Diagnostic Performance of Urinary Resveratrol Metabolites as a Biomarker of Moderate Wine Consumption

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Background: Nutritional biomarkers may be better measures of dietary exposure than self-reported dietary data. We evaluated resveratrol metabolites, potential biomarkers of wine consumption, in humans after moderate consumption of sparkling, white, or red wines.

Methods: We performed 2 randomized, crossover trials and a cohort study. In the first study, 10 healthy men consumed 30 g of ethanol/day as sparkling wine or gin for 28 days. In the second trial, 10 healthy women consumed 20 g of ethanol/day as white or red wine for 28 days. We also evaluated 52 participants in a study on the effects of a Mediterranean diet on primary prevention of cardiovascular disease (the PREDIMED Study). We used liquid chromatography–tandem mass spectrometry to analyze urinary total resveratrol metabolites (TRMs) and predictive values and ROC curve analyses to assess the diagnostic accuracy.

Results: We observed significant increases in TRMs [72.4 (95% confidence interval, 48.5–96.2; \(P = 0.005\)], 211.5 (166.6–256.3; \(P = 0.005\)], and 560.5 nmol/g creatinine (244.9–876.1; \(P = 0.005\)] after consumption of sparkling, white, or red wine, respectively, but no changes after the washout or gin periods. In the cohort study, the reported daily dose of wine consumption correlated directly with TRMs (\(r = 0.654; P < 0.001\)). Using a cutoff of 90 nmol/g, we were able to use TRMs to differentiate wine consumers from abstainers with a sensitivity of 72% (60%–84%); and a specificity of 94% (87%–100%).

Conclusions: Resveratrol metabolites in urine may be useful biomarkers of wine intake in epidemiologic and intervention studies.

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Epidemiologic studies have shown a negative correlation between moderate wine consumption and cardiovascular disease (1). In addition to ethanol, wine contains several minor compounds, such as polyphenols, that contribute to the differences observed between wine and distillates (2, 3). To date, no studies have been performed to determine biomarkers of wine consumption. Resveratrol (3,5,4′-trihydroxystilbene) and piceid (resveratrol-3-O-β-glucoside) are phenolic compounds present mainly in grapes and wine (4), and these compounds may have a role in the prevention of cancer, cardiovascular disease (1), and neurodegenerative diseases (5). In addition, they may be useful as biomarkers of wine consumption.

Biomarkers for epidemiologic and clinical assays have 3 distinct advantages over dietary data obtained by food frequency questionnaires (FFQs) (6, 7). One advantage is that biochemical markers of the intake of some nutrients are more precise than dietary assessment. Another advantage is that dietary data obtained by FFQ are often inadequate because of insufficient reporting of food com-

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* Nonstandard abbreviations: FFQ, food frequency questionnaire; LC-MS/MS, liquid chromatography–mass spectrometry; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; and TRMs, total resveratrol metabolites.
position. The third advantage is that biomarker analysis provides a more proximal measure of specific nutrient intake than do FFQ data because it is an integrated measure of the bioavailability and metabolism of the component.

Recent advances in analytical techniques have improved the effectiveness and expanded the possibilities of biomarker analyses. Tandem mass spectrometry increases the sensitivity and selectivity of measurement of the metabolites of some nutrients (8,9). Resveratrol metabolites could be the best nutritional biomarkers for wine consumption because trans-resveratrol-3-O-glucuronide has been reported to be the main resveratrol metabolite in human blood (10), urine (11), LDL (12), and target organs (13). Other phenolic metabolites previously used as biomarkers of food consumption include 4'-O-methylgallic acid (the main gallic acid metabolite) for tea (14), isofur-elic acid for coffee (14), and isoflavonoids for soy (15).

The aim of this study was to determine the concentrations of resveratrol metabolites in blood and urine in 2 different studies after 4 weeks of wine consumption and to evaluate their usefulness as potential biomarkers of wine intake in intervention studies. In addition, we analyzed baseline data from a cohort included in a large intervention study to assess the diagnostic performance of this biomarker in real-life conditions.

Material and Methods

STUDY PARTICIPANTS
Clinical trials. The 2 intervention studies were open, prospective, randomized, crossover, single-blinded clinical trials. The sparkling wine study (January to June 2005) included 10 healthy men [mean (SD) age, 28.2 (7.3) years; body mass index, 25.2 (1.3) kg/m²], and the wine study (September to December 2004) included 10 healthy women [mean (SD) age, 38.1 (9.2) years; body mass index, 24.1 (4.0) kg/m²]. All participants in both studies were healthy, and none reported any prior relevant disease.

COHORT STUDY
The PREDIMED (PREvencio con Dleta MEDiterránea) Study is a large, parallel group, multicenter, controlled, randomized 4-year clinical trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease (http://www.predimed.org). In the present study, we analyzed the baseline data of 52 consecutively admitted trial participants (30 men and 22 women admitted April to July 2005). Exclusion and inclusion criteria have been described previously by Estruch et al. (16). Twenty-nine participants (55.8%) reported a mean (SD) daily intake of 118.3 (112.3) mL of wine. Seven (13.5%) reported intermittent drinking, mostly during weekends, consuming a mean of 98.0 (28.7) mL of wine per week, and 16 participants (30.7%) did not drink. All but 2 (93%) of the daily drinkers reported to preferentially consume red wine, although 24% also reported drinking lower amounts of white wine and sparkling wine. The Institutional Review Board of the Hospital Clinic of Barcelona approved the 3 study protocols, and written informed consent was obtained from each participant.

STUDY DESIGN

Clinical trials. Both studies were carried out over a 16-week period. During the first 4 weeks, the participants did not drink any alcoholic beverages (first washout period). During the next 4 weeks, they underwent the first intervention, after which they underwent a second 4-week washout period. During the final 4 weeks, the participants underwent the second intervention.

In the sparkling wine study, the interventions consisted of the intake of 30 g of ethanol/day as sparkling wine (300 mL/day) or as gin (100 mL/day) in a random order during dinner. In the wine study, the volunteers consumed 20 g of ethanol/day as red wine (200 mL/day) or white wine (200 mL/day), also in a random order during dinner.

In both studies, diet was monitored before and after each intervention period by use of a 3-day food-and-drink recall questionnaire, which had been validated previously in our country (17). We converted the reported consumption into nutritional data with the Professional Diet Balance software (Cardinal Health Systems, Inc.). The clinical investigators and laboratory technicians did not know the sequence of the intervention.

Reports from the participants and the number of empty bottles returned showed adherence. We did not observe significant differences between nutrient intake, anthropometric variables, and energy expended in physical activity before and after the evaluated interventions.

Urine and serum samples were collected the morning after the interventions and washout periods after overnight fasting and were coded with random numbers and stored at −80 °C until analyses, which were performed with no knowledge of the clinical data.

Cohort study. At baseline, participants completed a 137-item validated FFQ (18) and the validated Spanish version (19) of the Minnesota Leisure Time Physical questionnaire. Data collected included information on drinking habits, such as amount, frequency, and type of alcohol intake. We took samples of fasting blood and morning urine from all participants. Energy and nutrient intakes were calculated from Spanish food composition tables (20). Urine samples were coded and stored at −80 °C until analyses. The clinical investigators and laboratory technicians were blinded to clinical data.

Reported daily consumption of the key food items and nutrients, as well as estimated energy expenditure from physical activity, were similar in the participants who drank wine daily, those who drank intermittently, and those who did not drink any kind of wine.
MEASUREMENT OF TOTAL RESVERATROL IN BEVERAGES BY HPLC WITH A DIODE ARRAY DETECTOR

We concentrated 5 mL of sparkling wine, white wine, or gin, under reduced pressure and protected against exposure to ultraviolet light, to a final volume of 2 mL. Wines were injected directly into the HPLC according to the previously described method (21). Results are reported as milligrams of total resveratrol consumed per day.

QUANTIFICATION OF RESVERATROL METABOLITES FROM HUMAN SAMPLES

We used liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described elsewhere (12) to analyze resveratrol metabolites extracted from urine and serum samples by solid-phase extraction. Briefly, urine samples (5 mL) were loaded on Oasis HLB cartridges (60 mg; Waters) that had been equilibrated. The cartridges were washed, and resveratrol metabolites were eluted with acidified methanol solution and ethyl acetate. The organic extract was evaporated under N2. The samples were redissolved with 100 μL of the mobile phase used for the LC initial conditions with taxifolin as internal standard and then analyzed in the LC-MS/MS system.

We identified and quantified resveratrol metabolites in urine and serum with an LC system (Perkin-Elmer s200) coupled to a triple-quadrupole mass spectrometer (API 3000; Perkin-Elmer Sciex) as described elsewhere (12). The intra- and interassay CVs for trans-resveratrol were 2.4% and 4.8%, respectively, and the analyses were performed in duplicate. All results for urinary resveratrol metabolites were corrected for urinary creatinine and are reported as nanomoles per gram of creatinine in the morning urine (11, 12). Urinary creatinine was assayed with the standard Jaffe (alkaline picrate) kinetic method (22). Serum (500 μL) was treated with 20 μL of orthophosphoric acid, vortex-mixed for 1 min, and processed by the same procedure.

STATISTICAL ANALYSIS

We used the standard statistical methods of the SPSS Statistical Analysis System, Ver. 11.5 (SPSS). Descriptive statistics with the mean (SD) were used for the baseline characteristics of the participants. Because the data were skewed (Kolmogorov and Levene tests), we used the Wilcoxon test for related samples to compare changes in outcome variables in response to each intervention period in both clinical trials. To exclude the presence of a carryover effect, we compared the observed outcome variables before both intervention periods. To compare groups in the cohort study, we used the 2-tailed t-test and ANOVA when indicated. We used Pearson correlations to examine associations between wine consumption and urinary excretion of resveratrol metabolites. To assess the accuracy of urinary resveratrol metabolite measurement for differentiating between wine consumers and nonconsumers, we calculated the sensitivity, specificity, positive (PPV) and negative predictive values (NPV), the likelihood ratio, and the ROC curve for the 2 randomized, crossover trials and the cohort study. With ROC curve analysis, we calculated a cutoff point that provided optimized sensitivity and specificity for the identification of wine consumers. Within- and between-group differences are expressed as means and 95% confidence intervals (CIs). All statistical tests were 2-tailed, and the significance level was 0.05.

RESULTS

RESVERATROL CONCENTRATIONS IN BEVERAGES

The amount of total resveratrol consumed per day in the clinical trials was 0.357, 0.398, and 2.56 mg for sparkling, white, and red wine, respectively. The content of resveratrol in gin was below the detection limits.

CLINICAL TRIALS

Sparkling wine study. After 28 days of dietary supplementation with 300 mL/day of sparkling wine, cis- and trans-resveratrol-3-O-glucuronides were found in the urine of all participants, whereas only very low concentrations of these metabolites were detected in the urine after the washout periods and the gin period. The mean concentrations of resveratrol metabolites in urine before and after each intervention are shown in Fig. 1. The amount of total resveratrol metabolites (TRMs) identified in this study increased by 72.4 nmol/g (95% CI, 48.5–96.2 nmol/g; P = 0.005) after sparkling wine consumption, whereas the concentration of these metabolites did not vary significantly after the gin period (Fig. 1). The order of interventions did not affect the results. No positive results were obtained when urine was checked for resveratrol aglycone, piceid, and sulfoconjugates. Serum concentrations of resveratrol and its metabolites were below the limits of detection in all participants evaluated.

White and red wine study. After 28 days of dietary supplementation with white or red wine (200 mL/day), trans- and cis-resveratrol-3-O-glucuronide were found in the urine of all participants, whereas only very low concentrations of these metabolites were detected in urine after the washout periods (Fig. 2). According to the TRM results, both metabolites increased by 211.5 nmol/g (95% CI, 166.6–256.3 nmol/g; P = 0.005) after white wine consumption and by 560.5 nmol/g (244.9–876.1 nmol/g; P = 0.005) after red wine intake. The differences between the changes observed after white and red wine intake significantly favored red wine [349.6 nmol/g (86.8–612.3 nmol/g); P = 0.005]. For all participants, no free resveratrol, piceid, or sulfoconjugates were detected in the urine, and resveratrol and its metabolites were below the limits of detection in serum.

BOTH CLINICAL TRIALS

We used ROC curves to assess the effectiveness of urinary resveratrol metabolite measurement as a biomarker for wine intake. The optimal cutoff point was 90 nmol/g,
which allowed differentiation of the washout and gin periods from the wine periods (Fig. 3): area under the curve = 0.985 (95% CI, 0.928–0.999); sensitivity = 93% (88%–99%); specificity = 98% (94%–100%); likelihood ratio = 46.7 (35.8–57.6); PPV = 95.6% (91.1%–100%); NPV = 85.3% (77.5%–93.1%).

COHORT STUDY
Participants who reported wine consumption had significantly higher urinary concentrations of trans- and cis-resveratrol-3-O-glucuronide than those who did not consume wine (Fig. 4). The mean (SD) urinary TRM concentration was 282.7 (305.2) nmol/g for participants who reported moderate daily wine consumption, a value that differed significantly from that measured in those who did not consume wine (P = 0.001) and those who consumed wine intermittently (171.4 nmol/g (44.5–298.2 nmol/g); P = 0.01; Fig. 3). Mean (SD) urinary TRM concentrations were 111.3 (69.1) nmol/g for participants who reported intermittent wine consumption, a value that differed from the concentration observed in abstainers [70.8 nmol/g (6.4–135.2 nmol/g); P = 0.035]. The reported daily wine consumption correlated directly with urinary concentrations of resveratrol glucuronides (r = 0.654; P < 0.001).

The cutoff of 90 nmol/g enabled differentiation of moderate wine drinkers from those who did not drink wine with an area under the ROC curve (Fig. 5) of 0.863 (95% CI, 0.739–0.942), a sensitivity of 72% (60%–84%), a specificity of 94% (87%–100%), a PPV of 96.4% (91.3%–100%), an NPV of 59.1% (45.7%–72.5%), and a likelihood ratio of 11.6 (2.9–20.3). The percentage of false negatives was higher in those who consumed wine intermittently than in those who consumed it daily (43% and 24%, respectively); consequently, the sensitivity was higher in those who consumed moderate amounts of wine daily (76%) than in those who consumed wine intermittently (57%).

In all participants, no free resveratrol, piceid, or sulfooconjugates were detected in the urine, and resveratrol and its metabolites were below the limit of detection in the serum.
Discussion

An ideal biomarker should be specific, have an adequate half-life, and provide good correlation between the measured value and exposure (7). The results of the current study indicate that resveratrol metabolites fulfill the criteria to be considered as a biomarker of wine intake.

The 2 intervention clinical trials, which included men and women, allowed assessment of a urinary concentration of resveratrol metabolites of 90 nmol/g as a cutoff to differentiate wine drinkers from non–wine drinkers: this cutoff had a sensitivity and specificity >90% and a PPV >95%. The usefulness of this biomarker was then tested in a cohort of 52 consecutively admitted participants in a large-scale feeding clinical trial, the PREDIMED Study. In real-life conditions, this biomarker had a sensitivity of 73%, a specificity of 93%, and a PPV of 96%. However, the NPV was 60% because of a high percentage of false negatives among intermittent drinkers. Thus, urinary concentrations of resveratrol metabolites are particularly useful as biomarkers of wine intake for moderate and regular drinkers, just as other phenolic compounds have been shown to be useful biomarkers for the intake of fruits, vegetables, tea, or coffee (14, 23). We selected resveratrol as a marker of wine intake because it is a characteristic polyphenol of grape and wine products. Although a few other foods contain resveratrol (24–27), the quantities in those foods are much lower than those observed in grape and wine products (4, 21, 28).

Resveratrol metabolites were detected in morning urine after moderate and regular wine intake. Resveratrol metabolism has been investigated extensively in preclinical studies using animals (11, 29, 30), but few studies have been performed in humans (10, 12, 30–33). In the current trials, resveratrol intake ranged from 0.0040 mg/kg (0.35 mg of total resveratrol) for those who drank sparkling wine to 0.041 mg/kg (2.56 mg of total resveratrol) for those who consumed red wine; these values are

![ROC Curve](image1)

Fig. 3. ROC curve of urinary TRM concentrations for wine consumption periods vs washout and gin consumption periods in the clinical studies.

![ROC Curve](image2)

Fig. 5. ROC curve of urinary TRM for discrimination of wine consumers from non–wine consumers in the PREDIMED Study.

![Graph](image3)

Fig. 4. Concentrations of urinary resveratrol glucuronides among non–wine consumers, intermittent consumers, and moderate daily consumers. Results are expressed as nmol of trans-resveratrol/g creatinine. Values are the means (SD; error bars) from 52 participants. □, trans-resveratrol-3-glucuronide; □, cis-resveratrol-3-glucuronide; □, sum of the resveratrol glucuronides. Significant differences: *, P < 0.05; **, P < 0.01; ***, P < 0.001 for difference from results obtained for samples from non–wine consumers (unpaired t-test). #, P < 0.01 for difference from values for intermittent wine consumers (unpaired t-test).
similar those reported in the literature (10, 30). These amounts cover the usual range of resveratrol intake from wine products (34, 35). To achieve high amounts of resveratrol, supplements must be taken (36). In the current trials, we were able to identify resveratrol metabolites in urine ~10 h after moderate intake of sparkling wine. Meng et al. (30) did not detect any resveratrol metabolites in the urine of volunteers after a comparable single dose of resveratrol, but the authors were able to quantify resveratrol metabolites in urine samples after a higher single dose (0.014 mg/kg). Because the accumulation of a metabolite increases after several days of ingestion (6, 37), our results suggest that urine analysis may be useful for determining regular wine intake. The metabolically active compounds could enter the urine when their concentrations in plasma increase and exceed the relevant renal threshold (38). Taking into account the bioactivity of wine phenols and comparing a single dose vs regular and moderate intake, Fisher and Hollenberg (39) observed an increased vascular response indicated by endothelial nitric oxide release over time. Furthermore, inclusion of resveratrol intake in a complex meal could increase or decrease the bioavailability of polyphenols (40).

We also observed interindividual variability in our intervention studies (Figs. 1 and 2). Similar results have been described previously for resveratrol in LDL particles (12), anthocyanins (41), isoflavones (42), and olive oil phenols such as tyrosol and hydroxytyrosol (43). Despite this variability among individuals, however, we observed a highly significant correlation between wine intake and urinary concentrations of the metabolites. Higher concentrations of resveratrol metabolites were found after higher resveratrol intake (when red wine was consumed). Likewise, a lower concentration of resveratrol metabolites was found after lower resveratrol intake (sparkling or white wine). After the washout periods as well as after gin intervention, very low concentrations of resveratrol glucuronide were detected in urine, possibly from previous intake of food with very low resveratrol content or interference from compounds with a similar structure, such as estrogens. Similar results have been observed after washout periods in studies investigating quercetin (44) and other polyphenols (45), as well as for hydroxytyrosol, a metabolite of dopamine, in an study of olive oil (46). Mean (SD) TRM concentrations did not differ significantly in response to various periods of no wine intake in our studies [43.4 (23.5), 51.5 (36.1), and 40.5 (33.6) nmol/g for sparkling, white, and red wine, respectively] as part of the larger PREMID Study.

Urinary resveratrol glucuronide is the main metabolite of resveratrol in humans (~97%) (30–32) and rodents (>90%) (29, 30), except in the study by Walle et al. (40%) (33). In studies of low resveratrol intake, only the glucuronidate form was detected (30). In the current studies, 2 monoglucurononides, trans- and cis-resveratrol-3-O-glucuronide, were identified after moderate wine consumption, findings similar to those of previous studies (11, 47).

Resveratrol sulfates were not detected in morning urine after wine intake. However, further investigation is needed in this regard. The sulfate form has been reported in human urine after administration of high resveratrol doses (33). In other studies, the free form of resveratrol was not detected in human urine after moderate wine consumption but was found after high doses were consumed (31, 32).

In the present study, resveratrol metabolites were also measured in serum. In these samples, however, no resveratrol metabolites were detected an average of 10 h after the consumption of wine. In previous studies, resveratrol has been detected in plasma after a minimum resveratrol intake of 0.357 mg/kg in a single dose (31, 32). Furthermore, the half-life of resveratrol in human plasma is 2 h, with the highest concentrations being recorded at 30 min (31, 32). Thus, plasma concentrations of resveratrol are not a useful marker for regular intake because plasma concentrations increase only after very recent intake.

In summary, we identified resveratrol metabolites in human urine after moderate wine intake, suggesting that these metabolites can be used as a biomarker of moderate wine intake in regular drinkers. This biomarker can also be used to exclude moderate wine drinking in abstainers but may be less effective in intermittent drinkers. Therefore, resveratrol metabolites may be used as a measure of compliance in interventional studies as well as an objective measure of wine consumption in epidemiologic studies.

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