Microbiota-derived resveratrol metabolites: new biomarkers of red wine consumption are inversely associated with inflammation in a longitudinal study of a Mediterranean population

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Objectives: To evaluate the association between urinary microbiota-derived resveratrol metabolites, which may serve as specific biomarkers of red wine consumption, and plasma circulating proinflammatory markers. **Design, settings, and participants:** One-year longitudinal study included 179 participants at high cardiovascular risk (mean age 69 years, 49% women) enrolled in the PREDIMED trial. **Measurements:** Plasma inflammatory biomarkers and urinary microbiota-derived resveratrol metabolites were analyzed using xMAP technology and high-performance liquid chromatography coupled to mass spectrometry, respectively. Receiver operating characteristic (ROC) analyses were performed to evaluate the reliability of urine resveratrol metabolites as biomarkers of red wine consumption, as reported in the food frequency questionnaires (FFQs) of the participants. The relationship between baseline values and 1-year changes in urinary microbiota-derived resveratrol metabolites and plasma levels of circulating inflammatory molecules were assessed. **Results:** ROC curves confirmed that urinary dihydroresveratrol glucuronide (DHRg) [AUC = 0.835] and sulfate (DHRs) [AUC = 0.803] metabolites are reliable and specific biomarkers of red wine consumption. Baseline urinary concentrations of DHRs were negatively associated with plasma levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) (-0.40 ng/mL per 1-SD increase, $p = 0.012$). After one year of follow-up, changes in urinary concentrations of DHRg also showed a negative association with plasma circulating sVCAM-1 levels (-0.39 ng/mL per 1-SD increase, p-value = 0.028). No significant associations were detected at baseline and after one year of follow-up when FFQ information of red wine consumption was used to perform the regression analysis with circulating inflammatory molecules. **Conclusions:** Light to moderate red wine consumption (10 to 20 grams of alcohol per day), which can be monitored by microbiota-derived resveratrol metabolites excreted in urine, is associated with lower plasma concentrations of sVCAM-1, an inflammatory biomarker related to atherosclerosis. Biomarkers of consumption offer advantages compared to FFQ data, since they provide objective and more accurate information about nutrient intake and metabolism. Without specific biomarkers of red wine consumption, no significant associations would have been found in the present study.

***Keywords*:** Anti-inflammatory; Antiaging; Biomarker; Healthy diet; Dihydroresveratrol; Bioactives; Health

***Abbreviations*:** BMI, body mass index; CVD, cardiovascular disease; CRP, C-reactive protein; DHRg, dihydroresveratrol glucuronide; DHRs, dihydroresveratrol sulfate; EVOO, extra virgin olive oil; FFQ, food frequency questionnaire; IL-1A; interleukin-1 alpha; IL-6, interleukin-6; IQR, interquartile range; MedDiet, mediterranean diet; METs, metabolic equivalent tasks; MCP-1, monocyte chemoattractant protein-1; NSAID, non-steroidal anti-inflammatory drug; PREDIMED, PREvención con Dieta MEDiterránea; ROC, receiver operating characteristic; ROS, reactive oxygen species; SD, standard deviation; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor alpha; TRMs, total resveratrol metabolites.

1. Introduction

Resveratrol, a (poly)phenol with manyfold health benefits (1), is found in some plant-based foods, such as grapes, berries, cocoa or nuts. Red wine is usually the main source of resveratrol in wine drinkers, which may also synergize with other phenolic compounds found in wine to produce their reported health benefits (2, 3).

Food intake was traditionally evaluated through food frequency questionnaires (FFQs), which suffer from methodological limitations and systematic errors. These include the under- or overestimation of the amount and frequency of food consumption, especially alcohol intake (4). Furthermore, FFQs do not consider interactions within the food matrix and with the gut microbiota (4, 5).

In this context, biomarkers of consumption can serve as a valuable tool to enhance the accuracy of FFQs. Tartaric acid, total resveratrol metabolites (TRMs), and gallic acid metabolites have been postulated as biomarkers of wine consumption (6–8). A recently published paper suggested anthocyanins and resveratrol derivates as the most selective biomarkers for regular wine consumption (9). Resveratrol is 10-fold higher in red wine. When wine is consumed, resveratrol is hydrogenated to dihydroresveratrol by gut microbiota prior to absorption (10). Dihydroresveratrol is metabolized mainly in the liver to dihydroresveratrol sulfate (DHRs) and dihydroresveratrol glucuronide (DHRg) (11). These microbiota-derived resveratrol metabolites can be quantified in urine and to date have not been assessed as specific biomarkers of red wine consumption in human intervention studies.

Light to moderate wine consumption is defined as up to one drink per day for women (1 drink ≈ 10 grams of alcohol) and two drinks per day for men (12). Although several studies suggest that moderate wine consumption, especially red wine, may reduce proinflammatory biomarkers (13, 14), some others have not found these benefits (15, 16), or even reported acute postprandial proinflammatory responses (17).

The aim of the present study was to corroborate the usefulness of microbiota-derived resveratrol metabolites excreted in urine as specific biomarkers of red wine consumption. Additionally, we evaluated the advantages of using these biomarkers over FFQ data to investigate associations between wine consumption and plasma circulating inflammatory molecules.

2. Methods

2.1 Study Design

This study included samples from 179 participants from the IDIBAPS and Navarra recruitment centers as part of the PREDIMED trial (www.predimed.es), from which additional data on inflammation biomarkers and urine metabolites were available, serving as one of the inclusion criteria for the present study. PREDIMED trial was a large parallel-group, multicenter, randomized, controlled intervention trial that involved 7,447 participants at high cardiovascular risk. Study design, inclusion and exclusion criteria and methodology were previously described (18, 19). After one year of intervention, FFQ data

from 6 participants were excluded as their energy intake was above predetermined limits, and 5 participants dropped out during the intervention.

2.2. Assessment of covariates

Food consumption was assessed using a semi-quantitative validated FFQ (20). Mediterranean diet (MedDiet) adherence was evaluated using a validated 14-item screener (21). Physical activity was assessed using a validated Spanish iteration of the Minnesota physical activity questionnaire, measuring metabolic equivalent tasks minutes per day (METs min/day) (22).

2.3. Inflammatory biomarkers

Plasma concentrations of soluble adhesion molecules were determined using Bio-Plex Pro Human Cytokine 17-plex Assay on the Luminex platform and analyzed with Bio-Plex ManagerTM Software. Five potential inflammatory biomarkers were determined: soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1).

2.4. Urinary dihydroresveratrol metabolites

DHRg and DHRs were determined in the first spot urine. 20 µL of urine were diluted 1:5 (v:v) with Milli-Q Water and formic acid at 0.05 % prior to analysis through LC– ESI–MS/MS with abscisic acid D6 as internal standard. Calibration curves of the aglycon form in synthetic urine were used for the quantification of the two dihydroresveratrol metabolites.

Chromatographic separation was performed on a Kinetex F5 100Å (50 x 4.6 mm x 2.6 µm) reverse column from Phenomenex® coupled to a SecurtiyGuard™ (UHPLC F5 4.6mm). High-resolution mass spectrometry was conducted on an Agilent 6560 Ion Mobility QTOF LC/MS working in negative mode, at the Separation Techniques Unit of the Scientific and Technological Centers (CCiTUB). The mobile phases were (A) 0.05% acetic acid in water and (B) 0.05% acetic acid in acetonitrile. The column temperature was maintained at 40ºC throughout the analysis. A 5 µL sample was injected into the chromatographic column, with a constant flow rate of 0.750 mL/min, using the following non-linear gradient: 1.7 min, 2% B; 4.7 min, 8%B; 6 min, 20%B; 7.3 min, 30%B; 7.4 min, 50%B; 8.7 min, 100%B; 10 min, 2%B. Agilent Mass Hunter Software was used to identify and quantify DHRg and DHRs.

2.5. Statistical analyses

Student's t-test was used to assess differences in quantitative variables and chi-square tests for categorical. Baseline values of inflammatory biomarkers in plasma (ng/mL) and dihydroresveratrol metabolites in urine (nmol/mL) were log-normalized and scaled to a mean of 0 and standard deviation (SD) of $\pm$1. Changes in these variables were calculated and were also normalized and scaled. Receiver operating characteristic (ROC) curve analysis was conducted using logistic regression, with wine (consumers/non-consumers) as the dependent variable and urinary dihydroresveratrol metabolites concentrations as the exposure variable. Multivariable-adjusted linear regression models were used to assess associations between baseline and one-year changes in urinary dihydroresveratrol

metabolites concentrations and inflammatory biomarkers per 1-SD increment. Adjustment models of increasing complexity were applied. Model 1 was adjusted for age (continuous, years) and sex (categorical, men/women); Model 2 was further adjusted for recruitment center (categorical, Barcelona/Navarra), body mass index (BMI) (continuous, kg/m^2), physical activity (continuous, METs-min/d), smoking habit (categorical, yes/no), diabetes (categorical, yes/no), hypercholesterolemia (categorical, yes/no) and hypertension (categorical, yes/no); and model 3 was additionally adjusted for total energy intake (continuous, kcal/d), intake of omega-3 fatty acids (continuous, g/d), statins intake (categorical, yes/no), aspirin consumption intake (categorical, yes/no), non-steroidal anti-inflammatory drugs (NSAIDs) intake (categorical, yes/no) and consumption of grapes and raisins (continuous, g/d). One-year changes were further adjusted for baseline levels of resveratrol metabolites (continuous, nmol/mL) and inflammatory molecules (continuous, ng/mL or pg/mL), and the intervention group (categorical, EVOO/nuts/low-fat).

All analyses were conducted with robust variance estimates to correct for intra-cluster correlations and two-sided significance was determined at $p < 0.05$. Analyses were performed using Stata 16.0.

3. Results

3.1. General characteristics of the participants

General characteristics of the participants are summarized in Table 1 and detailed in *Supplementary Table S1, S2, S3 and S4.* Plasma levels of circulating inflammatory molecules at baseline and after one year of follow-up are reported according to red wine consumption in *Supplementary Table S5.* As shown in Table 1, the mean age of participants was 68.9 years and 49.2% were female. As expected, based on the inclusion criteria of the PREDIMED trial, the mean BMI indicated overweight and the prevalence of cardiovascular risk factors was 49.7% for diabetes, 64.8% for dyslipidemia, and 81.6% for hypertension. Most participants were either non-smokers (85.5%) or had a low educational level (75.4%).

Table 1
Baseline characteristics of the participants (n = 179).

Age, years	68.9 ± 5.9
Sex, female	88 (49.2)
BMI, kg/m^2	29.5 ± 3.6
Overweight (BMI≥25kg/m^2), n (%)	162 (90.5)
Physical activity, METs-min/d	267.2 ± 214.0
Current smoker, n (%)	26 (14.5)
Medication, n (%)	
NSAIDS	12 (6.7)
Aspirin	42 (23.5)
Other antiplatelet drugs	37 (20.7)
Diuretics	38 (21.2)
Insulin	14 (7.8)
Antidepressants	42 (23.5)
ACE inhibitors	50 (27.9)
Oral hypoglycemic drugs	58 (32.4)
Vitamins or supplements	20 (11.2)
Educational level, n (%)	
Low	135 (75.4)
High & medium	44 (24.6)
Diabetes mellitus, n (%)	89 (49.7)

Dyslipidemia, n (%)	116 (64.8)
Hypertension, n (%)	146 (81.6)
Family history premature CHD, n (%)	43 (24.0)
Total energy intake, kcal/d	2353.8 ± 634.1
Intervention group, n (%)	
MedDiet supplemented with EVOO	60 (33.5)
MedDiet supplemented with nuts	63 (35.2)
Low-fat diet control group	56 (31.3)
Wine consumption (mL/d)	85.8 ± 135.4
Red wine consumption (mL/d)	62.1 ± 110.0
Grape & raisin consumption (g/d)	11.2 ± 20.0
Dihydroresveratrol glucuronide (nmol/mL)	1.1 ± 2.9
Dihydroresveratrol sulfate (nmol/mL)	1.6 ± 5.8
sVCAM-1 (ng/mL)	188.1 ± 51.9
sICAM-1 (ng/mL)	180.0 ± 72.6
IL-6 (pg/mL)	5.0 ± 3.2
TNF-α (pg/mL)	12.7 ± 20.2
MCP-1 (pg/mL)	39.3 ± 22.5

Data are given as mean ± standard deviation (SD) for continuous variables, and *n* (percentage) for categorical variables.

BMI, body mass index; d, day; EVOO, extra virgin olive oil; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; MedDiet, Mediterranean diet; METs, metabolic equivalent of tasks; NSAIDS, non-steroidal anti-inflammatory drugs; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor-α.

$p < 0.05$ were considered significant.

Student's t-test were used for continuous variables and a chi-square test was used for categorical variables.

3.2. Dihydroresveratrol metabolites as biomarkers of red wine consumption

ROC curves demonstrated that baseline urinary levels of DHRg and DHRs could predict red wine consumption, with an AUC = 0.7207 (95% CI: 0.644; 0.797) and AUC = 0.6489 (95% CI: 0.565; 0.733), respectively (*Figure 1A* and *Figure 1B*). After adjusting for potential confounders, the predictive value of DHRg and DHRs for red wine intake increased, with new values of AUC = 0.835 (95% CI: 0.775; 0.900) and AUC = 0.803 (95% CI: 0.736; 0.870), respectively (*Figure 1C and Figure 1D*).

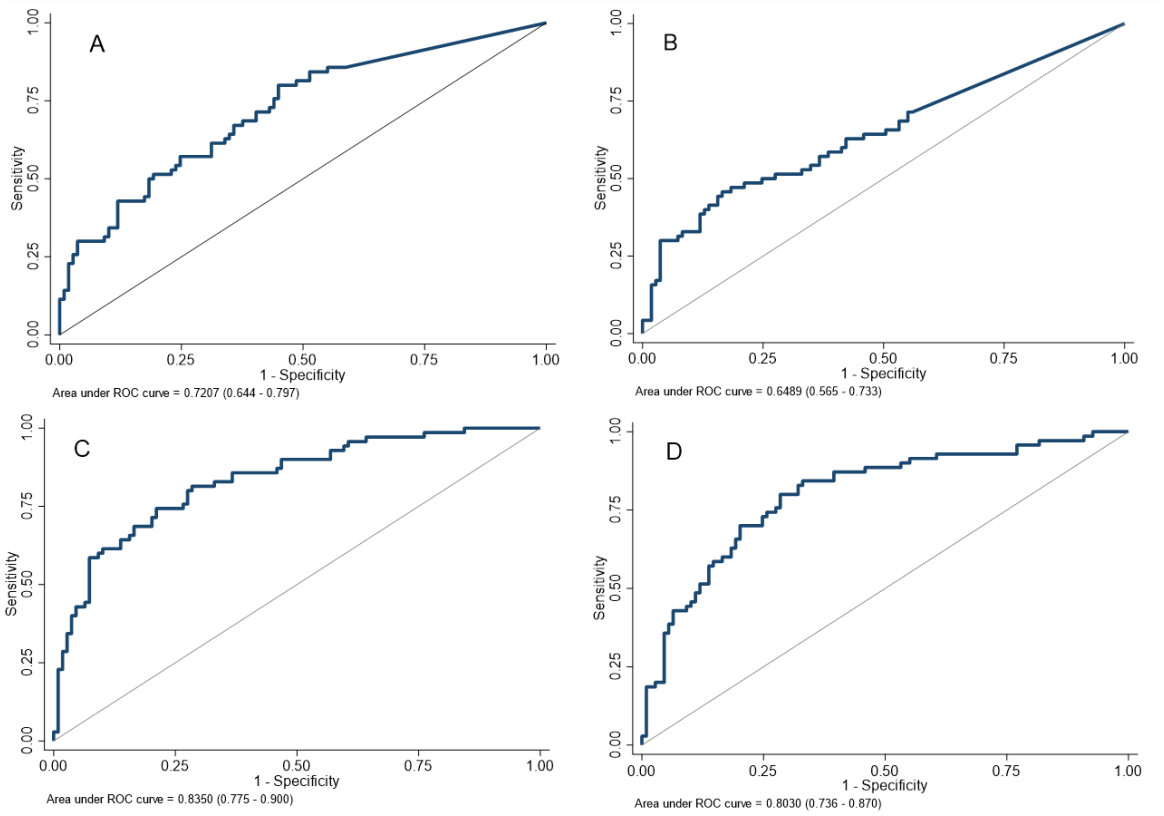

Figure 1. Receiver operating characteristic (ROC) curves for predicting baseline red wine consumption (yes/no) using baseline urinary dihydroresveratrol glucuronide (**A**) and sulfate (**B**). Adjustments for potential confounders were made to correct baseline urinary values of dihydroresveratrol glucuronide (**C**) and sulfate (**D**). Variables used for adjustments were age, sex, physical activity, smoking habit, BMI, diabetes, hypercholesterolemia, hypertension, energy intake, and consumption of grapes and raisins. The analyses were conducted with robust variance estimates to correct for intra-cluster correlation.

Multivariable-adjusted regression model between baseline values of red wine consumption and baseline urinary dihydroresveratrol metabolites revealed that higher urinary concentrations of DHRg and DHRs were present in the participants who consumed more red wine (βo = 0.707; β1 = 0.375; R-squared = 0.316; *p*-value = 0.002 and βo = 1.352; β1 = 0.312; R-squared = 0.268; *p*-value = 0.018, respectively). In contrast, when white wine consumption was modeled instead of red wine, no significant associations were observed for either DHRg or DHRs (βo = -2.290; β1 = 0.101; R-squared = 0.089; *p*-value = 0.239 and βo = -2.120; β1 = 0.079; R-squared = 0.091; *p*-value = 0.325, respectively).

3.3. Urinary resveratrol metabolites and circulating inflammatory molecules.

As illustrated in *Figure 2A*, after adjustment for potential confounders, participants with higher concentrations of DHRs at baseline showed significantly lower concentrations of circulating sVCAM-1 (–0.40 (95% CI: –0.66; –0.15) ng/mL per 1-SD increase, *p*-value = 0.012). No other significant associations were found with other circulating inflammatory biomarkers (*Supplementary Table S6*).

As shown in *Figure 2B*, after adjustment for potential confounders, participants with higher increments in urinary DHRg concentrations after one year of follow-up, presented significantly greater decreases in circulating sVCAM-1 concentrations (–0.43 (95% CI:

–0.73; –0.13) ng/mL per 1-SD increase, *p*-value = 0.016). No significant associations were found with other circulating inflammatory biomarkers (*Supplementary Table S7*).

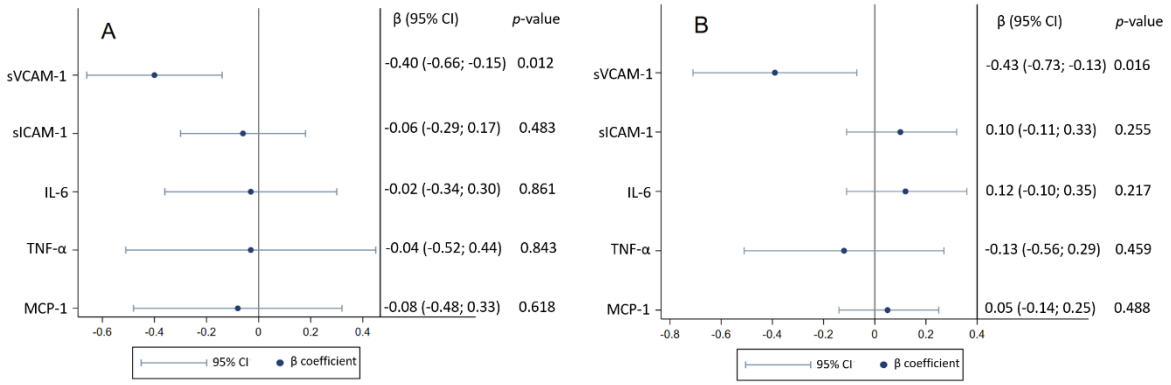

Figure 2. Multivariable-adjusted regression between baseline dihydroresveratrol sulfate in urine and inflammatory molecules (ng/mL per 1-SD increment) (**A**) and multivariable-adjusted regression between one-year changes in dihydroresveratrol glucuronide in urine and inflammatory molecules (ng/mL per 1-SD increment) (**B**). Regressions were adjusted for age, sex, recruitment center, intervention group (only **B**), physical activity, smoking habit, BMI, diabetes, hypercholesterolemia, hypertension, intake of energy, intake of omega-3 fatty acids, statins, aspirin, NSAIDs and consumption of grapes and raisins. Analyses were conducted with robust estimates of the variance to correct for intra-cluster correlation. sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; IL6, interleukin-6; TNF-α, tumor necrosis factor- a; MCP-1, monocyte chemoattractant protein-1.

3.4. Red wine consumption reported in FFQ and circulating inflammatory molecules.

Multivariable-adjusted regression was performed using red wine consumption reported in FFQ as continuous variable (mL/d) and circulating inflammatory molecules observing no significant associations between these two variables either at the baseline (*Figure 3A and Supplementary Table S8)* or after one year of follow-up (*Figure 3B and Supplementary Table S9)*. An inverse but not significant tendency was observed in case of sVCAM-1 at the baseline (–0.10 (95% CI: –0.61; 0.41) ng/mL per 1-SD increase, *p*-value = 0.623) and after one year of follow-up (–0.19 (95% CI: –0.50; 0.12) ng/mL per 1-SD increase, *p*-value = 0.162).

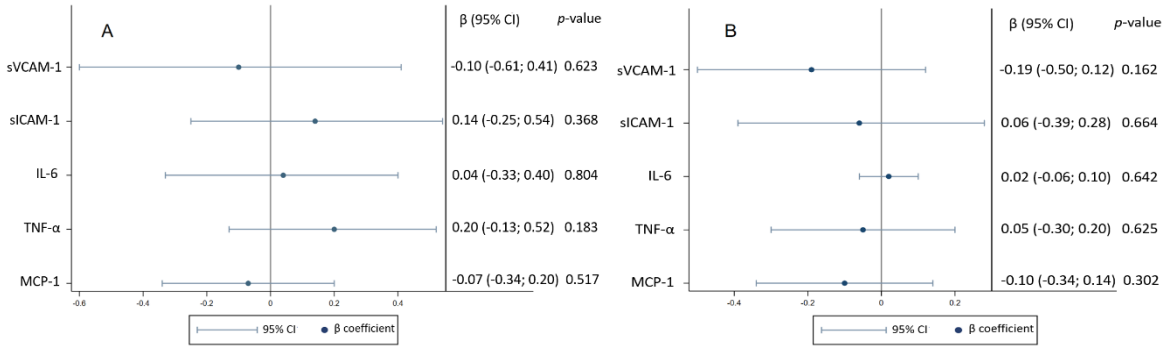

Figure 3. Multivariable-adjusted regression between baseline red wine consumption reported in FFQ as continuous variable (mL/d) and inflammatory molecules (ng/mL per 1-SD increment) (**A**) and multivariable-adjusted regression between one-year changes in red wine consumption reported in FFQ and inflammatory molecules (ng/mL per 1-SD increment) (**B**). Regressions were

adjusted for age, sex, recruitment center, intervention group (only **B**), physical activity, smoking habit, BMI, diabetes, hypercholesterolemia, hypertension, intake of energy, intake of omega-3 fatty acids, statins, aspirin, NSAIDs and consumption of grapes and raisins. Analyses were conducted with robust estimates of the variance to correct for intra-cluster correlation. sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; IL6, interleukin-6; TNF-α, tumor necrosis factor- a; MCP-1, monocyte chemoattractant protein-1.

4. Discussion

In this study of older adults at high cardiovascular risk we observed an inverse association between urinary microbiota-derived resveratrol metabolites and circulating sVCAM-1 levels. Moreover, we found a positive and significant association of red wine consumption and urinary microbiota-derived resveratrol metabolites.

In contrast with microbiota-derived metabolites, no significant associations were detected when FFQ-reported values of red wine consumption were used to perform the regression analysis with circulating inflammatory molecules. This outcome highlights the importance of using specific biomarkers of consumption instead of FFQ data, since food biomarkers provide objective and accurate information about nutrient intake, their interaction with microbiota and other food matrixes, absorption, and metabolism. Indeed, without DHRs and DHRg as specific biomarkers of red wine consumption, no significant associations would have been found in the present study. Interestingly, regression models between FFQ data and circulating sVCAM-1 showed an inverse association, particularly after one year of follow-up, although it remains non-significant. This supports the hypothesis that red wine consumption reported in FFQ was underestimated and can be corrected by using accurate and specific biomarkers as reported here.

Our results align with the anti-inflammatory outcomes of wine consumption reported in previous studies (13, 23). The specific correlation between red wine consumption and sVCAM-1 may be explained by the ability of red wine (poly)phenols to suppress VCAM-1 gene transcription, as demonstrated in molecular studies (24). Moreover, Sacanella et al. (25), who also observed the same effect in red wine, highlighted that while ICAM-1 is constitutively expressed by endothelial cells, VCAM-1 is only expressed on activated endothelial cells, providing an additional factor that could explain the observed effect.

It should be emphasized that positive health effects attributed to wine consumption have only been observed with light to moderate consumption during meals (26), in accordance with the MedDiet pattern, where main meals are complemented with one or two glasses of wine (19).

The novelty of the present study lies in the association between red wine consumption and urinary microbiota-derived resveratrol metabolites, with no significant associations with white wine. This high degree of specificity allows, for the first time, differentiation between red wine and other types of wines, which constitutes the main strength of the present study. In addition, longitudinal design and the use of biomarkers for both inflammation and specific biomarkers of red wine consumption further strengthen our work.

On the other hand, the small sample size of an older population at high risk of CVD represents a highly specific sample group and constitutes the main limitation of our study. Nevertheless, the sample size was sufficient to detect significant associations between microbiota-derived resveratrol metabolites in urine and plasma sVCAM-1 levels. Moreover, although the participants' data encompassed the most relevant variables that may impact on inflammation, kidney disease information was not available. In addition, we used spot urine samples instead of 24-hour urine, the latter being considered the gold standard for estimating nutrient intakes. Finally, we only examined inflammatory biomarkers associated with atherosclerotic plaque development, without assessing other pro-inflammatory molecules.

5. Conclusion

Our findings demonstrate that urinary DHRg and DHRs are reliable and specific biomarkers of red wine consumption, which present advantages compared to FFQ data. Levels of both resveratrol metabolites were inversely associated with circulating sVCAM-1, a recognized biomarker of atherosclerosis progression.

Conflicts of interest

R.M.L.R. reports fees from Cerveceros de España, UNIDECO, Adventia, Ecoveritas S.A, from the Fundación Dieta Mediterránea. R.E. reports fees from the Fundación Dieta Mediterránea, and Cerveza y Salud, Brewers of Europe, Fundación Cerveza y Salud, Instituto Cervantes, Lilly Laboratories, and Wine and Culinary International Forum. E.R. reports fees from California Walnut Commission, Alexion, Sociedad Española de Arteriosclerosis and Fundación Dieta Mediterránea.

Ethical standards

The study was conducted according to the guidelines of the Declaration of Helsinki. The Institutional Review Board (IRB) of the Hospital Clinic approved the study protocol. Controlled Trials number, ISRCTN35739639.

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Declaration of generative AI and AI-assisted technologies in the writing process

AI or AI-assisted technologies were not used in the writing process of this article.

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