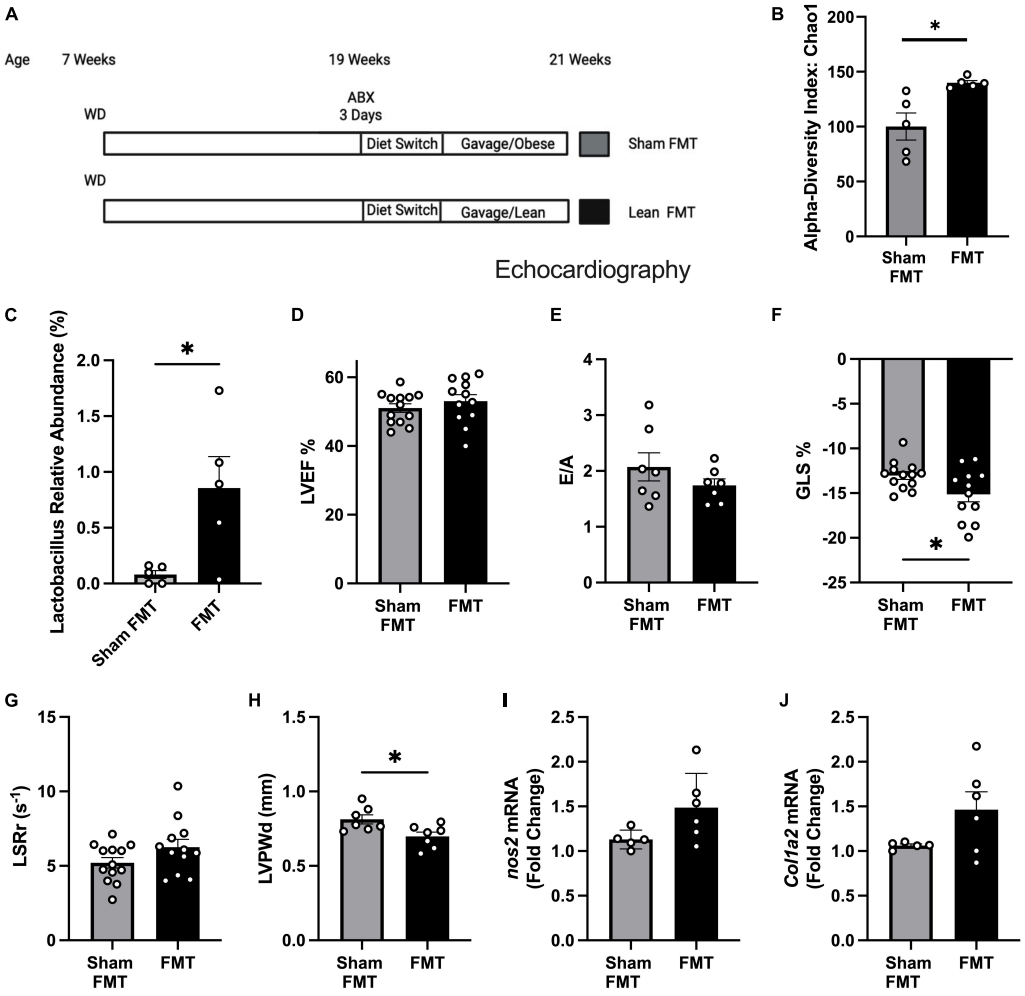

FIGURE 2
Fecal microbiome transplant (FMT) treatment improved diastolic dysfunction and cardiac hypertrophy in obese pre-HFpEF mice. **(A)** Experimental paradigm, C57BL/6J mice fed WD for 12 weeks, followed by broad-spectrum antibiotic treatment for 3 days mice were gavaged daily for 2 weeks with feces either from obese mice (sham FMT group) or from lean mice (FMT group), **(B)** alpha diversity index (Chao1), **(C)** *Lactobacillus* relative abundance (%), **(D)** representative echocardiography measurement of left ventricle ejection fraction (LVEF), **(E)** ratio between mitral E wave to A wave (E/A), **(F)** global longitudinal strain (%GLS), **(G)** longitudinal strain rate reverse (LSRr) (s^{-1}), **(H)** left ventricle posterior wall diameter during diastole (mm). **(I,J)** mRNA levels of *nos2* and *col1a2* in hearts of mice from different experimental groups. Statistical analysis was done using unpaired student's *t*-test. Data are mean ± S.E.M. (*$p < 0.05$, **$p < 0.005$).

whether tributyrin treatment decreased oxidative stress in the heart by measuring the levels of 4-HNE. We found the levels of 4-HNE to be significantly reduced with tributyrin treatment in obese pre-HFpEF mice (−0.1064 ± 0.04307) (unpaired *t*-test, $P = 0.0281$) (**Figures 4S, T**). These findings indicate the potential role of butyrate in the BCAAs metabolism pathway by increasing the levels of PP2Cm, decreasing p-BCKDH, which activates the degradation of BCAAs and in turn decreases oxidative stress in the heart.

Tributyrin upregulation of *ppm1k* occurred at least in part through histone deacetylase (HDAC) inhibition

It is well established that butyrate's effects are mediated either through (a) binding to G-protein coupled receptors (GPCRs); GPR43 (FFAR2), GPR41 (FFAR3) which activates downstream signaling pathways (32–34), or (b) epigenetic regulation through its histone deacetylase (HDAC) inhibition activity, as well as activation of histone acetyltransferases (HATs) which increases histone acetylation and gene expressions (35, 36). To test whether butyrate upregulates *ppm1k* through binding to its GPCRs GPR41/43, we performed RNAscope *in situ* hybridization of GPR41/43 on cardiac tissue sections as done previously (20) from wild-type mice and we found no staining in the heart compared to nodose ganglia, a GPR41/43 positive tissue (**Supplementary Figure 2**). These data rule-out the involvement of the receptors in butyrate's effect on heart function and hypertrophy. Next, we assessed whether HDAC inhibition may increase *ppm1k* mRNA level. We treated C2C12 cells with sodium butyrate and with the inhibitor of HDAC class I and II, Trichostatin A (TSA). We confirm the increase of *ppm1k* mRNA levels in butyrate treated cells compared to vehicle treatment (7.140 ± 1.838), and we observed that TSA also increased *ppm1k* expression compared to vehicle treatment (2.646 ± 0.2661) (unpaired *t*-test, $P = 0.003$, $P < 0.0001$, respectively) (**Figures 5A, B**). These data confirm that butyrate robustly regulates the levels of *ppm1k* in muscle cells and

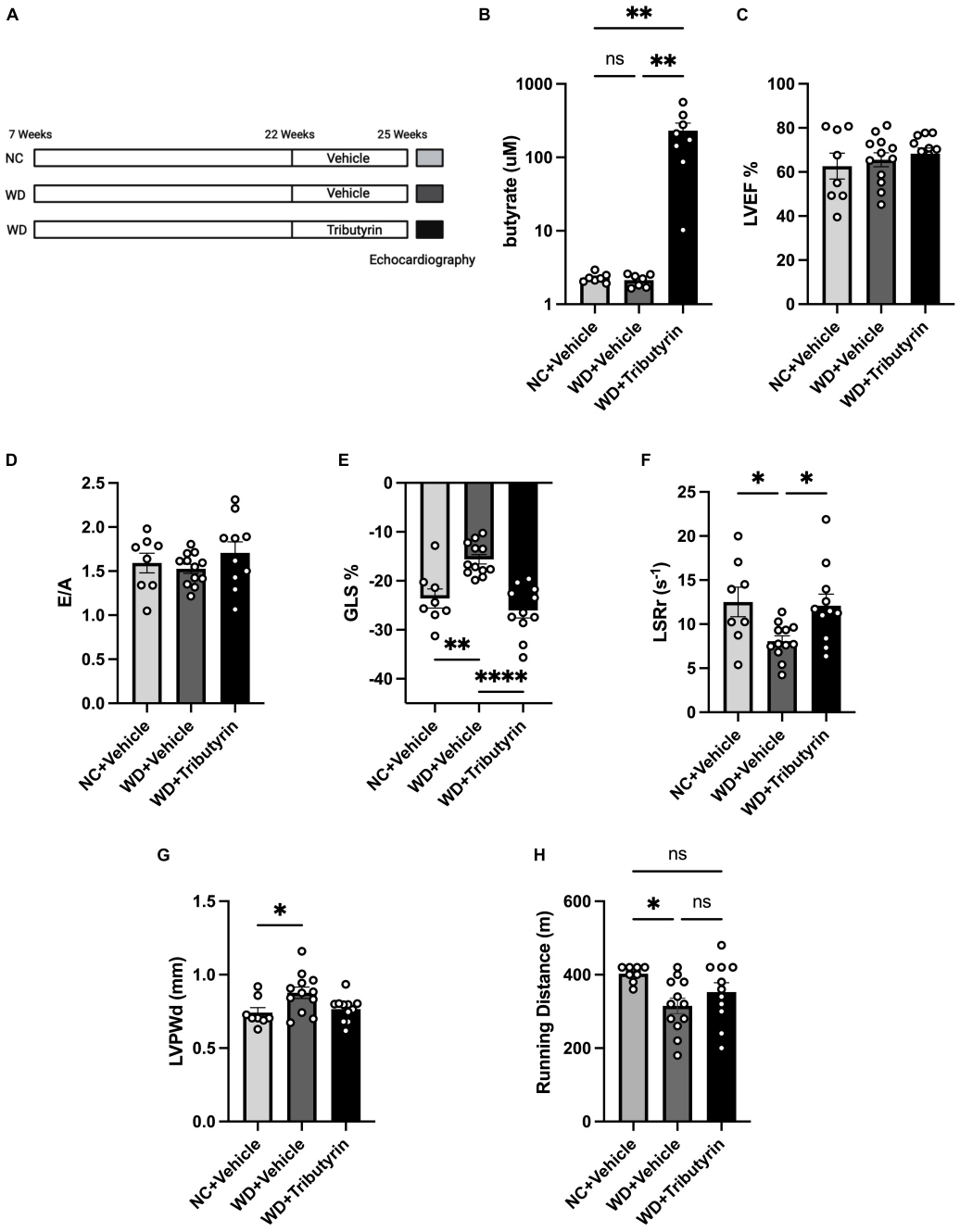

FIGURE 3
Tributyrin treatment improved early cardiac dysfunction and cardiac hypertrophy in obese pre-HFpEF mice. **(A)** Experimental paradigm, C57BL/6J fed WD or NC for 15 weeks were treated with Tributyrin using needle feeding for 3 weeks, followed by echocardiography and exercise exhaustion test. **(B)** Circulating levels of butyrate in serum (μM), **(C)** echocardiography measurements of LVEF (%), **(D)** ratio between mitral E wave to A wave (E/A), **(E)** global longitudinal strain (GLS) (%), **(F)** longitudinal strain rate reverse (LSRr) (S^{-1}), **(G)** left ventricle posterior wall diameter (mm). **(H)** Running distance during exercise exhaustion test (m). Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison test. Data are mean ± S.E.M. (*$p < 0.05$, **$p < 0.005$, and ****$p < 0.00005$), (n = 8–12 per group).

that butyrate HDAC inhibitor property may underlie these changes in gene expression.

Discussion

The role of gut microbiome and its metabolites in the development of cardiovascular diseases (CVDs) is an emerging area of research (27, 37–39). In this study we investigate the role of gut microbiome and its metabolite butyrate in obesity associated pre-HFpEF mouse model that shows early signs of cardiac dysfunction and hypertrophy. Using a specific model of obesity associated pre-HFpEF we aimed at harnessing the early changes that link obesity to the development of HFpEF. There are several animal models to try to mimic and replicate human HFpEF (40). However, most try to better understand the late stage of disease development and their findings focus on culprit or adaptative changes. By using an early stage of HFpEF with one specific risk factor which is

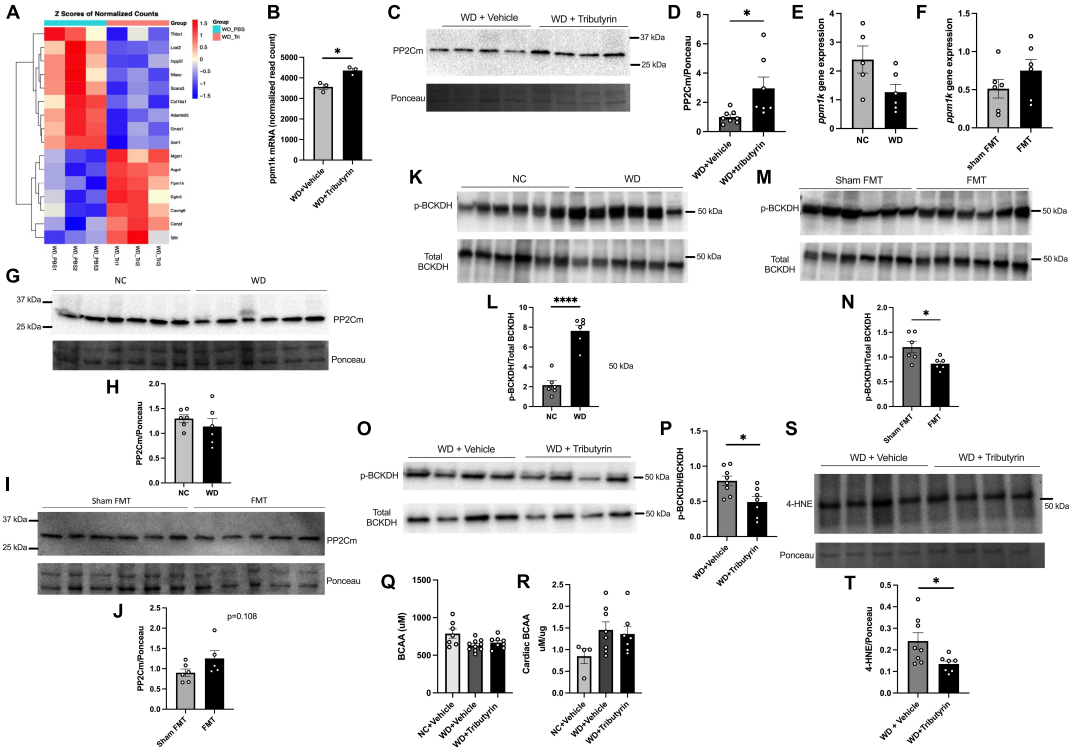

FIGURE 4

Tributyrin alters transcripts in the heart. **(A)** Topmost regulated transcripts after Tributyrin treatment in the heart (WD, WD + Tributyrin needle fed mice), FDR-adjusted $P < 0.1$ (n = 3 per group) (**Supplementary Datasheet 2**). **(B)** *ppm1k* mRNA normalized read count in WD and WD + Tributyrin treated mice (FDR-adjusted $P = 0.049515631$) **(C)** Immunoblot images of PP2Cm and total ponceau staining from hearts of WD fed mice treated with Tributyrin or vehicle. **(D)** Densitometric analysis of the ratio of PP2Cm to total protein staining bands, ($n = 7–8$ per group). **(E)** mRNA levels of *ppm1k* in heart from NC and WD fed mice ($n = 5–6$ per group). **(F)** mRNA levels of *ppm1k* in heart from sham FMT and lean FMT treated mice ($n = 6$ per group). **(G)** Immunoblot images of PP2Cm and total protein staining from NC and WD fed mice. **(H)** Densitometric analysis of the ratio of PP2Cm to total protein staining bands ($n = 6$ per group). **(I)** Immunoblot images of PP2Cm and total protein staining from sham FMT and FMT treated mice. **(J)** Densitometric analysis of the ratio of PP2Cm to total protein staining bands ($n = 5–6$ per group). **(K)** Immunoblot images of p-BCKDH, total BCKDH and total protein staining from hearts of NC and WD fed mice. **(L)** Densitometric analysis of the ratio of p-BCKDH/BCKDH protein bands ($n = 6$ per group). **(M)** Immunoblot images of p-BCKDH, total BCKDH and total protein staining from hearts of WD fed mice treated with sham FMT or FMT. **(N)** Densitometric analysis of the ratio of p-BCKDH/BCKDH protein bands ($n = 6$ per group). **(O)** Immunoblot images of p-BCKDH, total BCKDH and total protein staining from hearts of WD fed mice treated with Tributyrin or vehicle. **(P)** Densitometric analysis of the ratio of p-BCKDH/BCKDH protein bands ($n = 7–8$ per group). **(Q)** Serum BCAA levels in NC, WD and WD + Tributyrin treated mice ($n = 8–10$). **(R)** Cardiac BCAA levels in NC, WD and WD + Tributyrin treated mice normalized to total protein levels (n = 4–8). **(S)** Immunoblot images of 4-HNE and total protein staining from hearts of WD fed mice treated with Tributyrin or vehicle, **(T)** Densitometric analysis of the ratio of 4-HNE to total protein staining protein bands. Statistical analysis was done using unpaired student's *t*-test. Data are mean ± S.E.M. ($^*p < 0.05$), ($n = 7–8$ per group). Statistical analysis was done using unpaired student's *t*-test and one-way ANOVA followed by Tukey's multiple comparison test. Data are mean ± S.E.M. ($^*p < 0.05$, $^{****}p < 0.00005$).

obesity, we believe this could better help understand the early derangements that ultimately lead to the development of HFpEF. Using fecal microbiome transplantation (FMT) from lean mice, we were able to modulate gut microbiome composition in obese pre-HFpEF mice. In addition, we found FMT to improve early cardiac dysfunction and cardiac hypertrophy. Indeed microbiome alteration is a hallmark of obesity but similar derangements were also recently found in heart failure patients (21) and especially HFpEF (22, 23). Microbiota imbalance could be considered as a signature of heart failure, however, in this study we show for the first time that correction of this imbalance can improve cardiac mechanics. This could help stipulate that this derangement is a cause of HFpEF development. Further study in germ free mice could help better understand this causality. FMT treatment of obese mice led to an increase in circulating levels of the SCFA butyrate (19), and increased the levels of butyrate-producing bacteria *Lactobacillus*, which implied that FMT's improvement could occur due to SCFA butyrate. To confirm this, we treated obese pre-HFpEF mice with tributyrin, the triglyceride form of butyrate, and we were able to recapitulate FMT's effects on cardiac function and hypertrophy. Additionally, tributyrin improved exercise capacity, one of the key markers of HFpEF development. This finding can be explained by the improvement of cardiac mechanics but also opens a new area of investigation on the ability of skeletal muscles to use butyrate as a substrate for energy production. Indeed some studies have shown the potential for ketone ester to improve performance (41, 42), and more recently supplementation with the SCFA producer *Lactobacillus plantarum* was shown to increase triathlete performance (43). WD-fed mice had no significant changes in their body weight after tributyrin treatment compared to vehicle treatment, which indicates that improvements seen in cardiac function and exercise capacity was not due to weight loss but possibly due to a direct effect of FMT and butyrate on the heart. Therefore, we performed cardiac RNAseq analysis and found significant increase in *ppm1k* which regulates the rate limiting enzyme involved in branched chain amino acids (BCAAs) catabolic pathway after Tributyrin treatment. BCAAs are essential amino

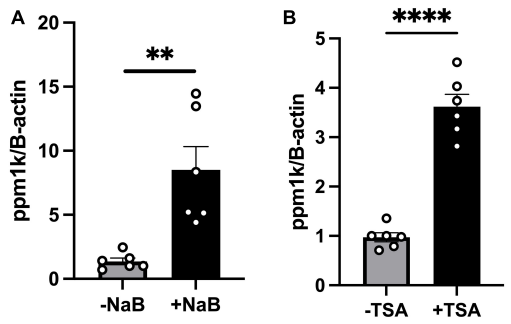

FIGURE 5
Tributyrin upregulation of *ppm1k* occurs at least in part through HDAC inhibition. **(A)** mRNA levels of *ppm1k* in C2C12 treated with 5mM sodium butyrate (NaB) or vehicle. **(B)** mRNA levels of *ppm1k* in C2C12 cells treated with 1 μM HDAC inhibitor Trichostatin A (TSA) or vehicle. Statistical analysis was done using unpaired student's *t*-test. Data are mean ± S.E.M. (**$p < 0.005$, ***$p < 0.0005$, and ****$p < 0.00005$), ($n = 6$ per group).

acids that include Valine, Leucine, and Isoleucine. They play a key role in protein synthesis, cell signaling and metabolism (44, 45). Accumulation of BCAAs has been shown in obesity and several obesity-associated cardiovascular diseases, which occurs due to an impairment in their catabolic pathway (13, 15, 16). This occurs mainly due to a deficiency in the presence and activity of branched-chain α-keto acid dehydrogenase (BCKDH) which is controlled by the activity of protein phosphatase 2Cm (PP2Cm) (28). A decrease in PP2Cm correlates with a decrease in the levels and activity of the unphosphorylated BCKDH which further leads to deficiency in the degradation and catabolism of BCAAs (13, 17, 18). This suggests that butyrate's improvement of early cardiac dysfunction, hypertrophy and exercise capacity could be mediated by enhancing the BCAAs catabolism pathway. We found the protein levels of PP2Cm and its encoding gene *ppm1k* to be significantly increased after Tributyrin treatment in obese pre-HFpEF. Concurrently, we found p-BCKDH to be significantly decreased in the heart. The regulation of *ppm1k* expression levels includes post-translational modifications such as phosphorylation, ubiquitination, and acetylation. Since butyrate is a well-known epigenetic regulator due to its histone deacetylation (HDAC) inhibition property, we tested whether butyrate's increase in *ppm1k* expression was through HDAC inhibition. We found the pharmacological HDAC inhibitor, Trichostatin A (TSA), to replicate at least in part butyrate's increase of *ppm1k* mRNA levels *in vitro*. This indicates that HDAC inhibition is one of the mechanisms by which butyrate increases *ppm1k* levels, but further work should be done to further understand the detailed effect of butyrate on *ppm1k* transcription. In addition, future work should be done to investigate whether butyrate can regulate *ppm1k* expression through activating HATs and increasing histone acetylation.

Although we found tributyrin treatment to increase PP2Cm and decrease p-BCKDH levels in the heart, we detected no change in the levels of serum or cardiac BCAAs. This is consistent with other findings of mice on HFD having no changes in their circulating BCAAs levels (46, 47). Tributyrin's increase in BCAA catabolic enzymes suggests that its role in improving cardiac function and hypertrophy in WD-fed mice is independent of changes in circulating BCAAs levels. It is still possible that changes in BCAAs occur in a cell specific manner and small differences could not be accurately measured in circulating or whole organ measurements. One of the main effects of BCAAs catabolic pathway defects is the increase in reactive oxygen species (ROS) production (18, 29, 47). Elevated levels of ROS is a known characteristic of obesity and HFpEF (1, 7, 48). Therefore, we measured the levels of 4-hydroxynonenal (4-HNE), a highly reactive and damaging lipid that is produced from lipid peroxidation and a marker of oxidative stress (31). We found the levels of 4-HNE to be significantly reduced in the heart after Tributyrin treatment in obese pre-HFpEF mice. This finding could imply that Tributyrin's effect on oxidative stress is through its change in PP2Cm and BCKDH expression, but future work would need to be done to measure the direct effect of increasing/decreasing PP2Cm and BCKDH expression and activity on ROS production.

Our study shows gut microbiome modulation and butyrate supplementation as a possible intervention to prevent the progression of obesity induced HFpEF. We identified the upregulation of BCAAs catabolic enzymes as a potential mechanism that mediates the effects of butyrate on cardiac function and hypertrophy, and a potential therapeutic target for the development of obesity associated HFpEF.

Materials and methods

Animals

Animal studies were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (49) and with the approval of the Loyola University Chicago Institutional Animal Care and Use Committee. Wild-type, C57BL/6J mice were obtained from Jackson Laboratories. Male and Female mice were fed NC (Teklad LM-485), while the experimental groups were fed WD (TD88137, Teklad Diets; 42% kcal from fat, 34% sucrose by weight, and 0.2% cholesterol total; Envigo).

FMT treatment cohort

After 12 weeks of WD, mice were given antibiotics for 3 days followed by gavage treatment. Mice were switched to NC diet for the first 5 days of gavage, then continued with WD for the remainder of the 2 weeks treatment. Sham FMT mice were gavaged with feces from obese mice, while FMT mice were gavaged with feces from lean mice. Fresh feces from donors were collected the morning of gavage, homogenized *via* vortexing, and fecal slurry supernatant was collected avoiding solid particles.

Tributyrin treatment cohort

At the end of week 15 on WD or NC, animals were needle-fed with Tributyrin (Sigma-Aldrich) or Vehicle 2 days on/2 days off for 3 weeks, at a dosage of 5g/kg of mice body weight.

Echocardiography

Echocardiography was performed using a Visual Sonics Vevo 3100 system equipped with MX550D transducer (Visual Sonics). Anesthesia was induced by isoflurane and measurements were

obtained from short-axis M-mode scans. Parameters collected include heart rate, stroke volume, LVEF, left ventricular fractional shortening, left ventricular end-diastolic diameter, left ventricular end-systolic diameter, left ventricular end-diastolic posterior wall, peak Doppler blood inflow velocity across the mitral valve during early diastole. At the end of the procedures all mice recovered from anesthesia without difficulties.

Speckle-tracking echocardiography and strain analysis

B-mode traces were acquired and used to calculate global longitudinal strain and longitudinal strain rate reverse peak using Vevo Strain software (Visual Sonics) and a speckle-tracking algorithm.

Exercise exhaustion test

After 3 days of pre-training for adjustment to the treadmill exercise, the exhaustion test was performed in all the experimental groups of mice. Mice ran on the treadmill staring at a speed of 5 m min^{-1} for 5 min. The treadmill speed was then increased by 1 m min^{-1} every minute until the mouse was exhausted. Continuation of running was encouraged by delivering a mild shock using an electric-stimulus grid. Exhaustion was defined as the inability of the mouse to return to running after 10 s of shock delivery. Running time was measured and calculated as total minutes ran by each mouse prior to exhaustion and running distance was calculated accordingly.

RNA isolation, cDNA library construction, and illumina sequencing

Total RNA was extracted from mice hearts using the RNAeasy isolation kit (Qiagen). The stranded mRNA-seq was conducted in the Northwestern University NUSeq Core Facility. Briefly, total RNA examples were checked for quality using RINs generated from Agilent Bioanalyzer 2100. RNA quantity was determined with Qubit fluorometer. The Illumina Stranded mRNA Library Preparation Kit was used to prepare sequencing libraries from high-quality RNA samples (RIN > 7). The Kit procedure was performed without modifications. This procedure includes mRNA purification and fragmentation, cDNA synthesis, 3′ end adenylation, Illumina adapter ligation, library PCR amplification and validation. Lllumina HiSeq 4000 sequencer was used to sequence the libraries with the production of single-end, 50 bp reads at the depth of 20–25 M reads per sample. The quality of DNA reads, in FASTQ format, was evaluated using FastQC. Adapters were trimmed and reads of poor quality or aligning to rRNA sequences were filtered. The cleaned reads were aligned to the reference genome using STAR [Dobin et al. (50)]. Read counts for each gene were calculated using htseq-count [Anders et al. (51)]. Normalization and differential expression were determined using DESeq2 [Love et al. (52)]. The cutoff for determining significantly differentially expressed genes was an FDR-adjusted *p*-value less than 0.05. A pathway analysis was performed on both gene lists using GeneCoDis [Tabas-Madrid et al. (53); Nogales-Cadenas et al. (54); Carmona-Saez et al. (55)] to identify pathways that are enriched with genes that are upregulated and downregulated.

Western blot analysis

Protein from frozen mouse hearts were prepared by lysis in ice-cold RIPA buffer (ThermoFisher, cat.89900) containing protease and phosphatase inhibitor (ThermoFisher, cat. A32959). Tissue was homogenized using bullet blender bead lysis kit (Next Advance), and protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermofisher, cat. 23225). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4–15% gradient gel (Bio-Rad, cat.4561086) and transferred to PVDF membrane using iBlot 2 transfer system (ThermoFisher). Protein expression was measured by chemiluminescence using ChemiDoc imaging system (Bio-Rad). Proteins were detected with the following primary antibodies: BCKDH (ThermoFisher, cat.PA5-97248), phospho-BCKDH (S293) (abcam, ab200577), PP2Cm (abcam, ab135286), 4-hydroxynonenal (abcam, ab46545) (14, 56, 57).

Cell culture and treatments

C2C12 mouse myoblasts cells were cultured in 1x DMEM (Corning) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified 5% CO2 atmosphere at 37°C. Cells were treated with sodium butyrate (Sigma-Aldrich) dissolved in water for 6 h at 5 mM concentration. For HDAC inhibition cells were treated with Trichostatin A (Tocris) dissolved in DMSO for 6 h at 1 μM concentration (58).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

mRNA was isolated from C2C12 cells with different treatments using TRIZOL reagent (ref protocol). cDNA was synthesized from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) was performed in triplicate for each sample using diluted cDNA (1:10), SYBR Green (Roche, Cat.04913850001), and 10 μM of forward and reverse primers specific for *ppm1k* (F: GAGTTATGCCCACCTGTCTGCA, R: CT GTCTCCAACACTGGCTACCA) and β-*actin* (F: ACCTTCTAC AATGAGCTGCG, R: CTGGATGGCTACGTACATGC). Samples were cycled 50 times as following (95°C 15 s, 60°C 1 min, measure fluorescence), using CFX96 Real-Time System (Bio-Rad, Cat.1855196). PCR amplification was followed by a melt curve analysis to verify uniformity of amplicon product. Gene expression was calculated and quantified relative to the housekeeping gene β-actin using the ΔΔ Ct method.

16S sequencing and diversity analysis

As described previously (19, 20) cecal contents were collected and DNA extracted using the QIAamp Powerfecal DNA Kit (Qiagen). qPCR was performed with universal 16S primers. The Loyola Genomic Core performed PCR of 16S rRNA V4-5 regions sequenced by the Illumina HiSeq4500 platform, as done previously (59);16S sequences were aligned using the Silva Taxonomy Annotation, and

files were uploaded to Microbiome Analyst for analysis (60) for analysis.[1]

Butyrate and BCAA serum measurements

Blood was isolated from mice, after decapitation under anesthesia, collected in Sarstedt microvette blood collection tubes, and centrifuged at 2,000 × *g* for 10 min for serum collection. The quantification and analysis of serum butyrate and BCAA was performed by the Mass Spectrometry Core in Research Resources Center of University of Illinois at Chicago. Serum samples were stored in the −80°C freezer prior to use and were thawed in water bath for 30 s. 10 ul of serum sample was taken for deproteinization and 40 ul methanol (MeOH) was added before vortexing for 2 min. The samples were incubated at 4°C for 30 min and vortexed again thoroughly. The samples were then centrifuged at 14,000rpm for 10 min and 30 ul of the supernatant was taken for derivatization. For derivatization, 30 ul of each standard solution or sample supernatant was mixed with 15 ul of 200 mM 3-NPH in 50% aqueous MeOH and 15 ul of 120 mM EDC in the same solution. The reaction was allowed to proceed for 30 min at 40°C. The reaction mix was then diluted with 350 ul of 10% MeOH. LC/MS analysis was performed on AB SCIEX 6500 QTRAP coupled with Agilent 1,290 UPLC system.

The LC/MS data files were processed using the AB Sciex MultiQuant software.

Cardiac BCAA measurement

Measurements were done following the BCAA colorimetric Assay protocol (BioVision, cat#K564). BCAA concentrations were normalized to total protein measured using BCA protein assay kit (ThermoFisher, cat#23225).

In situ hybridization

In situ hybridization for GPR41 (ACD Probe Cat#) GRP43 (ACD Probe#) was done as described previously (20) using a combination of chromogenic RNAScope (FastRed) and GFP immunohistochemistry.

Data availability statement

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (61) and are accessible through GEO Series accession number: GSE221195 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE221195).

Ethics statement

This animal study was reviewed and approved by Loyola University Chicago Institutional Animal Care and Use Committee.

1 https://www.microbiomeanalyst.ca/

Author contributions

JH and TC designed the experiments, performed the experiments, analyzed the data, wrote the manuscript, and reviewed the manuscript. RB designed the experiments, performed the experiments, and reviewed the manuscript. NE, CG, CW, and JJ performed the experiments and reviewed the manuscript. VM-A and GA designed the experiments, analyzed the data, wrote the manuscript, and reviewed 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1105581/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Log-transformed of *Lactobacillus* relative abundance in cecal contents from mice treated with sham FMT or lean FMT (n = 3–5). **(B)** SparCC analysis (correlation threshold > 0.5, $p < 0.05$) of 16S sequencing of cecal contents after sham FMT or lean FMT treatments.

SUPPLEMENTARY FIGURE 2
Chromogenic *in situ* hybridization staining of **(A)** *ffar2* mRNA (green) and **(B)** *ffar3* mRNA (green) in heart sections. **(C)** Chromogenic *in situ* hybridization staining of *ffar3* mRNA (green) in nodose ganglia (NG).

SUPPLEMENTARY TABLE 1
Summary table of DEseq2 analysis, data transformed using relative log expression.

SUPPLEMENTARY TABLE 2
Topmost regulated transcripts after Tributyrin treatment in the heart (WD, WD + Tributyrin needle fed mice), FDR-adjusted $P < 0.1$ ($n = 3$ per group).

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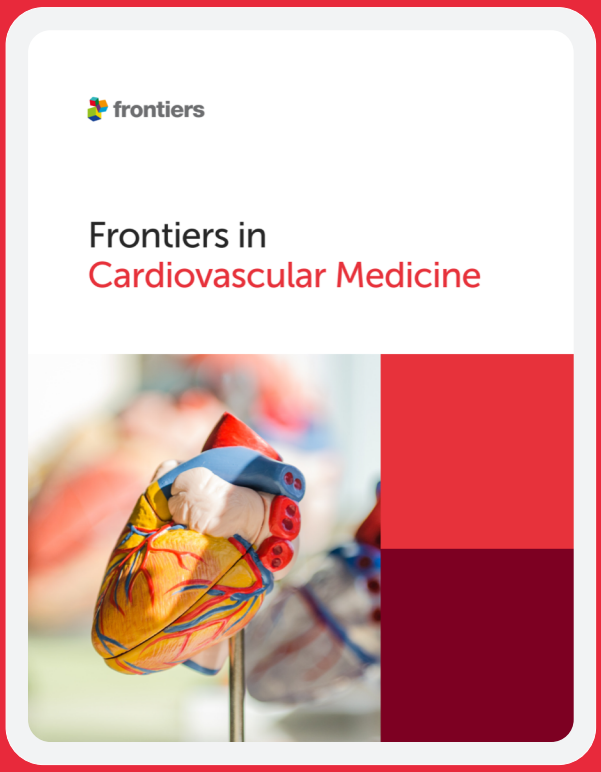

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