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Original Research

Plasma Metabolome Analysis Suggests That L-Arginine Supplementation Affects Microbial Activity Resulting in a Decrease in Trimethylamine N-oxide—A Randomized Controlled Trial in Healthy Overweight Adults with Cardiometabolic Risk Factors

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A B S T R A C T

Background: The effects of supplementation with L-arginine (L-arg), the precursor of nitric oxide (NO), on vascular and cardiometabolic health have largely been explored. Whether other mechanisms of the action of L-arg exist remains unknown, as arginine metabolism is complicated.

Objective: We aimed to characterize the effect of low dose L-arg supplementation on overall human metabolism both in a fasting state and in response to an allostatic stress.

Methods: In a randomized, double-blind, crossover study, 32 healthy overweight adults (mean age 45 y) with cardiometabolic risk (fasting plasma triglycerides >150 mg/dL; waist circumference >94 cm [male] or >80 cm [female]) were treated with 1.5 g sustained-release L-arg 3 times/d (4.5 g/d) or placebo for 4 wk. On the last day of treatment, volunteers consumed a high-fat meal challenge (900 kcal, 80% as fat, 13% as carbohydrate, and 7% as protein). Plasma was collected at fasting, 2, 4, and 6 h after the challenge, and the metabolome was analyzed by high-resolution liquid chromatography–mass spectrometry. Metabolic profiles were analyzed using linear mixed models–principal component analysis.

Results: The challenge meal explained most of the changes in the metabolome. The overall effect of L-arg supplementation significantly explained 0.5% of the total variance, irrespective of the response to the challenge meal ($P < 0.05$). Among the metabolites that explain most of the L-arg effect, we found many amino acids, including branched-chain amino acids, that were decreased by L-arg supplementation. L-arg also decreased trimethylamine N-oxide (TMAO). Other changes suggest that L-arg increased methyl demand.

Conclusions: Analysis of the effect of 4 wk of L-arg supplementation on the metabolome reveals important effects on methyl balance and gut microbiota activity, such as a decrease in TMAO. Further studies are needed to investigate those mechanisms and the implications of these changes for long-term health.

This trial was registered at clinicaltrials.gov as NCT02354794.

Keywords: TMAO, trigonelline, L-arg supplementation, LC/MS metabolomics, LIMM-PCA

Abbreviations: BCAA, branched-chain amino acid; GAA, guanidinoacetate; HF, high-fat; L-arg, L-arginine; LC-MS, liquid chromatography coupled with mass spectrometry; LiMM-PCA, linear mixed model coupled with principal component analysis; ML, maximum likelihood; NO, nitric oxide; REML, restricted maximum likelihood; RHI, reactive hyperemia index; SR-arg, sustained-release L-arginine; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

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Introduction

The physiological requirement for arginine is fulfilled by endogenous synthesis, and the contribution of dietary intake to arginine flux in the systemic circulation is relatively small [1,2]. However, there is supporting evidence that the intake of arginine from dietary protein (such as legumes, nuts, and seeds) may explain in part their effects on cardiometabolic risk. Arginine has been shown to be the major contributor to an amino acid pattern inversely associated with cardiovascular disease mortality [3]. The effects of L-arginine supplementation on vascular human health have been explored in the past decades in volunteers at cardiometabolic risk, with benefits on several outcomes such as blood pressure [4], platelet aggregation, endothelium-dependent vasodilation, monocyte-endothelial cell adhesion, insulin sensitivity, and even type 2 diabetes risk [5,6].

These effects are considered to be mediated by the action of endothelial nitric oxide (NO), of which the synthesis pathways from arginine are well understood [7–9]. NO is at the center of various physiological processes such as immunity [10] and endothelial function [11]. Altered NO synthesis/bioavailability has been implicated in the pathophysiology of vascular dysfunctions [12]. Altered NO signaling may be increased in stress states resulting from a high-fat (HF) challenge meal. In line with this hypothesis and extending previous findings in the acute setting [13], we previously showed that the transient decrease in endothelial function following a HF postprandial challenge could be alleviated by supplementation with low doses of sustained-release L-arg (SR-arg) in individuals with low plasma arginine [14]. However, the underlying mechanisms of action of SR-arg supplementation in these subjects remain to a large extent uncertain [15].

Indeed, beyond NO, arginine metabolism is at the crossroads of many important metabolic pathways, including those related to urea, creatine, polyamines, proline, glutamate, agmatine, methylated derivatives, and homoarginine [16,17], which may explain its many physiological effects.

Using plasma metabolomic analyses, we aimed to study the effect of SR-arg supplementation on the metabolism of overweight adults presenting cardiometabolic risk factors by combining estimated effects at fasting and under an allostatic stress (in response to a HF challenge meal).

We hypothesized that arginine supplementation has a broad imprint on the plasma metabolome. Metabolomics offer broad and strong insights into endpoints of metabolic fluxes in the entire body in response to a nutritional challenge [18]. We expected that metabolomics would provide new information about volunteers' phenotypic flexibility in response to the challenge test and in relation to the physiological effects of L-arg bioavailability. This study was also an opportunity to expand our understanding of secondary arginine metabolism and generate hypotheses substantiating its impact on cardiometabolic health in people at risk. To our knowledge, it is the first study investigating the repercussions of L-arg supplementation on the human metabolome.

Methods

Study

The trial was registered at clinicaltrials.gov (reference NCT02354794). The primary outcome was the assessment of endothelial function after a HF meal, as evaluated using flow-mediated dilation and Framingham reactive hyperemia index (RHI). The primary results have been published [14]. Briefly, the effects of SR-arg supplementation on postprandial endothelial function largely varied according to baseline fasting arginine concentration. SR-arg supplementation attenuated postprandial dysfunction only in individuals with low fasting plasma arginine (i.e., below the median of the study population). This result suggests that the benefits of arginine supplementation may be related to a decreased ability to mobilize endogenous arginine for NO synthesis during a postprandial challenge. Plasma metabolomics analysis was a secondary outcome measure of this trial.

Recruitment and study design has been described in full elsewhere [14]. In brief, the study was conducted on 36 (32 analyzed) healthy overweight ($25 < BMI < 30$ kg/m^2) adult volunteers (mean age 45 y) with the hypertriglyceridemic waist phenotype (waist circumference of >94 cm for male and >80 cm for female and a fasting triglyceride concentration of >150 mg/dL). We chose to include 36 subjects at study entry after an a priori power calculation for an effect size of 20% with 80% power on the main outcome (RHI), considering a ~10% attrition rate. Baseline characteristics of study volunteers are shown in Table 1.

This was a randomized, double-blind, 2-period crossover, placebo-controlled study with 4-wk treatment periods and a 4-wk washout period. We did not proceed to metabolomics analysis at the beginning and the end of the washout period to rule out any carry-over. However, we found no period effect and no evidence for a carry-over effect on plasma arginine concentrations. Supplemental arginine is disposed within 24 h as shown in a previous study using isotopic labeling [19], but arginine supplementation has a broad imprint on the plasma metabolite pool. However, a 4-wk washout duration is very cautious with regard

TABLE 1
Baseline characteristics of the participants at study entry[1]

	Total ($n = 32$)
Sex, male, %	73
Age, y	45 ± 8.9 (23–59)
Weight, kg	85.3 ± 11.3 (68.0–106)
Height, m	1.74 ± 0.103 (1.53–1.91)
Waist circumference, cm	101 ± 5.86 (94.0–112)
BMI, kg/m^2	28.0 ± 1.92 (23.9–31.5)
Plasma lipids	
Triglycerides, mmol/L	2.4 ± 1.6 (0.67–8.8)
Total cholesterol, g/L	2.1 ± 0.40 (1.2–3.1)
LDL cholesterol, g/L	1.2 ± 0.34 (0.65–2.2)
HDL cholesterol, g/L	0.4 ± 0.1 (0.2–0.8)
Glucose metabolism	
Plasma glucose, mmol/L	6.1 ± 0.69 (5.3–8.2)
Plasma insulin, pmol/mL	108 ± 87.3 (30.0–502)
Amino acids	
Arginine, μmol/L	77.6 ± 18.3 (41.2–136)
Isoleucine, μmol/L	70.3 ± 17.3 (53–87.6)
Leucine, μmol/L	128.9 ± 26.7 (102.2–155.6)
Valine, μmol/L	243.6 ± 43.9 (199.7–287.4)
BCAAs, μmol/L	442.8 ± 84.5 (358.2–527.3)

Abbreviations: BCAA, branched-chain amino acid; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Data were previously published in Deveaux et al. [14] except for amino acids other than arginine.

[1] All values are presented as mean ± standard deviation, with ranges in parentheses.

to the impact on the metabolome, and it should also minimize potential carry-over effects on gut microbiota [20,21].

The volunteers received a 1.5-g SR-arg supplement or a placebo 3 times/d (4.5 g/d) for 4 wk. The last day of each treatment period, volunteers were given a HF challenge meal, with a first intake of treatment (SR-arg or placebo) 2 to 3 h before the meal and a second intake 3.5 h after the meal. The daily arginine dose was chosen to be equivalent to the usual daily average arginine dietary intake in the French population. The composition of the SR-arg and Placebo are detailed in Supplemental Table 1. In brief, for the placebo, the 500 mg arginine in the Arginine capsules was replaced by 300 mg microcrystalline cellulose. To assess their compliance, the participants were asked to return any unused capsules upon arriving at each final session at the end of each arm (L-arg or Placebo). Fasting plasma arginine was 45% higher after L-arg supplementation compared with placebo (Supplemental Table 2), supporting good compliance from participants.

The HF meal consisted of heavy cream (30% fat ultra-high temperature sterilized liquid cream, Carrefour), white sugar (Beghin-Say), and milk protein (total Milk Protein Isolate, Ingredia). Meals were presented in the form of a "whipped cream" dessert. The total energy value of the meals was 900 kcal with 80% of energy as fat, 13% as carbohydrate, and 7% as protein.

Blood samples were collected in EDTA vacutainers at the fasting state and 2, 4, and 6 h postprandial. Blood samples were centrifuged, and the plasma was then aliquoted in microcentrifuge tubes and stored at −80°C until metabolomics analysis.

High-resolution liquid chromatography–mass spectrometry (LC-MS)

The method was described in full elsewhere [22], and details can be found in the supplemental material.

Data processing and metabolite identification

All the raw data generated by the LC-MS were converted to mzXML by ProteoWizard (Version 2.0, ProteoWizard, Palo Alto, USA), then processed by XCMS (Scripps Research, San Diego, USA). Identification of the metabolites was performed using an in-house database referencing more than 1300 metabolites with their chromatographic retention time acquired both with C18 and hydrophilic interaction liquid chromatography columns, together with their exact mass obtained in positive and negative ionization mode including their adducts and neutral losses, but also MS/MS spectra when acquired. These led to level 1 or 2 identification.

Statistical analysis

Statistical analyses of metabolomics data from crossover designs are very complex. First, the untargeted MS method used to generate metabolomics data results in high dimensional data, with much more features (retention time and mass/charge number ratio) than volunteers. Furthermore, spectral features are colinear. Second, crossover studies that describe effects of a challenge meal on metabolomic postprandial changes result in repeated measurements on volunteers, collected at different time points, and data can be unbalanced. Therefore, measures on the same volunteer are not independent in such designs. To take full advantage of measurements in whole sets of metabolites, the application of multivariate methods can be warranted instead of univariate analyses used to tackle problems due to the large number of features and collinearities between spectral features. With the progress of the omics field, promising statistical methodologies are now available. For example, several approaches based on a combination of analysis of variance and multivariate methods have been developed to analyze data from complex study designs, as summarized in Lépine et al. [18]. In Martin et al. [23], these methods were further generalized to unbalanced designs and to linear mixed models in the LiMM-PCA (linear mixed model-principal component analysis) method, enabling the inclusion of both fixed and random effects.

The method is further described in the Supplemental Methods. Briefly, the LiMM-PCA consists of 5 steps, as follows.

1. In the first step, PCA is used to reduce data dimensionality and to have orthogonality between features: the number of principal components, m^*, is automatically computed to explain 99% of variability in the original data. "Raw" MS intensities, y^*, are replaced by volunteers' scores on the principal components that are retained for the following steps.
2. A LiMM is then fitted for each principal component y^* to study the relationship between metabolic response and experimental factors in the second step. In this study, we used the following model:

$$y_j^* = \beta_1 Treatment + \beta_2 Time + \beta_3 Period + \beta_4 Order + \beta_5 Arg_{level} + \beta_6 TreatmentxTime + \alpha_1 Volunteer + \varepsilon_j \quad (1)$$

with $j = 1 \ldots m^*$, Treatment (placebo/SR-arg supplementation), Time (0, 2, 4 and 6h postprandial), Period (first or second 4-wk treatment period), Order (refers to the order in which the treatments were given: Placebo then SR-arg or SR-arg then Placebo), Arg_{level} (Baseline plasma Arginine: "low"/"high" depending if they are under or above the median basal plasma arginine), and Treatment × Time were fixed factors. Volunteer was a random factor, and ε correspond to residuals. β and α parameters were estimated using the maximum likelihood (ML) and covariance matrices based on the restricted ML (REML).

3. In the third step, the response matrix is decomposed into a sum of fixed and random effect matrices, one each for fixed and random effects.
4. Then, effects importance and significance are computed in the fourth step. Importance is the proportion of the total variance of the data explained by each model term (fixed and random effects). Significance is based on a generalized log likelihood ratio test. Finally, effect matrices are represented based on PCA.
5. In the last step, PCA is applied on matrices to obtain scree, loading, and score plots.

Results

Figure 1 shows the study flow chart and is from the original article [14]. LC-MS metabolomics data were generated from plasma samples of 32 volunteers submitted to the 2-treatment

(Placebo/SR-arg supplementation) periods (1 at 4 wk, 2 at 12 wk) at 4 times (0, 2, 4 and 6 h postprandial). One MS spectrum was missing because of a sample loss, resulting in unbalanced data.

PCA was first applied on Pareto-scaled data for dimension reduction and orthogonalization. The first principal component almost explained 40% of the total variability: spectra from samples collected at 1 h postprandial were discriminated from spectra collected later. Scores and diagnostic plots (see Supplemental Figure 1) enabled detection of 11 outliers (among 255). These observations were removed for subsequent analysis. This PCA showed that 17 out 226 principal components explained

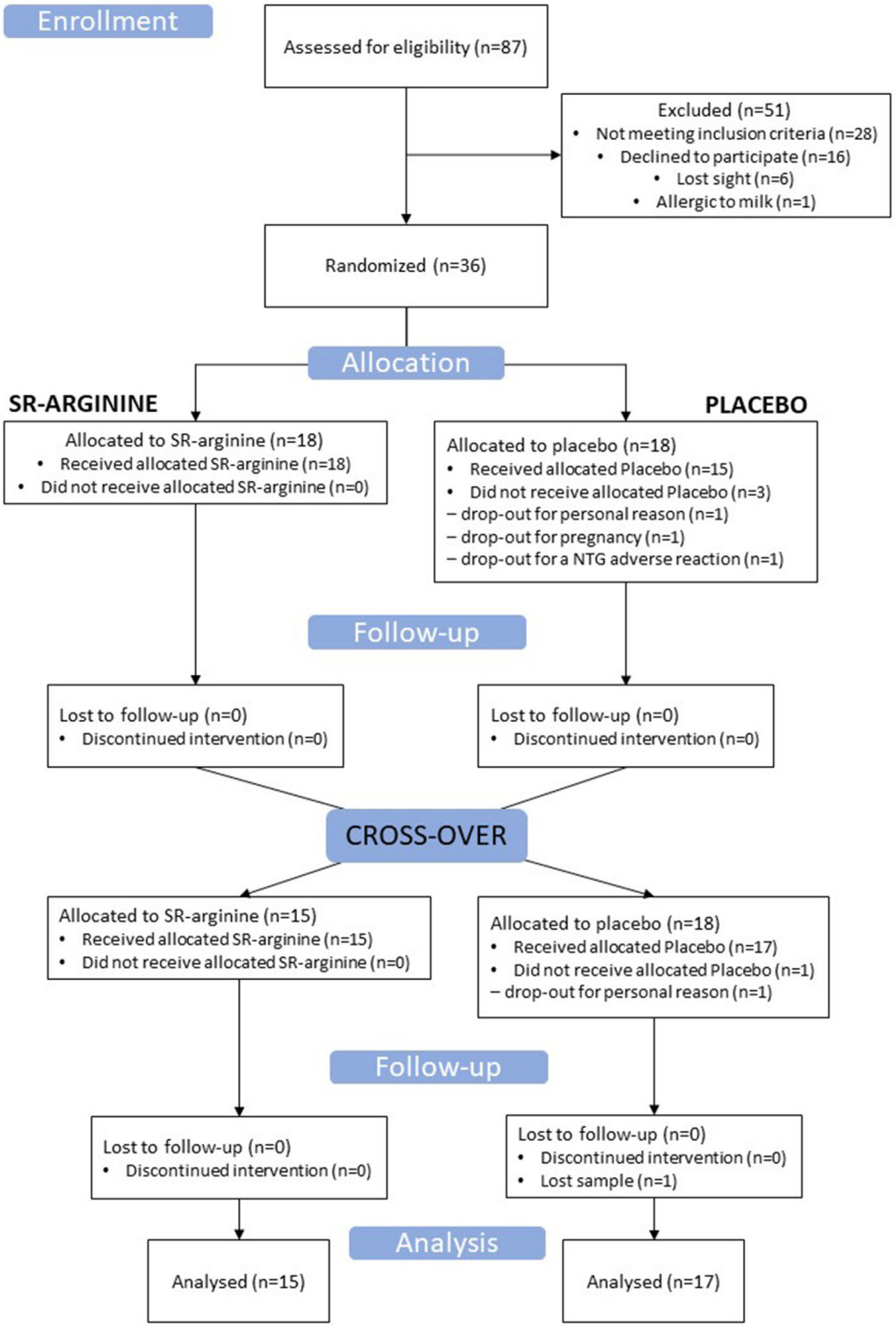

FIGURE 1. Study flow chart. Adapted from Deveaux et al. [14]. NTG, nitroglycerin; SR, sustained release.

99% of the total variability of the data. For following steps of the LiMM-PCA method, only these 17 principal components were kept.

In the second step, parallel parameters of Equation *1* were estimated using the REML method of the model for the 17 principal components. According to visual inspection, the residuals (see Supplemental Figure 2) were approximately normal. This step resulted in the multivariate model, which was decomposed into the effect matrix in the third step as described in Supplemental Methods (Equations *2* and *3*).

In step 4, effects importance was computed and their significance tested. Table 2 presents the percentage of total variability explained by each effect. Plasma metabolome was significantly affected by the time factor ($P < 0.001$) with the fasting period being clearly distinct from the postprandial period. Time accounted for about 40% of the variance in the dataset, showing that the response to the challenge meal represented the largest imprint on the metabolome. Interindividual variability explained 10% of the total variance, while the other factors had a marginal contribution (<1%). The results were not significantly different depending on the period or the order of the treatments. Basal arginine level did not have a significant effect on the metabolic response to treatment.

SR-arg treatment had a significant ($P < 0.05$) but relatively small effect on the plasma metabolome because it only explained 0.5% of the total variance. This effect did not differ after the challenge meal (time × treatment interaction) so the effect of L-arg applied similarly at fasting and during the allostatic stress (as induced by the meal challenge). Note that half of the total variability of the model was not explained by the controlled experimental factors.

In the last step, for each effect included in the LiMM (see Equation *1*), we obtained scree, score, and loading plots (Figure 2).

For the significant effects (Treatment and Time), we observed that the first principal component explained most of the total variability: 100% for the Treatment effect (with only 1 principal component built as this effect had 2 levels) and 98.8% for the Time effect (with 3 principal components built as this effect had 4 levels).

The score plot in panel A shows a clear separation between Treatment levels. The first principal component of PCA on Time clearly discriminated samples collected 1 h postprandial from samples collected later. A trend was observed on the second principal component, but this principal component explained a negligible part of the variability.

PCA scores of the residuals, which account for variability explained by other factors than those included in Equation *1*, did not show any outlier, meaning that we had excluded all the "extreme" spectra.

The loading plots showed which spectral features were important in separating individuals according to factor levels. We observed that among the 227 annotated metabolites, few had a high loading value. MS features with an absolute loading value > 0.05 (see Table 3) were further investigated for the Treatment effect only as we believe that the effect of time might be confounded with instrumental bias between fasting and postprandial samples. In other words, LiMM-PCA is based on linear combinations of all metabolites included in the data matrix, the importance of each metabolite being represented by its weight in the linear combination. This means that all metabolites are modulated by arginine supplementation. The lower the weight, the less important is the metabolite, meaning that some metabolites are impacted but to a lesser extent. We have chosen to present the main (high loading) metabolites contributing to these variations.

Few metabolites contributed to separate volunteers according to treatment (SR-arg compared with Placebo). We found high contributions of betaine, L-phenylalanine, leucine, and isoleucine (Table 3). Interestingly, several microbial metabolites contributed to the L-arg effect with important intensity variations between the 2 treatment modalities, indicating that the associated metabolic pathways were particularly responsive to L-arg. It was particularly striking for trimethylamine N-oxide (TMAO) and trigonelline, the intensities of which were reduced by about 21% and 44%, respectively, after SR-arg supplementation.

TABLE 2
Percentage of explained variance and bootstrapped *P* value

Effect	Percentage of total variance	*P* value
Order	0.2	0.55
Period	0.23	0.59
Residuals	48.21	—[1]
Time	39.66	< 0.001
Treatment	0.54	0.02
Treatment × Time	0.63	0.56
Volunteer	9.96	—[1]

Values were determined using 1000 randomly drawn samples, of the effects analyzed by the LiMM-PCA method applied on Pareto-scaled mass spectra of plasma samples ($n = 244$), corresponding to step 4 of the method (Effect importance and significance).

[1] Significance was not tested for random effects.

Discussion

Our findings bring unique insights into the effects of manipulating L-arg intake on the human metabolome, explored both in the fasting state and following a HF challenge test. The composition of the plasma metabolite pool is determined by the interplay of diet, lifestyle, environment, gut microbiota, and host genetics. The fact that arginine supplementation explains only a tiny fraction of the total variation of the blood metabolome in the study population was expected. In contrast, time after the ingestion of the challenge meal explained 40% of the total variance, which is not surprising because the ingestion of a challenge meal high in fat and sugar is by definition challenging metabolism via a very large number of metabolic and physiological pathways, and this is captured in the data that precisely concern the fasting–postprandial transition (blood samplings at 0, 2, 4, and 6h after the meal). In contrast, arginine is a single molecule at a relatively low dose that has been chronically administered. Thus, it was expected that the footprint left by arginine supplementation would be small in relation to the overall metabolome. Combining LC-MS metabolomics and a HF challenge test was a very useful strategy to characterize the treatment effect on the volunteers' capacities to handle nutritional stresses. Moreover, the innovative statistical analysis method (LiMM-PCA) that we applied further allowed us to disentangle the medium-term effects from complex postprandial effects in the data set, revealing a significant effect of SR-arg

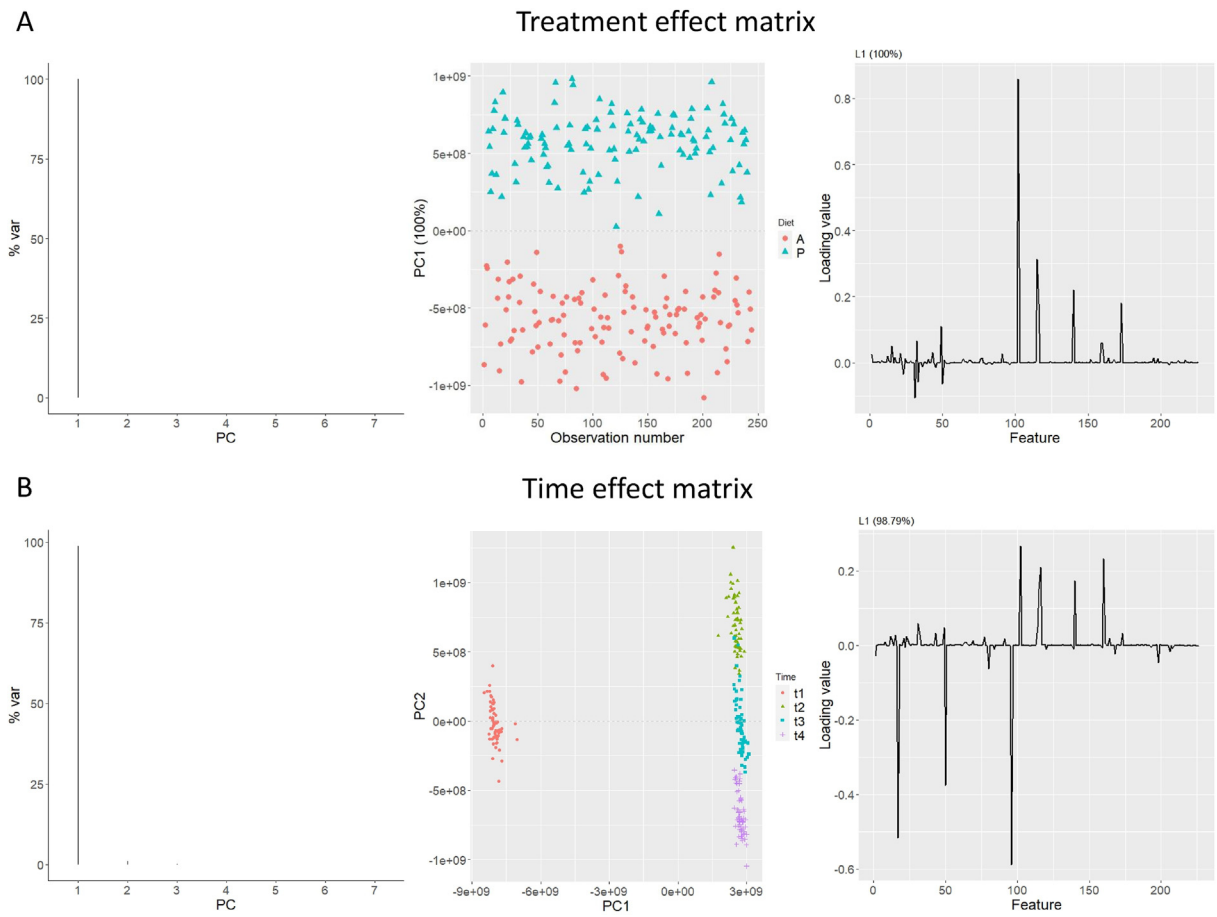

FIGURE 2. LiMM-PCA method applied on Pareto-scaled mass spectra of plasma samples (n = 244 observations), showing PCA of the effect matrices of all fixed and random effects (from panel A to panel H). For each panel, the left figure is the scree plot (showing percentage of explained variability by successive principal components); the middle figure is the score plot [showing projection of individuals onto the subspace spanned by the first (and second) principal components; and the right figure is the loading plot (showing weight of mass spectral features for the first and second principal components)]. A and P are the treatments (Arginine and Placebo); t1, t2, t3, and t4 are the timepoints (fasting, 2 h, 4 h, and 6 h after the challenge meal); J2 and J4 are the first and second study periods of measurements; AP and PA are the orders of the treatments (Arginine then Placebo, and Placebo then Arginine); H and L are the levels of baseline concentrations of arginine (High, i.e., above study sample median, or Low, below study sample median). LiMM, linear mixed method; PCA, principal component analysis.

supplementation, which was present irrespective of the effect of the challenge test and therefore pertained to the overall metabolic response of the overweight adults presenting cardiometabolic risk factors.

In this study, we thought that the effects of L-arg on the metabolome might be different when assessed at the fasting state or following a HF meal, which deeply and acutely challenges the metabolic capacities of the volunteers. However, this was not the case, as we found no treatment × time interaction. This suggests that the effects of the arginine treatment as revealed by our metabolomic analyses were rather chronic effects, with no impact on the metabolic pathways that are acutely altered by the challenge meal, in particular for those pointed out as resulting from a modulation of the microbiota.

Indeed, the fact that the chronic effects of the L-arg supplementation remained significant over the fasting and the postprandial conditions supports the robustness of the observations. This further allowed us to restrain the noticed changes to a limited number of metabolites and provide new hypotheses about the physiological/pathophysiological effects of dietary/supplemental arginine. In this regard, of particular interest are decreases in both branched-chain amino acids (BCAAs) and TMAO. Other metabolites, namely trigonelline, stachydrine, and betaine, offer a hypothesis for underlying mechanisms.

Overall, our metabolomics findings are consistent with a study in growing pigs [24] that also found betaine, tyrosine, lysine, TMAO, and BCAAs as key metabolites underlying the effect of arginine supplementation. However, the effects were at variance. Plasma BCAA increases have been related to many metabolic dysregulations, the most explored being type 2 diabetes mellitus and insulin resistance. The elevation of circulating BCAAs has been related to impaired glucose metabolism in adipose tissue that could activate the mTOR pathway, contributing to the development of insulin resistance [25,26]. Here, we did not find statistically significant differences in HOMA-IR according to SR-arg supplementation [14]. Plasma BCAA concentrations give limited information on BCAA turnover, and stable isotope studies would be needed to discuss further the relationship between BCAA metabolism, microbiota, and insulin resistance.

The decrease in TMAO with L-arg supplementation is an important finding that may be useful to further characterize the

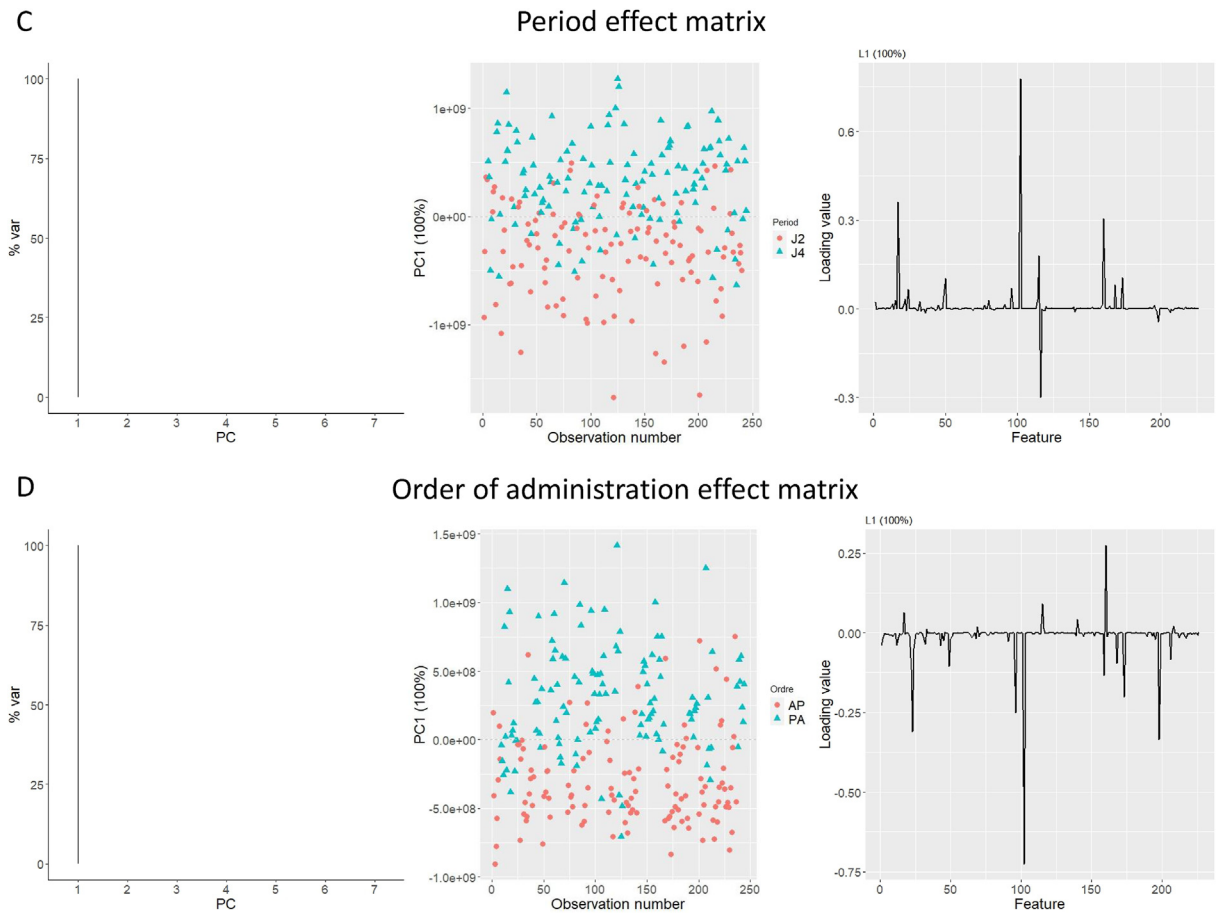

FIGURE 2. (*continued*).

physiological effect of arginine. Higher circulating levels of TMAO is associated with all-cause mortality [27] and has been proposed as an important cardiovascular disease risk factor [28, 29]. Therefore, our results suggest that some of the classically observed benefits of L-arg supplementation may actually operate in part by changes in TMAO.

TMA (trimethylamine) is a metabolite synthesized by the gut microbiota from choline and choline end-products such as L-carnitine or betaine. Then, TMA is oxidized in the liver into TMAO by the FMO3 enzyme [30]. TMAO is known to be increased by meat intakes [31], which provide L-carnitine, and it has been associated with animal protein intake [32]. However, we found no literature data reporting about a relation between arginine intake and TMAO levels.

Microbiota can biosynthesize niacin, and this production is increased following a plant diet [33]. Trigonelline is the methylated form of nicotinic acid, and it is also likely biosynthesized by the gut microbiota and possibly the liver via the metabolism of tryptophan to niacin or through the conversion of S-adenosylmethionine to S-adenosylhomocysteine in the methionine cycle [34]. Indeed, the nicotinate methyltransferase (trigonelline synthase) enzyme is not detected in animal samples [35], whereas it has been shown that nicotinic acid is directly methylated to trigonelline in the human body [36].

Interestingly, like TMAO, trigonelline [37,38] has been associated with glucose homeostasis and tolerance and is a signature metabolite for obesity [34,39] and all-cause mortality [40]. This is not clearly established at present if TMAO is a marker or is directly increasing mortality by itself, given that observational studies have strong limitation in causal inference, the results in animal studies are conflicting, and that the effects could also depend on the population (healthy, at risk, or with overt disease) [41]. More research is needed to determine if reducing plasma TMAO concentration is actually beneficial to health.

Since we found that L-arg supplementation decreased plasma TMAO and trigonelline, and, in our crossover, closely controlled setting, changes in dietary intakes were restricted to arginine supplementation, our finding points to a change in the activity of the gut microbiota [42]. Although the mechanisms by which arginine supplementation may modulate TMA synthesis are not known, we suggest a few possible mechanisms.

A first mechanism is that L-arg supplementation might affect gut microbiota composition by supporting gut functions. High-TMAO producers have a higher Firmicutes to Bacteroidetes phyla [43,44], and this ratio has also been associated with obesity [45] and western diets [46] in contrast to long-term plant-based diets [47]. Since the volunteers of this study had a western diet style and cardiometabolic risk factors [48], they may have had a microbiota dysbiosis at study entry. The fact that the volunteers of this study presented higher plasma BCAAs compared with healthy individuals would also be consistent with dysbiosis because high microbial BCAA synthesis has been associated with dysbiosis [49], even though high BCAAs have been related to many other metabolic dysregulations, and the link with the microbiota is not the most explored hypothesis.

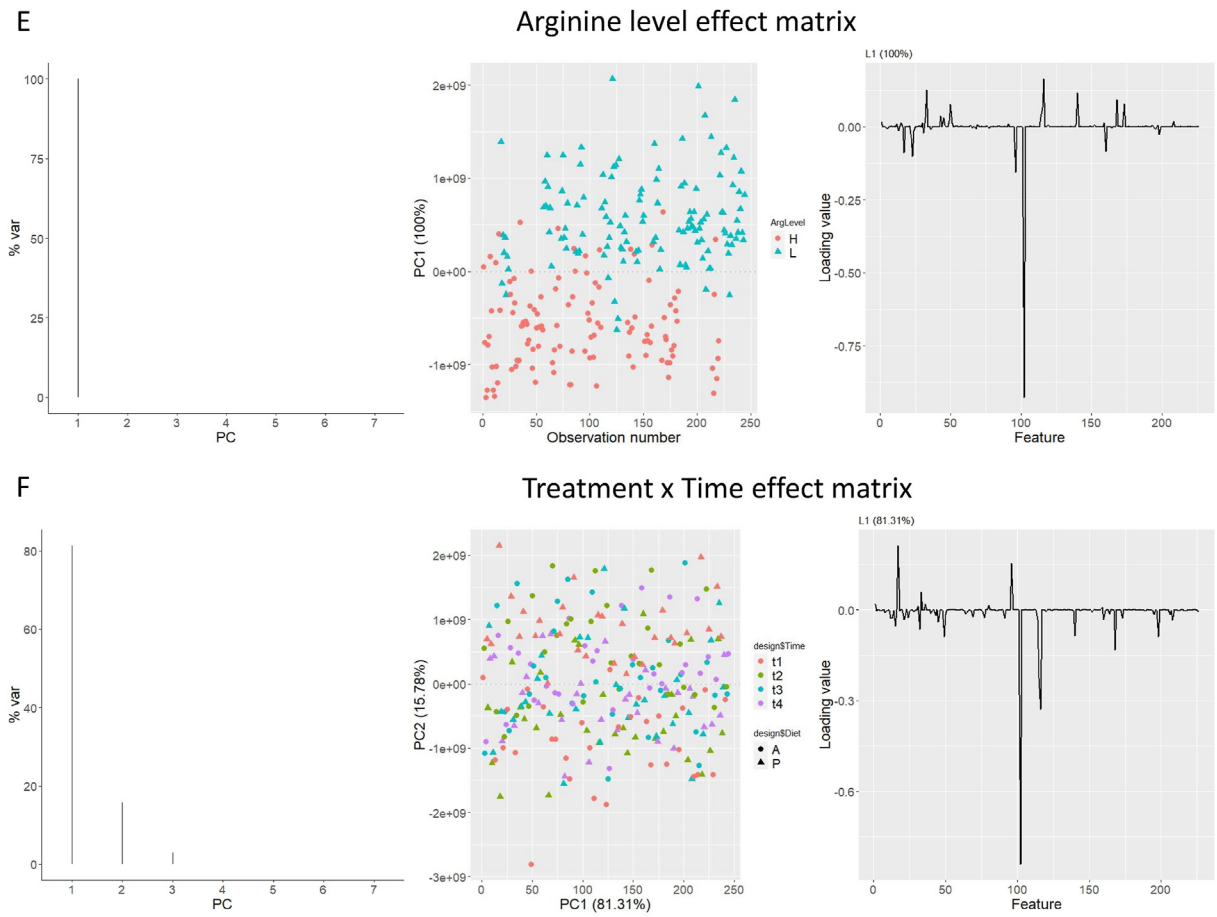

FIGURE 2. (*continued*).

Arginine is also known to play an important role in intestinal physiology as a regulatory molecule in intestinal protein turnover, barrier function, and immuno-inflammatory pathology, for example [50–53]. Effects of a higher arginine availability to the enterocytes could result in a higher synthesis of polyamines [54] or increase in intestinal NO synthesis [50,55]. Actually, NO derived from dietary L-arg supplementation has been shown to regulate gut microbial community in animal models [56,57], which could be compatible with the decrease in plasma TMAO concentrations observed here.

A second potential mechanism, which we think is more likely, is that the L-arg effect could be mediated by changes in methyl balance. Since arginine is the key substrate for the synthesis of creatine, via guanidinoacetate (GAA), creatine synthesis exerts an appreciable demand on arginine metabolism and one-carbon metabolism [58]. Indeed, GAA is used as one of the main methyl acceptors in the methionine cycle to such an extent that it could account for more than 70% of all endogenous methylation reactions in creatine-free diets [59,60]. L-arginine supplementation alone has been reported to increase plasma GAA concentration and the ratio of homocysteine-to-methionine, suggesting increased methylation demand and one-carbon flux in this pathway [61, 62].The decrease in plasma betaine concentration after L-arg supplementation would be in line with higher methyl transfer, with GAA becoming a preferential methyl acceptor. Thus, a decrease in trigonelline synthesis from nicotinic acid in the liver [34] would explain its lower plasma concentration in this study.

Limitations

Since the different microbial metabolites associated with SR-arg in this study, namely TMAO, trigonelline, and stachydrine, can also have a dietary origin, the observed changes might reflect an exposure bias rather than a specific effect of the supplementation. Although the diet of the volunteers was not controlled, they were instructed to maintain the same diet and lifestyle during the trial. In addition, we applied a blinded, placebo-controlled, randomized crossover design to our study, which further points to a specific effect of the L-arg treatment. However, we cannot strictly rule out that the placebo, which contained some cellulose to replace SR-arg, may have affected gut microbiota, even if the level of cellulose intake was low compared with total fiber intake. Nevertheless, because additional cellulose would have been expected to have a favorable impact on the microbiome, it cannot explain the changes reported after treatment compared with placebo.

Overall, the size effect of L-arg is very small because it represents only 0.5% of the variability, so one might suspect that the clinical significance is low. However, the total variability in the metabolome includes dramatic changes following a HF challenge meal, which overwhelmed the effect of arginine. The specific effect of L-arg on changes of discriminant metabolites remains important, especially for risk factors such as TMAO (21% decrease), which may lead to long-term health repercussions.

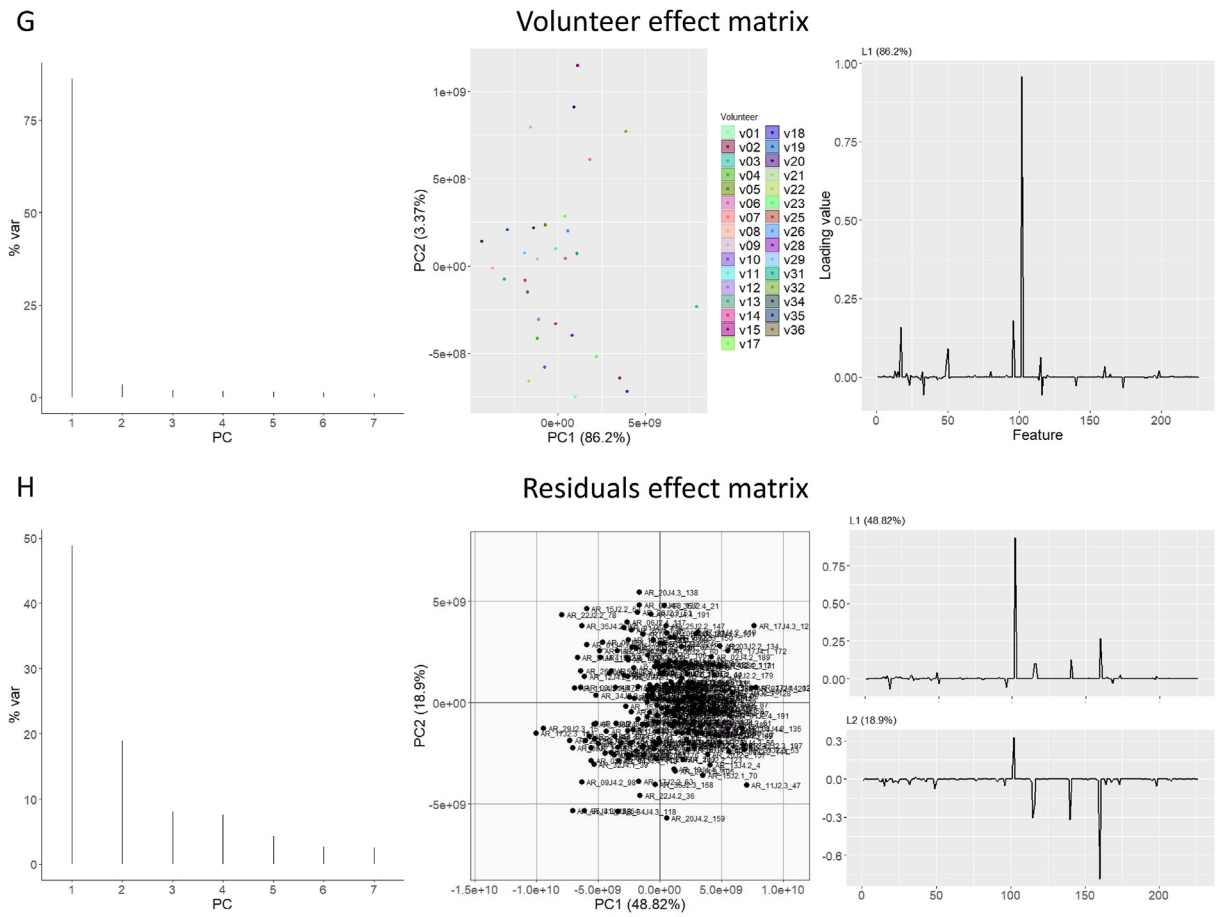

FIGURE 2. (*continued*).

TABLE 3
Identification of metabolites that explain the treatment effect (L-arg compared with placebo), with their respective contributions and effect sizes

Metabolite name	PubChem Compound ID (CID)	Sample size *n* arginine sample/ *n* placebo sample	Contribution to the effect (Loadings: L-arg vs. placebo)	Effect size (changes in mean plasma intensities, L-arg vs. placebo, %)
2-aminophenol	5801	123/120	+0.09	+ 13.4%
Betaine	247	123/120	-0.85	- 8.2%
Isoleucine	6306	123/120	-0.15	- 5.0%
Leucine	6106	123/120	-0.24	- 7.7%
L-lysine	5962	123/120	-0.06	- 13.8%
L-phenylalanine	994	123/120	- 0.33	- 8.5%
L-tyrosine	6057	123/120	-0.06	- 12.5%
P-cresol-sulfate	4615423	123/120	+0.11	+ 15.7%
Stachydrine (proline betaine)	115244	123/120	+0.06	+ 13.8%
Trigonelline	5570	123/120	-0.06	- 43.5%
Trimethylamine N-oxide (TMAO)	1145	123/120	-0.15	- 13.0%
Tryptophan	6305	123/120	-0.12	- 6.0%
Valine	6287	123/120	-0.06	- 1.6%

Conclusion

In this crossover study, we deciphered the effect of a nutritional dose of SR-arg supplementation on the metabolome. We revealed that L-arg affects amino acid metabolism, including BCAAs, which have been consistently reported together as important features of the metabolic signatures of many cardiometabolic diseases. It also decreased TMAO levels, which is supported by a growing body of research as an important cardiovascular disease risk factor. Changes in TMAO, stachydrine, and p-cresol sulfate levels suggest a mechanism related to the microbiota activity and possibly an interaction with intestinal function. Part of the effect of L-arg may stem from an increase in methyl demand.

More generally, our findings warrant further study to precisely characterize the effect of L-arg supplementation on the

specific metabolic pathways as revealed here and to appreciate their contribution to the physiological effects of L-arg.

Author contributions

The authors' responsibilities were as follows—FM: designed the clinical study; FM, AD: implemented the clinical study; CT, JCM: metabolomics data acquisition; MTF: data analysis and results; HF, SP, LD, JCM, FM: study design; LD, FM: interpreted the results; LD: wrote the manuscript, with assistance from MTF and FM; LD: had primary responsibility for final content; and all authors: read and approved the final manuscript.

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Data availability

Data described in the manuscript and analytic code will be made available upon request pending application and approval. Further inquiries can be directed to the corresponding author.

Conflict of interest

The authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cdnut.2023.102038.

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