

# A red wine intervention does not modify plasma trimethylamine *N*-oxide but is associated with broad shifts in the plasma metabolome and gut microbiota composition

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## ABSTRACT

**Background:** Gut microbiota profiles are closely related to cardiovascular diseases through mechanisms that include the reported deleterious effects of metabolites, such as trimethylamine *N*-oxide (TMAO), which have been studied as diagnostic and therapeutic targets. Moderate red wine (RW) consumption is reportedly cardioprotective, possibly by affecting the gut microbiota.

**Objectives:** To investigate the effects of RW consumption on the gut microbiota, plasma TMAO, and the plasma metabolome in men with documented coronary artery disease (CAD) using a multiomics assessment in a crossover trial.

**Methods:** We conducted a randomized, crossover, controlled trial involving 42 men (average age, 60 y) with documented CAD comparing 3-wk RW consumption (250 mL/d, 5 d/wk) with an equal period of alcohol abstinence, both preceded by a 2-wk washout period. The gut microbiota was analyzed via 16S rRNA high-throughput sequencing. Plasma TMAO was evaluated by LC-MS/MS. The plasma metabolome of 20 randomly selected participants was evaluated by ultra-high-performance LC-MS/MS. The effect of RW consumption was assessed by individual comparisons using paired tests during the abstinence and RW periods.

**Results:** Plasma TMAO did not differ between RW intervention and alcohol abstinence, and TMAO concentrations showed low intraindividual concordance over time, with an intraclass correlation coefficient of 0.049 during the control period. After RW consumption, there was significant remodeling of the gut microbiota, with a difference in  $\beta$  diversity and predominance of *Parasutterella*, *Ruminococcaceae*, several *Bacteroides* species, and *Prevotella*. Plasma metabolomic analysis revealed significant changes in metabolites after RW consumption, consistent with improved redox homeostasis.

**Conclusions:** Modulation of the gut microbiota may contribute to the putative cardiovascular benefits of moderate RW consumption. The low intraindividual concordance of TMAO presents challenges

regarding its role as a cardiovascular risk biomarker at the individual level. This study was registered at clinicaltrials.gov as NCT03232099. *Am J Clin Nutr* 2022;116:1515–1529.

**Keywords:** gut microbiota, metabolomics, trimethylamine *N*-oxide (TMAO), coronary artery disease, wine, redox

## Introduction

Recognition of the roles of the gut microbiota in the pathophysiology of cardiovascular diseases (CVDs) (1) and of diet in influencing the microbiota's composition, function, and interaction with the human host (2, 3) has increased. Trimethylamine *N*-oxide (TMAO), a gut microbiota-dependent metabolite of dietary quaternary ammonium compounds, largely choline and carnitine, has been widely reported to correlate with CVD and major adverse cardiovascular events (MACEs). Different taxa within the gut microbiota metabolize these dietary precursors to trimethylamine (TMA), which is converted to TMAO by flavin monooxygenase enzymes in the liver. Plasma TMAO is associated with atherosclerotic burden and is independently predictive of myocardial infarction, stroke, and cardiovascular death (4).

Different interventions have been proposed to modify plasma TMAO concentrations, such as dietary modifications related to choline content (5), through modulation of the gut microbiota with dietary prebiotic supplements (6, 7) and medical interventions that manipulate the gut microbiota. For instance, experimental studies using a structural analogue of choline, 3,3-dimethyl-1-butanol (DMB) (8), and the gut microbiota-targeting inhibitor iodomethylcholine (9) have been shown to nonlethally

inhibit the formation of TMA and TMAO and reduce TMAO concentrations.

One potential influencer of the gut microbiota and plasma TMAO is red wine (RW). RW has been proposed to have health-promoting effects. Moderate RW consumption is correlated with a lower incidence of cardiovascular events (10), cancer (11), and overall mortality (10, 12), which is mainly attributed to its polyphenolic components. The main phenolic compounds in wine are flavonoids, particularly flavan-3-ols, flavonols, and anthocyanins (13). Other nonflavonoid compounds, such as hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, and stilbenes, are also key components in wine. The polyphenol chemical structure, the food matrix, and enterohepatic circulation can influence polyphenol bioavailability and absorption, and a large percentage of polyphenols is not absorbed in the small intestine. Consequently, ~90% of polyphenols reach the colon intact, where they can exert a regulatory function and probably act as prebiotics; there, they serve as fuel for bacterial fermentation and are accessible to a large proportion of the gut bacteria (14). Therefore, some beneficial effects of RW might occur through alterations of the gut microbiota (15), fecal metabolome (16), and plasma metabolites (17). For instance, the grape polyphenol proanthocyanidin induces intestinal blooms of *Akkermansia muciniphila*, and this genus is associated with improved metabolic health (18). Furthermore, Le Roy et al. (19) reported increased gut microbiota  $\alpha$  diversity associated with RW consumption in 3 independent human cohorts. In

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The raw Illumina read data for all stool samples have been deposited in the NCBI SRA repository under the BioProject ID PRJNA726242. The metabolomic data sets for this study can be found in the MetaboLights repository under the public URL <https://www.ebi.ac.uk/metabolights/editors/study/MTBLS310/>. Other data supporting the findings of the study are available in this article and its Supplemental Information files or from the corresponding authors upon request.

Supplemental Figures 1–5, Supplemental Methods, Supplemental Tables 1–8, and Supplemental Results are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/ajcn/>.

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Abbreviations used: ACE, angiotensin-converting enzyme; ASV, amplicon sequence variant; AUDIT, Alcohol Use Disorders Identification Test; CAD, coronary artery disease; CVD, cardiovascular diseases; DBS, dried blood spot; DMB, 3,3-dimethyl-1-butanol; ICC, intraclass correlation coefficient; IPA, indole propionate; MACE, major adverse cardiovascular event; PPP, pentose phosphate pathway; RW, red wine; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide.

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addition, RW contains DMB, a structural analogue of choline, which may inhibit TMA formation in the gut and decrease plasma TMAO concentrations, according to a rodent study (20). In this context, the biological activity of polyphenols in the circulatory system may also depend on the ability of the gut microbiota to transform them into bioavailable metabolites. This highlights the importance of assessing both microbiota and plasma metabolites when a given intervention is tested.

However, the relations between the gut microbiota, TMAO, and plasma metabolomics in patients with coronary artery disease (CAD) remain understudied. The present study aimed to assess the effects of short-term, moderate RW consumption on the gut microbiota and plasma TMAO in 42 male patients with CAD compared with those of alcohol abstinence in the same individuals. In addition, it also sought to elucidate the effects of RW on the plasma metabolome in a subset of 20 patients.

## Methods

### Trial design

This was a randomized, controlled (1:1) crossover trial composed of two 3-wk interventions: one involved the consumption of RW, 250 mL a day, 5 d a week, and the other involved alcohol abstinence. Each intervention was preceded by a 2-wk washout period, without consumption of any alcoholic beverages, fermented foods, prebiotics, or probiotics. The trial was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as NCT03232099 and was conducted from August 2016 to May 2018.

### Patients

Patients were recruited from the Heart Institute, University of São Paulo, São Paulo, Brazil, and from advertisements in local newspapers and on local radio stations. Eligible patients with established CAD were men who were aged between 46 and 69 y, had a BMI (in kg/m<sup>2</sup>) <30, and were stable and asymptomatic. Only men were selected with the intention of homogenizing our sample since both alcohol metabolism (21) and TMAO metabolism may differ by sex; the activity of flavin monooxygenase 3 (FMO3) has been reported to be higher in female mice than in male mice (22). Established CAD was defined as a diagnosis at least 30 d before the beginning of the study of myocardial infarction, angiographic evidence of  $\geq 50\%$  stenosis of 1 or more epicardial vessels, coronary angioplasty, or coronary artery bypass grafting. Importantly, the included patients had no evidence of acute coronary syndrome (cardiac troponin T concentration, <0.1  $\mu\text{g/L}$ ), coronary angioplasty, or coronary artery bypass grafting at least 30 d prior to protocol initiation. The exclusion criteria included antibiotic treatments (<2 mo before the start of the study), heart failure (New York Heart Association functional class  $\geq \text{II}$ ), renal failure (creatinine clearance <30 mL/min by the Cockcroft–Gault formula), hepatic failure (thrombocytopenia, reduced serum albumin, and prolonged prothrombin time), digestive tract cancers, inflammatory bowel diseases, obstructive biliary diseases, prior digestive surgeries (cholecystectomy, gastrectomy or colectomy), and diabetes mellitus or the use of antidiabetic drugs. Patients who self-reported to be regular RW drinkers

(defined as a previous consumption of at least 100 mL RW a day) were not included in the study. Patients were also required to have an Alcohol Use Disorders Identification Test (AUDIT) score of 7 or less. The AUDIT (23) score is used to identify individuals with hazardous or harmful alcohol consumption behavior; scores up to 7 suggest a low risk of alcohol abuse. The Scientific Committee of the Heart Institute (Instituto do Coração—InCor-HCFMUSP) and the Institutional Ethical Committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo approved the study protocol (SDC 4257/15/084—CAAE 57379216.0.0000.0068). All patients gave written informed consent before participating in the study.

### Sample size

For sample size calculation, we assumed that the TMAO concentration would decrease by 1.0  $\mu\text{M}$  after the RW intervention and that there would be a within-patient standard deviation of 1.5. These assumptions were made according to Tang et al. (24). We assumed a type I error of  $P < 0.05$  and an 80% test power, and it was estimated that 38 patients would be necessary. Thus, considering a 10% loss rate during the intervention, it was found that 42 patients should be included; the calculation was performed with Sample Size Calculators ([http://hedwig.mgh.harvard.edu/sample\\_size/js/js\\_crossover\\_quant.html](http://hedwig.mgh.harvard.edu/sample_size/js/js_crossover_quant.html)) (25).

### Randomization

The random allocation sequence of patients was generated by the Random Integer Generator (random.org) at the time of patient inclusion in the study, as shown in the Consort Flow Diagram in **Supplemental Figure 1**. The random allocation sequence was implemented using the participant IDs matched with the allocated sequences.

### Interventions

The patients were randomly assigned to start either a 3-wk intervention with RW or a 3-wk intervention with alcohol abstinence. RW was provided for all patients at the beginning of the respective intervention period. Ibravin (Brazilian Wine Institute) produced and supplied RW made with the Merlot grape variety. Embrapa (Brazilian Agricultural Research Corporation) assessed the quality of the wine and released it for consumption after their technical approval. The wine used was a Merlot from the 2014 vintage, bottled in August 2016 in 250-mL bottles and customized for the study. The Merlot variety was chosen because it is one of the grapes that is best adapted to the soil and climate of Rio Grande do Sul state, Brazil, where the samples were customized. The RW dosage of 250 mL, with  $\sim 24$  g of alcohol, was selected because this amount of RW has been shown to be associated with beneficial effects (26) and has been used in several previous studies investigating the effects of RW on the gut microbiota (15, 27). For men, moderate alcohol consumption can be defined as the ingestion of 1–3 standard drinks per day or the equivalent of 10–30 g of alcohol (28).

The RW sample had an alcohol content (% v) of 12.75, a total acidity (mEq/L) of 95.58, and a volatile acidity (mEq/L) of 7.44. It contained 109 mg/L total anthocyanins (DescSO<sub>2</sub>) and 2155 mg/L total polyphenols in catechin. Patients received 21 labeled bottles and written guidance to drink 250 mL/d, 5 d a week, for 3 wk. They were also given a dietary recall

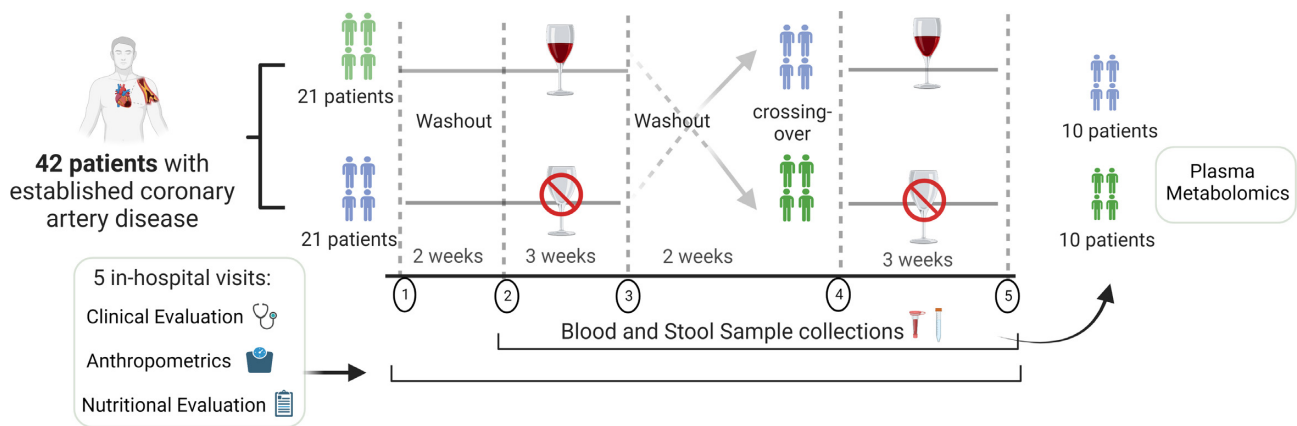
form to record their daily wine consumption. After the RW intervention period, the patients returned the 21 empty RW bottles and completed the consumption diary.

### Overview of protocol visits and measures

After written informed consent was obtained and randomization was performed, the patients returned 5 times for in-hospital visits consisting of clinical and nutritional assessments, anthropometric measurements, and sample collection. On the first visit, there was no sample collection, and a trained nurse or physician interviewed the patients; recorded their vital signs, weight, height, and waist circumference; and gave instructions about the stool sample collection that would be carried out at home 1 d prior to each of the subsequent 4 visits. In addition, during the first visit, patients underwent nutritional assessment by a trained dietitian, who provided instructions about completing the 3-d dietary recall and advised patients to maintain their diet and physical activity routine. Patients also received written and verbal instructions regarding the 2-wk washout period, during which they were instructed to avoid any alcoholic beverages, fermented products, prebiotics, or probiotics. After the 2 washout periods and the 2 intervention periods, 4 hospital visits were carried out, during which blood sample collection, clinical interviews, and nutritional evaluations were performed. A schematic view of the study design is shown in the **Figure 1**. The patients brought stool samples that were collected at home 1 d before each visit. The primary endpoint was to assess changes regarding gut microbiota composition and plasma TMAO concentrations between RW consumption and alcohol abstinence groups. For biochemical analysis, TMAO analysis, microbiota analysis, and metabolomic analysis, the assessors who collected the outcome data and the data analysts were blinded to the assigned intervention.

### Dietary intake assessment and compliance

A trained dietitian conducted an in-person interview at every in-hospital visit, focusing on the completion of the dietary diaries and adherence to the dietary guidelines, that is, avoidance of other polyphenol-rich foods, any alcoholic beverages other than the provided RW, prebiotics, probiotics, and fermented products and maintenance of a similar diet pattern throughout the study. In this context, there were written instructions to avoid the consumption of beer, grapes or grape juice, berries or berry juices, yogurt, kombucha, kefir sauerkraut or other fermented veggies, and any products with added fiber or synthetic prebiotics, such as inulin, fructooligosaccharides, or fiber dairy. Patients were also instructed to call or e-mail the dietitian if they had any doubts about the consumption of these products. These measures aimed to decrease the interference of changes in the habitual diet other than RW on the gut microbiota. Patients completed a 3-d dietary recall; they were asked to recall their food consumption in the past 24 h, including all food and drink consumed, for 3 d (2 weekdays and 1 weekend day) at the end of both interventions. The dietary assessment was recorded manually and was dietitian-led reviewed after returning. The daily intake of total energy, macronutrients, and micronutrients was calculated according to the Brazilian Food Composition Table—TACO (29) and the



**FIGURE 1** Study design. A total of 42 patients with established coronary artery disease were randomly selected for 1 of the 2 groups in the crossover study involving red wine (RW) consumption for 3 wk or alcohol abstinence for 3 wk. All patients were evaluated over 5 in-hospital visits for anthropometrics and clinical and nutritional assessments. After an initial evaluation, there was a 2-wk washout period when patients were instructed not to consume alcoholic beverages, fermented foods (yogurt, kombucha, soy lecithin, kefir, sauerkraut, and other fermented veggies), synthetic prebiotics (insulin, fructooligosaccharides), fiber, dairy, food polyphenols (grapes, grape juice, cranberries, strawberries), and probiotics. Samples were collected after the first 2-wk washout period, and patients were randomly assigned for a 3-wk intervention with RW consumption (250 mL/d, 5 d/wk) or 3 wk of alcohol abstinence. After these 3 wk, new blood and stool samples were collected, and another 2-wk washout period was implemented. Then, new samples were collected, and patients crossed over: the group that received RW was instructed to abstain from alcohol for 3 wk, and the group that abstained from alcohol in the first 3 wk received RW. After the second 3-wk period, stool and blood samples were collected in both groups. In 20 randomly selected patients (10 from each group), plasma metabolomics was analyzed after the 2 washout periods and after the intervention with RW and abstinence.

second Brazilian Food Composition Table (<http://www.fcf.usp.br/tbca/>).

### Interruption criteria

Patients were periodically evaluated with respect to safety. In addition to hospital visits, patients were encouraged to contact the study staff by phone or e-mail if they experienced any adverse health-related impacts of the interventions. In the case of RW intolerance—limiting headaches, dizziness, gastrointestinal complaints, or other forms of intolerance—patients were excluded. If there were any MACEs such as myocardial infarction, stroke, heart or digestive tract surgery, or the use of antibiotics or antidiabetic drugs, the protocol was interrupted.

### Metagenome profile

Patients received verbal and written instructions regarding the collection of stool samples. They were advised to collect the samples using a collection tube with DNA-stabilizing buffer (STRATEC Biomedical AG) 1 d prior to the visit to the hospital or on the same day of the visit. The samples were immediately stored in provided coolers at 4°C and then transported to the hospital, where they were immediately frozen at −20°C until analysis. Total DNA was extracted from the intestinal microbiota in 200 mg of stool with the PSP Spin Stool Plus DNA kit (STRATEC Biomedical AG). To profile the gut microbiota composition, the hypervariable region (V3–V4) of the bacterial 16S rRNA gene was amplified following the Illumina 16S Metagenomic Sequencing Library Preparation guide (30), which uses the sequences 338F-5'-TCGT CGGC AGCG TCAG ATGT GTAT AAGA GACA GCCT ACGG GNGG CWGC AG-3' and 785R-5'-GTCT CGTG GGCT CGGA GATG TGTA TAAG

AGAC AGGA CTAC HVGG GTAT CTAA TCC-3' (2 × 300 bp paired-end reads with an insert size of ~550 bp).

### Gut microbiota bioinformatic analysis

The DADA2 tool was used to analyze the fastq sequences to recover single-nucleotide resolved amplicon sequence variants (ASVs) from the amplicon data, according to Magro et al. (31). The total number of ASVs was 1106. To remove the adapter sequences at the 5' end, the trimLeft option was set to 17 and 21 (forward and reverse reads, respectively). The data were preprocessed, removing possible contaminants (mitochondrial and chloroplast sequences) and filtering infrequent features (features with <10 read counts and present in <2 samples). The taxonomic assignment was performed with the naive Bayesian classifier method implemented in DADA2 using the SILVA database as a reference (version 132). The  $\alpha$  diversity was evaluated with the richness, Simpson, and Shannon indexes, and  $\beta$  diversity was assessed with sparse partial least squares regression-discriminant analysis. All gut microbiota data were compared through paired analyses.

### Integration analysis of gut microbiota and plasma metabolomics data

Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO) R package (32) version 6.3.2 (<http://mixomics.org/mixdiablo/>) was used to evaluate and integrate these 2 data sets. DIABLO is a multiomics integrative method that seeks correlated information across different data types by selecting a subset of molecular features while discriminating between multiple phenotypic groups. It maximizes the common or correlated information between multiple omics (multiomics) data sets while identifying the key omics variables. DIABLO is

a component-based method (or a dimension reduction technique) that transforms each data set into components maximizing the correlations between components and the phenotype of interest. The first step in DIABLO analysis involves choosing the number of components. The correct number of components can be assessed by evaluating the model performance across all the specified numbers of components.

This analysis aimed to simultaneously identify correlated and discriminant microbiota and plasma metabolomics features able to distinguish the RW and alcohol abstention interventions in the cohort of patients. We evaluated the model across 5 components. The assessment was performed using the *perf* function, setting the cross-validation method to *leave-one-out* (validation = "loo") (32). During this process, the assignment of each new observation to the final predicted class was performed using different prediction distances (maximum, centroids, or Mahalanobis). The performance plot (Supplemental Figure 2) shows that the error rate decreased from 1 to 2 components.

In our data set, we found that the Mahalanobis distance (a multivariate distance metric that measures the distance between a point and a distribution) yielded the best accuracy, indicating that the optimal number of components was 2. The next step was to tune the number of features selected per data set and per component (*keepX* parameters) using the *tune.block.splsda* function. The optimal number of variables was selected within a grid of possible *keepX* variables (from a minimum of 5 to a maximum of 30 features). For each number of *keepX* variables, the function assesses the model's performance to discriminate between the 2 groups using leave-one-out cross-validation. The model identified 6 taxa for the first component and 5 for the second component, as well as 9 metabolites for the first component and 5 for the second component. These selected features were considered representative of the multiomics biomarkers that better discriminated the RW and alcohol abstention interventions.

### TMAO assessment

During the protocol, after a 12-h overnight fast, all 42 patients had blood samples collected at every 1 of 4 examination visits. After collection, the blood samples were stored in the refrigerator, separated into serum and plasma, placed in Eppendorf tubes, and immediately stored at  $-80^{\circ}\text{C}$  until analysis. All blood sample analyses were performed in only 2 batches to ensure low variability.

### Assessment of TMAO intraindividual variability

To assess TMAO intraindividual variability, we randomly selected 10 patients for 4 additional visits (twice a week) after the protocol was finished. After a 12-h fast, blood samples were collected and analyzed by 3 techniques. Patients were advised not to modify medication, diet, or exercise. The assessments of variability were performed using 3 different collection techniques: 1) blood samples were collected, separated into serum and plasma, placed in Eppendorf tubes, and immediately frozen at  $-80^{\circ}\text{C}$  after collection; 2) blood samples were collected, separated into serum and plasma, placed in Eppendorf tubes, and

refrigerated for 2 h after collection and then frozen at  $-80^{\circ}\text{C}$ ; or 3) blood samples from the fingertips were used for dried blood spot (DBS) analysis.

### TMAO quantification

The method used was described by Wang et al. (33). TMAO was extracted from a 50- $\mu\text{L}$  plasma sample using 200  $\mu\text{L}$  methanol (containing the internal standard TMAO-d9 at 10  $\mu\text{M}$ ) to denature the proteins after mechanical agitation (vortex). The plasma samples were centrifuged to precipitate the proteins, and an aliquot of the supernatant was collected for injection into the LC-MS/MS system. The analytical curve (from 0.25 to 200  $\mu\text{M}$  TMAO) and control samples (0.5, 5, and 100  $\mu\text{M}$  TMAO) were prepared through the addition of TMAO ( $\mu\text{M}$ ) to a 5% bovine serum albumin (Sigma-Aldrich) aqueous solution containing 0.7% sodium chloride (w/w). TMAO and TMAO analytical standards were purchased from Sigma-Aldrich. The DBS samples were obtained from 2 drops of whole blood collected on Whatman Paper 903 (Protein Saver Card; GE Healthcare) after fingertip pricking. The DBS cards were dried at room temperature for 1 h. To extract TMAO from the DBS samples, two 3.0-mm circular "punches" were obtained from the DBS card and placed in a 1.5-mL Eppendorf tube, and 150  $\mu\text{L}$  75% methanol/25% water (v/v) solution containing a 1.0- $\mu\text{M}$  concentration of the internal standard TMAO-d9 was added. The tube was mixed with a vortex for 10 s, ultrasonicated for 3 min, vortexed for another 10 s, and then centrifuged at  $10,000 \times g$  for 3 min at  $4^{\circ}\text{C}$ . An aliquot of the extracted solution was transferred to a 96-well plate (or vials) and injected into the LC-MS/MS system. The analytical curve (from 0.25 to 200  $\mu\text{M}$  TMAO) and control samples (0.5, 5, and 100  $\mu\text{M}$  TMAO) were prepared through the addition of TMAO ( $\mu\text{M}$ ) to whole blood (lyophilized form; ControlLab) without the presence of TMAO. The blood was collected on a card following the TMAO extraction procedure and then analyzed. To quantify TMAO and its internal standard TMAO-d9 in the extracted plasma and DBS samples, liquid chromatography (Agilent 1260; Agilent Technologies) coupled to a hybrid triple quadrupole linear ion trap mass spectrometer (3200 QTRAP; SCIEX) was used. The compounds were separated in the LC system using an Atlantis HILIC Silica column (100  $\times$  3.0 mm, 3  $\mu\text{m}$ ; Waters Corp) maintained at  $35^{\circ}\text{C}$  and the following mobile phases: (A) aqueous solution containing 10 mM ammonium formate (LC-MS grade; Sigma-Aldrich) and (B) 90% acetonitrile/10% water (v/v) solution containing 10 mM ammonium formate. A 10- $\mu\text{L}$  sample volume was injected, and the compounds were eluted through the column using the following gradient conditions at a flow rate of 500  $\mu\text{L}/\text{min}$ : from 0 to 1 min, 0% mobile phase (A); from 1.0 to 5.20 min, 90% (A) to 10% (B); from 5.20 to 7.50 min, maintained at 90% (A); and from 7.50 to 11.5 min, returned to the initial 0% condition (increased the flow to 600  $\mu\text{L}/\text{min}$ ) and allowed to equilibrate for an extra 1.0 min before reinjection. The TMAO/TMAO-d9 retention time was 6.15 min. After separation, the compounds were ionized by an electrospray ionization (Turbo V; SCIEX) source in positive mode at 5200 V with the nebulizer gas at 45 psi, heater gas at 50 psi, and curtain gas at 15 psi and analyzed in the MS/MS system by multiple reaction mode optimized for the following *m/z* transitions: 76 (precursor ion)

> 58 (product ion) and 85 > 66 for TMAO and TMAO-d9, respectively.

### Plasma metabolomics

Using a random integer generator (random.org), 20 of the 42 patients were randomly assigned to have their plasma metabolomic profiles analyzed. The sample size for this subanalysis was determined using the program MetSizeR (34). From the blood samples collected at the 4 visits, 20 patients and 80 samples were selected for untargeted plasma metabolomic analysis. The samples were stored at  $-80^{\circ}\text{C}$  and assayed with an untargeted evaluation by ultra-HPLC–MS/MS (Waters ACQUITY) coupled with a HESI-II Thermo Scientific Q-Exactive Orbitrap (35,000 mass resolution) performed by Metabolon. Brief methodologic details are provided in the **Supplemental Methods**.

### Fasting blood sample biochemical analyses

Blood samples of the 42 patients were collected on all 4 examination days after a 12-h fast. Blood sample analyses were performed on the day of collection. At every visit, we analyzed fasting concentrations of plasma glucose, triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, alanine aminotransferase, aspartate aminotransferase, serum albumin, serum bilirubin, serum lactate dehydrogenase,  $\gamma$ -glutamyl transferase, prothrombin time, high-sensitivity C-reactive protein, IL-6, complete blood count, creatine, urea, and serum lipopolysaccharides. These tests were performed on a Dimension EXL analyzer (Siemens Healthcare), except for troponin I, which was measured using a chemiluminescence immunometric assay (ADVIA Centaur-XP; TnI-Ultra; Siemens Healthineers). Blood counts of total hemoglobin, leukocytes, neutrophils, lymphocytes, and other immune cells (including monocytes, mast cells, basophils, and eosinophils) were obtained using a Sysmex XN-2000 automated hematology analyzer (Sysmex America). Creatinine clearance was estimated using the Cockcroft–Gault equation, and the Friedewald equation was used to estimate LDL cholesterol.

### Statistical analysis

All statistical analyses were performed using either SPSS v.25 software for Windows (SPSS, Inc.) or R (R version 4.0.3; R Project for Statistical Computing). Variables were assessed by descriptive measures, according to the type of variable (qualitative or quantitative). For qualitative variables, frequencies and percentages were calculated. Normality of the distribution was assessed through the Shapiro–Wilk test. Data that followed a normal distribution were compared with a 2-sided paired Student *t* test. Data that did not follow a normal distribution were compared using a paired Wilcoxon test. The level of significance adopted for testing all hypotheses was 5%. The correlations between TMAO measurements and urea, creatinine, glomerular filtration rate, lipid profile measurements, macronutrients, and micronutrients were calculated using the Spearman rank correlation test. The generalized estimating equation method was used to evaluate the pattern of TMAO

measurements within an individual longitudinally in the different intervention groups (RW and abstinence) and in relation to the use of angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, and diuretics. This verified the effect of time with an independent variable of interest for the measure assessed over time. We wanted to observe whether there were differences between groups, between times, and for the interaction between the variables and time. Paired comparisons were performed using the Bonferroni test. We investigated the intraindividual concordance of plasma TMAO via repeated-measures analysis with the intraclass correlation coefficient (ICC).

## Results

### Crossover intervention

Fifty-seven patients were initially included, of whom 15 were excluded. Among the excluded patients, 8 withdrew from the protocol, 2 used antibiotics, 1 had hypertriglyceridemia on the first laboratory examination, and 1 had a leukemia diagnosis and was referred to the Hematology Clinic. One patient used metformin during the protocol, 1 patient had gouty arthritis, and 1 patient had an acute myocardial infarction shortly after randomization. Ultimately, 42 male patients completed the protocol (more details about patient enrollment are displayed in the CONSORT Flow Diagram, Supplemental Figure 1). The patients' baseline characteristics are shown in **Table 1**. All patients had CAD documented by coronary angiography or a clinical event and were clinically stable, and their ages ranged between 46 and 69 y; they were overweight on average, with a median BMI of 27.1, and they had a mean waist circumference of 98.0 cm. Blood pressure, lipid profiles, and glycemic profiles were well controlled. All the volunteers tolerated RW ingestion well. According to the patients' records of RW consumption, there were no missed dosages, and all the proposed dosages were ingested during the 3-wk intervention period. During the RW period compared with the alcohol abstinence period, there were no significant changes in blood pressure, heart rate, or BMI. In addition, there were no significant effects on high-sensitivity C-reactive protein, IL-6, lipopolysaccharides, glucose, or the glomerular filtration rate. However, after RW consumption, there were increases in HDL cholesterol, LDL cholesterol, and total cholesterol concentrations. In addition, liver enzyme concentrations did not change, although decreases in prothrombin time ( $P = 0.004$ ) and the platelet count ( $P = 0.02$ ) were observed (**Supplemental Tables 1 and 2**).

### There were no significant differences in dietary patterns during the protocol

Patients underwent nutritional evaluation at 5 in-hospital visits and completed a 3-d dietary recall at the end of each intervention period (abstinence or RW). Patients were compliant with the nutritional guidance and maintained the same food ingestion pattern, according to both dietary recalls. Except for the inclusion of alcohol and its calories during the RW intervention period, there were no significant differences in other macronutrient or micronutrient consumption

**TABLE 1** Baseline participant demographic and clinical data<sup>1</sup>

Variable	All participants (N = 42)
Age, y	60.4 ± 5.4
SBP, mmHg	119.8 ± 16.0
DBP, mmHg	72.3 ± 9.3
Heart rate	68.1 ± 9.3
BMI, kg/m <sup>2</sup>	27.1 (25.2–28.3)
Waist circumference, cm	98.0 ± 8.1
Current smoker	3 (7.1)
Former smoker	23 (54.7)
Hypertension	21 (50.0)
Dyslipidemia	18 (42.9)
Physical activity	23 (53.8)
5–10 METs.h/wk	7 (30.4)
10–40 METs.h/wk	10 (43.5)
>40 METs.h/wk	6 (26.1)
Physical inactivity	19 (45.2)
History of myocardial infarction	28 (66.7)
>50% stenosis of epicardial artery	25 (59.5)
CABG	13 (31.0)
PCI	19 (45.2)
Medications	
β-Blockers	30 (71.4)
ACE inhibitors	21 (50.0)
ARBs	10 (23.8)
CCBs	3 (7.1)
Aspirin	37 (88.1)
Clopidogrel	10 (23.8)
Statins	38 (90.5)
PPIs	8 (19.0)
Diuretics	
Total cholesterol, mg/dL	143 (127–170)
High-density lipoprotein, mg/dL	46 (39–54)
Low-density lipoprotein, mg/dL	77 (66–97)
Triglycerides, mg/dL	85 (66–111)
Apolipoprotein A, g/L	1.37 (1.22–1.51)
Apolipoprotein B, g/L	0.7 (0.62–0.86)
Lipoprotein a, mg/dL	60.5 (4.3–91.3)
Fasting glucose, mg/dL	90 (86–100)
GFR, mL/min/1.73 m <sup>2</sup>	85.5 (72–99)
hsCRP, mg/L	1.01 (0.58–2.77)
IL-6, pg/mL	0.75 (0–4.8)
LPS, EU/mL	0.76 (0.63–0.81)

<sup>1</sup>Data are presented as the mean ± SD, median (interquartile range), or n (%). ACE, angiotensin-converting enzyme; ARBs, angiotensin-receptor blockers; CABG, previous coronary artery bypass grafting; CCBs, calcium channel blockers; DBP, diastolic blood pressure; GFR, glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; LPS, lipopolysaccharides; METs, metabolic equivalents; METs.h/wk, METs-h/wk score = (MET level × hours × times/wk); PCI, previous percutaneous coronary intervention; PPIs, proton pump inhibitors; SBP, systolic blood pressure.

between the 2 intervention periods (Table 2). Moreover, the dietitians reviewed all patients' dietary records and confirmed the absence of self-reported intakes of the prohibited study foods according to our dietary guidelines: polyphenol-rich foods, prebiotics, probiotics, and fermented products during the washout and interventions periods. The dietitians reviewed the written recall records for RW consumption and confirmed no consumption of alcoholic beverages during the abstention period and no consumption of other beverages (other than the provided RW) during the RW intervention period. Finally, correlations of nutrients described in the dietary recalls and gut microbiota modifications can be found in the **Supplemental Results**.

### Alterations in the gut microbiota could be used to distinguish RW consumption

After a 2-wk washout period, the gut microbiota profile observed after RW consumption was modified compared with that after the abstention period. The β diversity showed a clear separation between groups (Figure 2A), and the gut microbiota profile could be used to distinguish RW consumption. In contrast, α diversity did not change after RW consumption (Supplemental Figure 3). After RW consumption, there was a predominance of *Bacteroides*, *Ruminococcaceae*, *Roseburia*, and *Prevotella*. *Parasutterella* was the most prominent genus for differentiating the gut microbiota composition during the RW period (Figure 2B). Three stool samples from 3 different

**TABLE 2** Food recall data quantification for the 42 patients during the 2 periods of the study<sup>1</sup>

Variable	All patients (N = 42)		P value
	Abstention	Red wine	
<b>Macronutrients</b>			
Total energy intake, kcal	1995.5 ± 608.8	2263.5 ± 566.0	0.039 <sup>2</sup>
Protein, g	97.2 ± 34.3	99.7 ± 28.1	0.616 <sup>2</sup>
Lipids, g	62.7 ± 24	63.6 ± 23.5	0.688 <sup>3</sup>
Carbohydrates, g	258.4 ± 92.6	251.9 ± 83.5	0.651 <sup>2</sup>
Fiber, g	23.7 ± 12.8	23.2 ± 11.3	0.849 <sup>3</sup>
Alcohol, g/kcal	—	24.0/180	—
<b>Micronutrients</b>			
Cholesterol, g	326.1 ± 156.7	354.9 ± 137.8	0.246 <sup>2</sup>
Calcium, g	611.6 ± 428.8	497.5 ± 305.9	0.061 <sup>3</sup>
Iron, g	10.6 ± 9.1	17.8 ± 55.0	0.446 <sup>3</sup>
Sodium, g	1603.7 ± 784.1	1560.6 ± 761.7	0.778 <sup>2</sup>
Potassium, g	2509.1 ± 904.4	2346.6 ± 764.0	0.164 <sup>2</sup>
SFA, g	22.5 ± 9.8	22.0 ± 8.5	0.446 <sup>3</sup>
MUFA, g	18.5 ± 7.8	18.9 ± 9.3	0.797 <sup>2</sup>
PUFA, g	9 ± 4.8	9.7 ± 5.1	0.491 <sup>2</sup>

<sup>1</sup> Average 3-d dietary recalls were collected at the end of each intervention period. Values are presented as the mean ± SD per day.

<sup>2</sup> Samples followed a normal distribution, according to the Shapiro–Wilk test. Therefore, comparisons were performed with a paired *t* test.

<sup>3</sup> Samples did not follow a normal distribution, according to the Shapiro–Wilk test. Therefore, comparisons were performed with a paired Wilcoxon test.

No differences were observed in macronutrient or micronutrient consumption between periods.

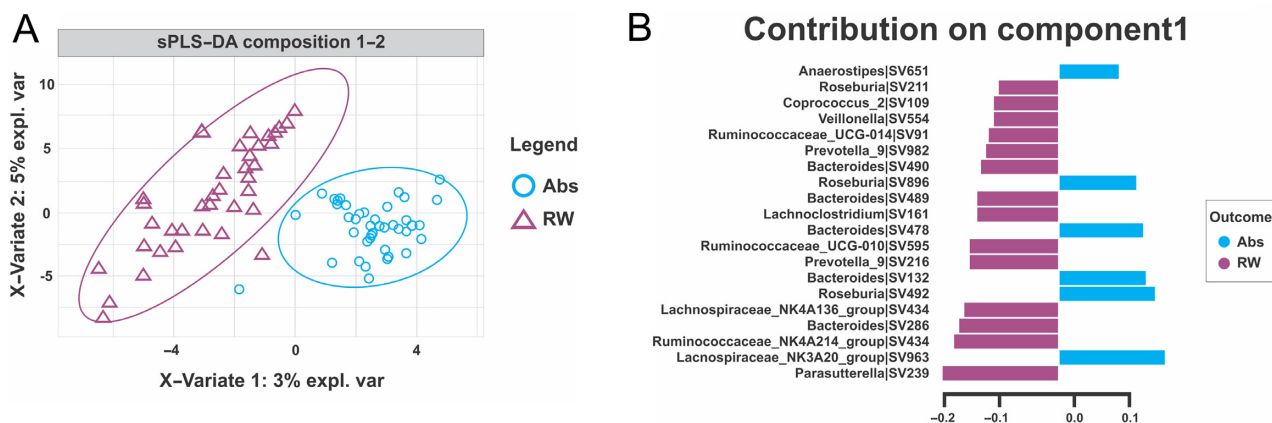
patients were removed from the analysis due to low read counts, with <10 reads per sample (31). In addition, we investigated correlations of gut microbiota taxa with clinical, biochemical, and nutritional variables, as shown in **Supplemental Figure 4**. A full description of these findings can be found in the Supplemental Results.

### Plasma TMAO analysis

Plasma concentrations of TMAO after the consumption of RW did not differ significantly compared with those following the abstention period (**Table 3**). There was also no evidence of a

carryover effect, as **Table 3** shows no significant differences between the beginning of the abstention period and the beginning of the RW period. In addition, TMAO concentrations varied substantially in the samples collected at both the beginning and the end of the abstention period: the ICC between these 2 sets of samples was 0.049 (95% CI: 0.255, 0.345; *P* = 0.377).

In 10 participants selected for assessment of the reproducibility of the plasma TMAO measurements, high concordance was observed among the 3 different sample techniques (**Supplemental Table 3**), with an ICC of 0.915 (95% CI: 0.861, 0.951;



**FIGURE 2** Sparse partial least squares–discriminant analysis (sPLS-DA). (A) The plot of the 2-component sPLS-DA model showed stool sample clustering according to red wine consumption (RW) or not (Abs). The percentage of variance captured for each principal component (x-axis for the first component and y-axis for the second component) for each study period (RW compared with abstention). The sPLS-DA plot is based on the relative abundance of bacterial taxa in the gut microbiota from the Abs group (blue circle) or RW group (purple triangle) and their 95% confidence ellipses. (B) The contribution plot indicates each genus's contribution to the first component of sPLS-DA. Genus contribution ranked from the bottom (most important) to the top. The colors blue (Abs) and purple (RW) indicate the group in which the genus is most abundant. The horizontal graduation line represents the variance explained by the single genera.



**TABLE 3** Fasting TMAO plasma concentrations at baseline, after the 2-wk washout period, and at the end of each 3-wk intervention period measured in all the patients ( $N = 42$ )<sup>1</sup>

Intervention	Before intervention	After intervention	$P$ value <sup>2</sup>
Abstinence	3.95 (2.64–6.78)	4.99 (3.08–9.59)	0.390
Red wine	4.58 (2.81–8.39)	3.37 (2.63–7.66)	0.710
$P$ value <sup>3</sup>	0.258	0.600	

<sup>1</sup>Values are presented as the median (IQR). The data did not follow a normal distribution, according to the Shapiro–Wilk test; therefore, a paired Wilcoxon test was employed to compare groups. TMAO, trimethylamine *N*-oxide.

<sup>2</sup>Paired Wilcoxon test assessing the effect of the intervention (either red wine consumption or abstinence).

<sup>3</sup>Paired Wilcoxon test comparing the abstinence period and red wine consumption groups before and after their respective interventions.

$P < 0.001$ ). The highest concordance was between immediate freezing and refrigeration (ICC of 0.993) (**Supplemental Table 4**). In contrast, there was a low concordance between repeated measures of TMAO (**Table 4**), indicating significant intermeasurement variability for the same individual over time.

There was an inverse, weak correlation of plasma TMAO with renal function (Supplemental Results). Regarding nutrient intake (**Supplemental Table 5**) during the RW period, plasma TMAO was positively correlated with protein intake ( $\rho: 0.478$ ,  $P = 0.025$ ) and cholesterol intake ( $\rho: 0.336$ ,  $P = 0.045$ ). There was no significant evidence indicating associations between the use of ACE inhibitors or diuretics, TMAO concentrations, and RW intervention (**Supplemental Table 6**). Regarding the relations between TMAO and gut microbiota, during the abstinence period, TMAO was weakly correlated with *Roseburia* abundance and negatively correlated with *Lachnospiraceae* abundance. After RW consumption, TMAO plasma concentrations were positively correlated with the abundance of the genus *Bacteroides* (**Supplemental Figure 5C**).

### Metabolites in several metabolic pathways were modified after RW consumption

In untargeted plasma metabolomics, 39 metabolites changed significantly following the RW intervention (**Figure 3B**), as listed in **Supplemental Table 7** (no indication of carryover effects, **Supplemental Table 8**). The metabolites with significant changes likely represented changes in the pathways of amino acids, lipids, carbohydrates, and vitamins and cofactors (**Figure 3A**). Metabolites that significantly changed after RW consumption compared with the abstinence period were identified by the paired Wilcoxon signed-rank test for nonparametric data and a paired  $t$  test for parametric data.

### Pentose phosphate, ascorbate, and aromatic amino acid pathways were modified after RW intervention

The pentose phosphate pathway (PPP), the glucuronate/ascorbate pathway, and aromatic amino acid metabolism exhibited several interconversions (**Figure 3C**). The concentrations of several metabolites in these pathways increased. After RW, there was a significant increase in the concentrations of sedoheptulose, arabinose, ribitol, arabitol, and xylitol, components of the PPP nonoxidative branch. The increase in the concentration of ribitol, an important metabolite in the PPP and an integral part of riboflavin (vitamin B-2), might indicate modulation of the riboflavin metabolism pathway.

In the glucuronate/ascorbate pathway, there was a significant increase in the concentration of gulonate, which is an obligatory intermediate in ascorbic acid generation.

In addition, there were alterations in microbiota-associated products of aromatic amino acids (i.e., tryptophan, tyrosine, and phenylalanine). After RW consumption, the concentration of indole propionate (IPA), a microbial-derived metabolite of tryptophan, significantly increased. The concentrations of the tyrosine metabolites gentisate and homovanillate also increased, but the concentration of thyroxine decreased. RW consumption also increased the concentration of 1-carboxyethylphenylalanine, which is derived from phenylalanine metabolism.

### Amino acid metabolites changed after RW ingestion

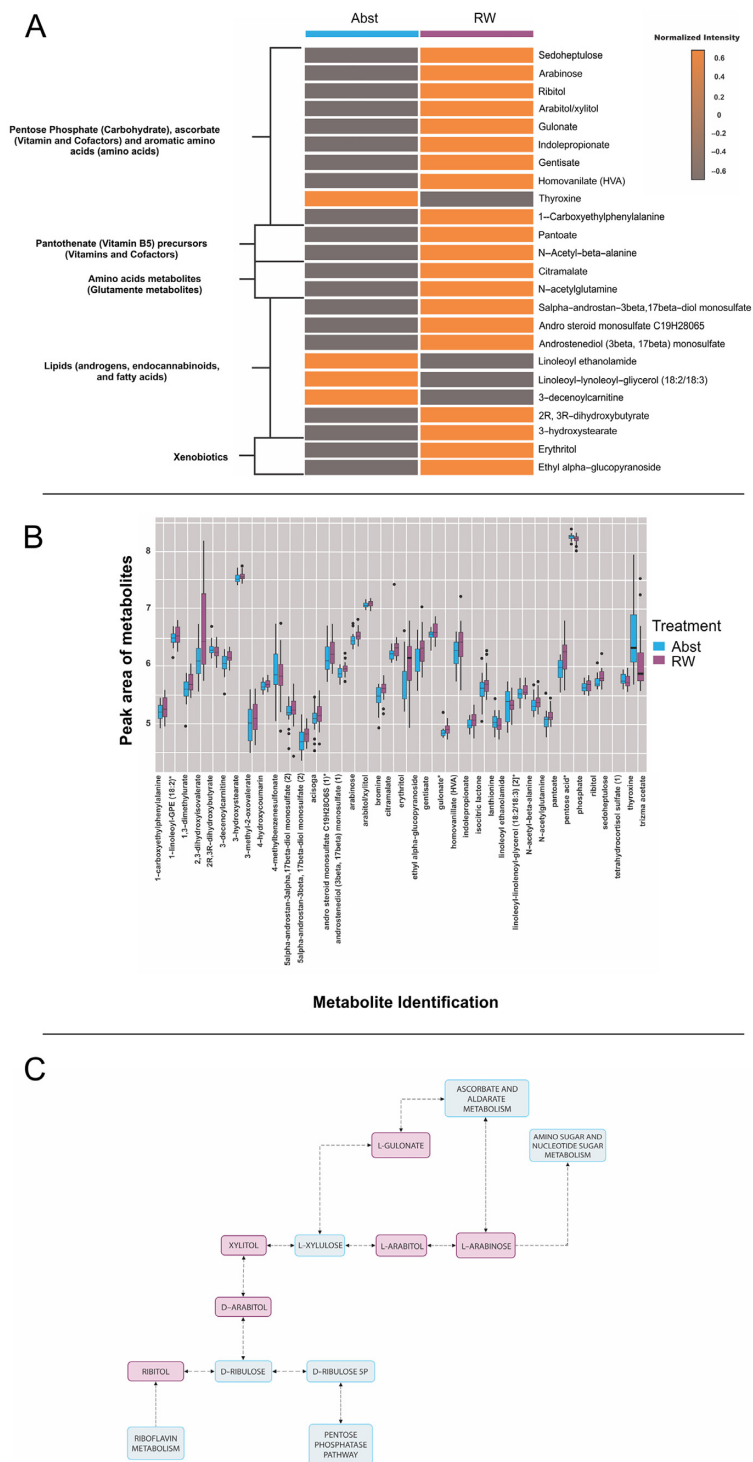
After RW consumption, the concentrations of the glutamate metabolites citramalate and *N*-acetylglutamine were elevated. Concentrations of the branched-chain amino acid metabolites 3-methyl-2-oxovalerate and 2,3-dihydroxyisovalerate also increased. Moreover, the concentrations of lanthionine, a

**TABLE 4** Assessment of TMAO intraindividual variability ( $n = 10$ )<sup>1</sup>

Technique	ICC	95% CI	$P$ value <sup>2</sup>
Freezing	0.224	−0.047, 0.627	0.060
Refrigeration	0.208	−0.058, 0.613	0.072
DBS	0.219	−0.051, 0.623	0.063

<sup>1</sup>The intraclass correlation coefficient for each trimethylamine *N*-oxide (TMAO) sample collection technique in 10 patients among 4 sample collection times showed a similar low concordance of TMAO values. DBS, dried blood spot; ICC, intraclass correlation coefficient.

<sup>2</sup>Under the null hypothesis, the intraclass correlation coefficient is equal to zero.



**FIGURE 3** Untargeted plasma metabolome alterations in 20 subjects. (A) Heatmap showing metabolites that were significantly different between the red wine (RW) and abstinence (Abst) periods. According to the metabolic pathways and biochemical functions, the graph highlights the most clinically relevant metabolites that were significantly different between the 2 periods of the study ( $P < 0.05$ , without adjustments for multiple comparisons). (B) Box-and-whisker plot of the distribution of the discriminating metabolites that were significantly altered after RW consumption compared with the abstinence period. (C) Pentose and glucuronate interconversions adapted from Kyoto Encyclopedia of Genes and Genomes pathway analysis (62). In red are the metabolites that were significantly increased after RW, and in blue are the putative pathways these metabolites are involved with. Arrows indicate the direction of the reaction and reversible and irreversible reactions, which are indicated by bidirectional and unidirectional arrows, respectively. Bold lines indicate activation or interaction. Dashed lines indicate an indirect link or unknown reaction.

metabolite in cysteine metabolism, and acisoga, a metabolite in polyamine metabolism, were increased after RW consumption.

### Lipid metabolites, including androgens, endocannabinoids, and fatty acids, were altered after RW consumption

Compared with abstinence, after RW consumption, there were increases in the concentrations of the androgens 5 $\alpha$ -androstan-3 $\beta$ , 17 $\beta$ -diol monosulfate (2), androsteroid monosulfate C19H28O6S (1)\*, and androstenediol (3 $\beta$ ,17 $\beta$ ) monosulfate (1). Furthermore, we detected a reduction in the concentrations of the endocannabinoid metabolite linoleoyl ethanolamide and its intermediate linoleoyl-linolenoyl-glycerol (18:2/18:3) [2]\* with RW consumption. In addition, after RW consumption, the concentrations of fatty acid metabolites were modified, as shown by a decrease in the 3-decenoylcarnitine concentration and increases in 2R,3R-dihydroxybutyrate and 3-hydroxystearate concentrations.

### Xenobiotic metabolites after RW ingestion

After RW consumption, the concentrations of erythritol and ethyl  $\alpha$ -glucopyranoside were significantly increased compared with those observed after abstinence. In addition, there was an increase in the concentration of 1,3-dimethylurate, a metabolite of theophylline, in the xanthine pathway.

### Pantothenate (vitamin B-5) precursor concentrations increased post-RW consumption

After RW consumption, pantoate and N-acetyl- $\beta$ -alanine concentrations significantly increased compared with those observed after abstinence. B complex vitamins are vital for several human metabolic processes and are directly absorbed in the upper digestive tract but are also derived from gut microbiota metabolism.

### Integration of plasma metabolomics data and gut microbiota analysis adequately separated the RW period and the abstinence period

For 20 randomly selected patients, we used the DIABLO platform for multiomics integrated data to simultaneously identify correlated and discriminant microbiota and plasma metabolomics features able to separate the RW and alcohol abstinence interventions in the cohort of patients. The microbiota data set included 586 taxa, while the metabolomic data set consisted of 39 metabolites. The first components from each data set were highly correlated, indicating a high discriminative power of each component to separate the different groups, RW and abstinence, with a correlation index of 0.85. Metabolites and taxa in the first component are displayed in Supplemental Figure 5. The optimally selected key predictors included several taxa of gut microbiota (i.e., *Streptococcus*, *Blautia*, *Ruminococcaceae*, *Bacteroides*, and *Prevotella*) and metabolites in amino acid pathways, lipid pathways, and cofactor pathways, such as pantoate. The agreement among all data sets is shown in the arrow plot (Figure 4A), which indicates that the 2 omics (microbiota

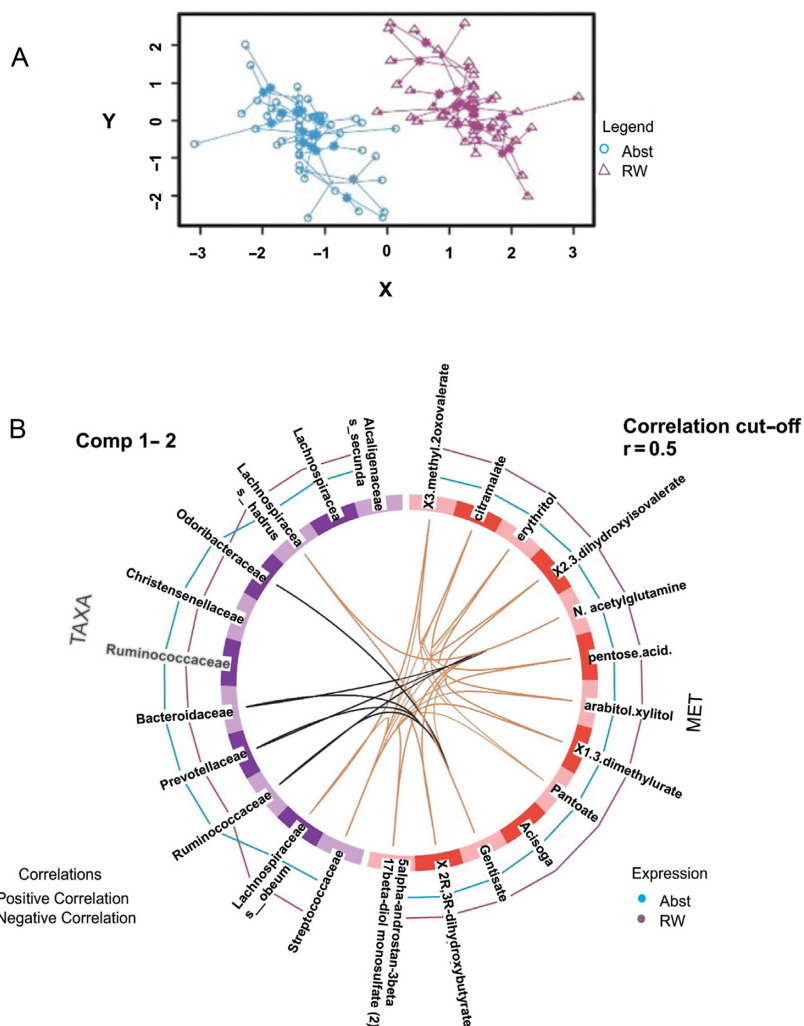
and metabolomics) data sets can distinguish the 2 conditions. The taxa and metabolites varied during the different interventions, and strong correlations among key predictors also contributed to a significant separation between the RW and abstinence groups (Figure 4B).

## Discussion

This study showed that in male patients aged between 46 and 69 y (median BMI of 27.1) with stable CAD, short-term, moderate RW consumption did not reduce TMAO concentrations. In parallel, the gut microbiota was modified after RW, as well as the plasma metabolome, particularly pathways affecting amino acids, vitamins and cofactors, lipids, and carbohydrates. In addition, plasma TMAO displayed high intraindividual variability.

Previous studies suggested that dietary modifications could alter plasma TMAO in humans, with mixed results. Higher concentrations of TMAO were found in omnivores than in vegetarians because exposure to dietary L-carnitine promotes the production of TMAO (35). However, in females, a 4-wk diet rich in animal protein (36) did not increase TMAO plasma concentrations. Nevertheless, in a randomized trial with healthy older men, there was an increase in TMAO concentrations with higher dietary protein intake (5). Another dietary modification that could modulate TMAO is RW, which is rich in polyphenols, including resveratrol, which was shown to decrease the plasma TMAO concentration in mice (20); moreover, RW contains DMB, a possible choline competitor for the gut microbiota (37). However, this study did not show a similar response in humans. Similarly, in obese individuals at risk for insulin resistance, consumption of polyphenols (tea or cocoa flavanols) did not reduce fasting TMAO concentrations (38).

Our TMAO analysis was very rigorous, involving a strict method, several measures, and a clear assessment of intraindividual variability that corroborated the analysis. Thus, it is very unlikely that the TMAO variation observed was due to methodologic issues. In addition, the high intraindividual variability of TMAO was unlikely to be explained by medication use, renal function, diet, or the gut microbiota profile. In this context, there was a negative correlation of TMAO plasma concentrations with the abundances of *Lachnospiraceae* and *Blautia* (Supplemental Results), gut bacterial taxa that have been implicated in TMAO formation (39, 40), as well as a positive correlation with the abundances of *Bacteroides* and *Roseburia*. These results contrast with the literature, as members of *Bacteroidetes*, such as *Roseburia* and *Bacteroides*, would not be capable of converting choline to TMA in the gut (40). Hence, the main factors that could possibly influence TMAO concentrations were investigated and did not explain the observed variations (1, 40–42). Further longitudinal evaluation and additional replicable measurement techniques for TMAO may be required to establish its role as an individual cardiovascular risk biomarker. For instance, Wu et al. (39) found that an oral carnitine challenge test may have better efficacy than fasting plasma TMAO for identifying the TMAO-producing phenotype. Moreover, recent studies highlighted the importance of gut microbiota intraindividual variability in long-term scenarios and revealed that unidentified influences or intrinsic dynamics of the



**FIGURE 4** Correlation matrix and multiomics data integration analysis. (A) In the arrow plot, the arrow origin indicates the centroid among all data sets for a given sample, and the tips of the arrows indicate the location of that sample in each block. These graphs highlight the agreement among all data sets at the sample level when modeled with DIABLO. The 2 omics (microbiota taxa and metabolomics) data sets performed well in separating the 2 interventions: red wine (RW) and abstinence (Abst). (B) The line outside each circle indicates the group with which each feature is associated [taxa, on the right side of the circle or metabolites (MET), on the left side]. The purple line represents biomarkers associated with the RW period, whereas the blue lines represent those associated with the abstinence period. The higher the line is, the higher the discrimination power of the feature. The line inside the circle represents the correlation between the taxa and the metabolites (an orange line indicates a positive correlation, and a black line indicates a negative correlation). The correlation cutoff was set to 0.5.

community members of the gut microbiota could be responsible for these variations (43, 44). These overlooked variations could explain the dissimilarities in gut microbiota profiles or gut microbiota-dependent metabolites, such as TMAO.

In addition, analysis of the gut microbiota after the RW intervention, compared with that after the alcohol abstinence period, revealed a predominance of *Ruminococcaceae*, several *Bacteroides* species, and *Prevotella*. In a crossover trial, Queipo-Ortuño et al. (15) also documented increases in the abundances of *Bacteroides* and *Prevotella* among other genera in 10 healthy volunteers who consumed 272 mL of RW/d for 20 d. In contrast to the work by Chadaideh et al. (45), who found that proanthocyanidins induced *A. muciniphila* growth in mice fed a high-fat diet, we did not detect the presence of *A. muciniphila* in our study, possibly because fat consumption was low in our population. Of particular interest, *Parasutterella*, based

on discriminant analysis, was the main genus responsible for alterations in the composition of the gut microbiota after RW consumption. *Parasutterella* is a genus considered beneficial for fiber digestion, succinate production, salutary aromatic amino acid metabolism, and bile acid metabolism (46). In line with this, the increase in *Parasutterella* abundance is associated with some of the features found in plasma metabolomic analysis after RW consumption. In particular, there were alterations in tryptophan and tyrosine metabolism because increases in IPA, gentisate, and homovanillate concentrations were observed. *Parasutterella* is also a member of the Proteobacteria phylum, which includes predicted ascorbate-producing genera that could modify the cellular redox state (47). In accordance with this, an increase in the concentration of gulonate, a key cofactor in the glucuronate/ascorbate pathway, was noted. In addition, the group formed by *Ruminococcaceae*, *Bacteroides*, and *Prevotella* is part

of the core microbiota in humans; this group was previously described as one enterotype identifier and is associated with high taxa abundance (48). Taken together, these alterations in gut microbiota taxa suggest changes after RW ingestion, which could possibly be related to some of the alterations observed in the plasma metabolome.

RW has long been proposed to reduce oxidative stress. For example, previous analysis showed that RW could reduce oxidative stress in rodent colonic mucosa (11), and in healthy males, RW increased plasma and LDL polyphenol concentrations and enhanced antioxidant activity (49). Our findings are in line with these proposed redox effects of RW. We found plasma changes that indicate modulations in redox signaling, such as increases in the concentrations of gulonate (50), metabolites in the PPP (51), and ribitol, an integral part of riboflavin, which has recently been studied as an important redox pathway (52). Another putative beneficial influence on redox homeostasis was the increase in the concentrations of the tyrosine metabolites gentisate and homovanillate, derived from polyphenols with redox capability (53). In parallel, we noted metabolite alterations that could be beneficial for insulin resistance and type 2 diabetes mellitus, such as an increase in the concentrations of the tryptophan metabolites IPA (54), glucopyranosides (55), erythritol, and ethyl  $\alpha$ -glucopyranoside and a decrease in long-chain acylcarnitine concentrations (56). Furthermore, RW consumption might also beneficially influence energy expenditure and metabolism, as suggested by the decrease in endocannabinoid concentrations (57, 58) and the increase in pantothenate precursor concentrations (59). Importantly, these changes were not significantly correlated with gut microbiota alterations in mediation analysis (DIABLO). Although the mediation analysis of the gut microbiota and the plasma metabolome could clearly separate the 2 intervention periods in this study, we cannot be sure that there was a causal association among these variables.

This study has some limitations. RW intake was not confirmed by direct sample tests but through self-reported consumption and the return of empty bottles. In addition, because we investigated the effects of RW only in men for a short duration, our results may not reflect the long-term effects of RW consumption and thus may not be generalizable to the overall population. In contrast, we minimized any influence of between-subject variability by using a crossover design. Another limitation is that it is not possible to ensure that the energy metabolism changes occurred solely because of polyphenolic or RW influences on the gut microbiota. Alcohol intake may also have influenced energy metabolism, affecting hepatic lipid metabolism, such as fatty acid uptake and de novo lipid synthesis (60, 61). Notably, important confounders that have been frequently mentioned in other investigations of RW, such as genetics and socioeconomic status, were primarily controlled in this design. In addition, in a free-living intervention study, we were able to note no significant differences in dietary consumption between the intervention periods on average. Except for calories added from the alcohol consumption, a similar dietary pattern was present in dietary recalls during the RW and in abstinence periods, suggesting that RW consumption was the only dietary change between groups. Moreover, although the number of calories increased during the RW intervention, there were no changes in weight, waist circumference, or glycemic profile. Finally, in contrast to our initial hypothesis, we observed no evidence of decreases in plasma TMAO concentrations,

partly because of their intraindividual variability. Further studies with larger sample sizes, longer durations, and additional repeated measures are needed to validate the reported findings, especially with regard to any false-positive findings of metabolite changes.

In summary, our findings indicate that TMAO measurements are highly variable, which may hinder clinical follow-up at the individual level. In addition, TMAO concentrations were not correlated with RW intake. Furthermore, our data indicate that the RW intervention is associated with remodeling of the gut microbiota and significant changes in the plasma metabolome. Although changes in the gut microbiota may have mediated the physiologic effects of RW consumption, these data remain hypothesis-generating and pave the way for future research, possibly with longer follow-ups and different populations.

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The authors' responsibilities were as follows—PLDL, FRML, and PL: conceived the trial; EAH: conducted the trial supervised by PLDL and participated actively in writing the manuscript; DF: contributed to the trial design and supervised the statistical analysis; MJAS and AS: contributed to microbiota profiling of stool samples, were involved in the generation of microbiota data, and contributed to data interpretation; NV and WJFL: performed the gut microbiota bioinformatics analyses; NV: integrated gut microbiota information and results of the plasma metabolomic analysis; AMAM and CRCP: performed metabolomic quality control analyses and statistical analyses for metabolomics; RSG: performed statistical analysis of the TMAO data and supervised other statistical work; DOM: performed nutritional analysis and interpretation; EAH, PLDL, FRML, and DF: performed the integrative data analyses and overall data interpretation; and all authors: revised and approved the final manuscript.

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Dr. Libby's interests were reviewed and are managed by Brigham and Women's Hospital and Mass General Brigham in accordance with their conflict-of-interest policies. The other authors report no conflicts of interest.

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