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Protective effect of homovanillyl alcohol on cardiovascular disease and total mortality: virgin olive oil, wine, and catechol-methylathion[1–3]

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ABSTRACT

Background: Hydroxytyrosol is a phenolic compound that is present in virgin olive oil (VOO) and wine. Hydroxytyrosol-related foods have been shown to protect against cardiovascular disease (CVD).

Objective: We investigated the associations between hydroxytyrosol and its biological metabolite, 3-*O*-methyl-hydroxytyrosol, also known as homovanillyl alcohol (HVAL), with CVD and total mortality.

Design: We included 1851 men and women with a mean ± SD age of 66.8 ± 6 y at high risk of CVD from prospective cohort data. The primary endpoint was a composite of myocardial infarction, stroke, and death from cardiovascular causes; the secondary endpoint was all-cause mortality. Twenty-four-hour urinary hydroxytyrosol and HVAL and catechol-*O*-methyltransferase (*COMT*) rs4680 genotypes were measured.

Results: After multivariable adjustment, all biomarkers were associated, as a continuous variable, with lower CVD risk, but only HVAL showed a strong inverse association (HR: 0.44; 95% CI: 0.25, 0.80) for the comparison between quintiles. Only HVAL, as a continuous variable, was associated with total mortality (HR: 0.81; 95% CI: 0.70, 0.95). Individuals in the highest quintile of HVAL compared with the lowest had 9.2 (95% CI: 3.5, 20.8) and 6.3 (95% CI: 2.3, 12.1) additional years of life or years free of CVD, respectively, after 65 y. Individuals with the rs4680GG genotype had the highest HVAL concentrations ($P = 0.05$). There was no association between *COMT* genotypes and events or interaction between *COMT* genotypes and HVAL concentrations.

Conclusions: We report, for the first time to our knowledge, an independent association between high urinary HVAL concentrations and a lower risk of CVD and total mortality in elderly individuals. VOO and wine consumption and a high metabolic COMT capacity for methylation are key factors for high HVAL concentrations. The association that stems from our results reinforces the benefits of 2 key components of the Mediterranean diet (wine and VOO). This trial was registered at www.predimed.es as ISRCTN35739639. *Am J Clin Nutr* doi: 10.3945/ajcn.116.145813.

Keywords: cardiovascular, homovanillyl alcohol, hydroxytyrosol, traditional Mediterranean diet, virgin olive oil

INTRODUCTION

Hydroxytyrosol is a polyphenol present in free (as a simple phenolic compound) and mainly conjugated forms (secoiridoids) in 2 key components of the traditional Mediterranean diet (TMD)[21]: olive oil [particularly virgin olive oil (VOO)] and wine. Both TMD and olive oil consumption have been shown to

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[3] Supplemental Material 1 and 2, Supplemental Figure 1, and Supplemental Tables 1–9 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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[21] Abbreviations used: COMT, catechol-*O*-methyltransferase; CVD, cardiovascular disease; HVAL, homovanillyl alcohol; MOHTyr, methyl hydroxytyrosol; PREDIMED, Prevención con Dieta Mediterránea; TMD, traditional Mediterranean diet; TOHTyr, total hydroxytyrosol; VOO, virgin olive oil.

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be protective against cardiovascular disease (CVD) and total mortality (1–5). Hydroxytyrosol and its related phenolic compound tyrosol represent 70–80% of the total polyphenol VOO content (6). In 2011, the European Food Safety Authority released a health claim for the benefits of the daily ingestion of olive oil rich in hydroxytyrosol for preventing LDL oxidation. The panel considers that to bear the claim, 5 mg hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) in olive oil should be consumed daily within the context of a balanced diet (7).

Epidemiologic studies support that light-to-moderate alcohol consumption (10–20 g/d) may reduce the risk of CVD and all-cause mortality (8). Among other polyphenols, hydroxytyrosol and tyrosol are also present in wine. Within the framework of the PREDIMED (Prevención con Dieta Mediterránea) study (ISRCTN35739639), we reported a direct dose-dependent association between hydroxytyrosol urinary concentrations and wine or alcohol consumption in individuals at high risk of CVD (9). We recently reported (10) that alcohol, particularly red wine, can promote an endogenous hydroxytyrosol generation at moderate concentrations. Biological concentrations of hydroxytyrosol obtained after moderate red wine consumption were higher than those that, provided by VOO ingestion, had been proven to have protective effects against risk factors for CVD in human clinical trials (11, 12).

Hydroxytyrosol is absorbed from VOO in a dose-dependent manner with respect to the polyphenol content of the olive oil (13). The main biological metabolite of hydroxytyrosol is the product of the catechol-*O*-methyltransferase (*COMT*) enzyme, 3-*O*-methyl-hydroxytyrosol, also known as homovanillyl alcohol (HVAL) (14, 15). In experimental studies, hydroxytyrosol is one of the strongest antioxidant, antiproliferative, proapoptotic, antiplatelet, and anti-inflammatory polyphenols (16). In addition, several clinical trials have shown the benefits of hydroxytyrosol-rich olive oils on risk factors for CVD (11, 12, 17, 18). Circulating biomarkers are always subject to some degree of homeostasis, absorption, distribution, or metabolism. Metabolic influences seem to be especially relevant for the formation of hydroxytyrosol from tyrosol and for the conversion of hydroxytyrosol to HVAL (10, 14). To our knowledge, no prior studies have evaluated how biological concentrations of hydroxytyrosol and HVAL relate to CVD and total mortality. We hypothesized that urinary hydroxytyrosol and HVAL could be associated with lower fatal and nonfatal CVD events and all-cause mortality, and, in the case of HVAL concentrations, genotypes of *COMT*, the enzyme that catalyzes the *O*-methylation of various compounds such as catechol estrogens and dietary polyphenols, could be involved (15).

METHODS

Design and population

PREDIMED is a parallel-group, randomized, multicenter controlled feeding trial aimed at assessing the effects of a TMD in the primary prevention of CVD. Details of the recruitment method and study design have been described elsewhere (4, 19). Eligible participants included 7447 community-dwelling men and women from Spain aged 55–80 y free from CVD at enrollment but at high risk. The participants had either type 2 diabetes mellitus or ≥3 major risk factors: smoking, hypertension, dyslipidemia, overweight or obesity, or a family history of premature CVD. Eligible participants were randomly assigned to 1 of 3 dietary intervention groups, 2 TMD groups supplemented with extra VOO or mixed nuts or to a control (low-fat) diet. Yearly study-clinic evaluations were performed by trained personnel and included a physical examination, diagnostic testing, blood sampling, and questionnaires on health status, medical history, and lifestyle. All participants provided written informed consent, and the study protocol was approved by the institutional review boards of the participating centers. In this work, we performed observational analyses of pooled study treatment arms.

Study measures

We measured hydroxytyrosol and HVAL in a random sample of 1851 of the 7447 participants with the use of stored urine samples from the initial visit, which was considered the baseline year for this analysis. The analyses herein were conducted in these participants assuming the design of an observational cohort with a median follow-up of 4.8 y and controlling for relevant confounding factors. Hydroxytyrosol and methyl hydroxytyrosol (MOHTyr) were measured with the use of gas chromatography–mass spectrometry. Limits of detection and quantification for MOHTyr and hydroxytyrosol were 1.85 and 5.60 and 1.60 and 4.80 ng/mL, respectively. Relative SDs of low, medium, and high control urine samples for 15, 30, and 60 ng MOHTyr/mL were 8.3%, 6.4%, and 8.1%, respectively, and those for 21, 42, and 98 ng hydroxytyrosol/mL were 7.1%, 4.7%, and 2.0%, respectively.

See **Supplemental Material 1** for details of cohort sampling and hydroxytyrosol and HVAL measurements. At the initial visit a 137-item validated semiquantitative food-frequency questionnaire (19) was administered to calculate energy intake and nutrients. CVD risk factors, anthropometric variables, blood pressure, and laboratory measures were evaluated with the use of standardized procedures, and alcohol use, physical activity, and adherence to the TMD were evaluated with the use of validated questionnaires (20–22). Total hydroxytyrosol (TOHTyr) was calculated as the sum of hydroxytyrosol plus HVAL.

Endpoints

The primary endpoint was a composite of myocardial infarction, stroke, and death from cardiovascular causes. The secondary endpoint was all-cause mortality. We used 4 sources of information to identify endpoints: repeated contacts with participants, contacts with family physicians, a yearly review of medical records, and consultation of the National Death Index. All medical records related to endpoints were examined by the Endpoint Adjudication Committee, whose members were blinded to the study group assignments. Only endpoints that were confirmed by the committee were included in the analyses. The criteria for adjudicating primary and secondary endpoints are detailed in Supplemental Material 1.

Genotyping of the *COMT* locus

Genomic DNA was extracted from buffy coat with the MagNaPure LC DNA Isolation Kit (Roche Diagnostics). The

rs4680 (G>A) polymorphism in the *COMT* gene was genotyped on a 7900HT Sequence Detection System (Applied Biosystems) with the use of a fluorescent allelic discrimination TaqMan assay. The calling rate was 98%. This genetic polymorphism resulting from the G for A substitution at codon 158 of the *COMT* gene led to a Val to Met substitution. The minor allele frequency for the A allele was 0.47.

Statistical analysis

We calculated HRs and their 95% CIs for the composite CVD endpoint and separately for all cause-mortality. Potential confounders identified in univariate analyses were included in the models. Cox proportional hazard models were adjusted for age, sex, center, education, current smoking, waist circumference, physical activity, diabetes, and dyslipidemia and further by VOO, wine, and vegetable consumption. We performed a sensitivity analysis for the association of HVAL with CVD and total mortality by group of intervention (**Supplemental Tables 1–6**). We evaluated nonlinear associations with the use of cubic splines (23). Parametric survival estimates, or years free of CVD, were assessed with the use of the Weibull accelerated time model with age as the response variable (23). The difference between expected age obtained by the model at a specific quintile minus the expected age at the first quintile was the estimation of gained years of life (24). Regression dilution bias was also evaluated (**Supplemental Material 2**). The interaction with the type of dietary intervention used in PREDIMED was also tested in the models.

In genotyping analyses, differences between normal continuous variables were tested with the use of ANOVA, and *P* values were corrected with the use of Tukey's procedure for multiple comparisons. Differences between nonparametric variables were tested with the use of the Mann-Whitney *U* test (*P* values corrected with the Benjamini-Hochberg method) and between dichotomous variables with the chi-square test (including Fisher's exact test). Analyses used 2-tailed estimations of significance. $P \leq 0.05$ was considered to be statistically significant. The Kaplan-Meier method was used to estimate the cumulative survival, and differences between genotypes were tested with a log-rank test. We used the Cox proportional hazards model to adjust for age, sex, and other potential confounding factors. Multiplicative and additive interactions between genotype and hydroxytyrosol and MOHTyr were tested. The additive interaction was assessed with the use of the estimated relative excess risk for interaction (25, 26). We estimated interaction on an additive scale between continuous determinants in a logistic regression model.

CIs of relative excess risks for interactions were calculated with the use of the bootstrapping methodology. Statistical significance was defined as $\alpha \leq 0.05$. Analyses were performed with the use of R version 3.1.0 (R Foundation).

RESULTS

At baseline, 49.8% of the participants were women, and the mean ± SD age was 67 ± 6 y. In univariate analyses, urinary hydroxytyrosol, HVAL, and TOHTyr were related to sex, BMI, and physical activity and alcohol, wine, VOO, and vegetable consumption (**Table 1**).

TABLE 1
Baseline characteristics of urinary hydroxytyrosol, homovanillyl alcohol, and total hydroxytyrosol[1]

	Hydroxytyrosol						Homovanillyl alcohol						Hydroxytyrosol					
	Q1	Q2	Q3	Q4	Q5	*P*-trend	Q1	Q2	Q3	Q4	Q5	*P*-trend	Q1	Q2	Q3	Q4	Q5	*P*-trend
Participants, *n*	363	362	363	362	362		354	354	354	354	353		346	346	346	346	346	
Age, y	66.9 ± 6.5[2]	66.5 ± 6.3	67.0 ± 6.3	67.1 ± 5.8	66.3 ± 6.1	0.497	67.6 ± 6.0	66.8 ± 6.0	67.1 ± 6.1	67.1 ± 6.3	66.4 ± 6.2	0.037	67.3 ± 6.2	67.0 ± 6.2	67.3 ± 6.2	67.0 ± 6.0	66.4 ± 6.1	0.084
Men, %	40.2	46.7	43.3	54.7	63.3	<0.001	33.1	49.2	47.5	57.9	60.3	<0.001	35.8	43.1	46.8	57.5	61.8	<0.001
Education > high school, %	6.2	8.4	8.3	7.5	10.3	0.103	8.0	5.7	6.5	10.5	11.5	0.012	7.6	7.3	8.1	7.5	11.1	0.121
Current smoking, %	20.9	28.2	24.0	24.0	26.3	0.326	16.7	23.7	25.7	26.8	29.2	<0.001	18.2	24.6	27.5	24.3	27.5	0.013
Diabetes mellitus, %	52.3	55.0	49.3	57.6	50.0	0.388	56.2	51.4	52.5	52.8	48.4	0.093	56.9	53.5	48.6	56.1	47.1	0.045
Hypertension, %	80.7	82.3	79.1	80.4	77.9	0.253	81.1	80.5	82.5	78.5	77.6	0.187	80.3	82.9	78.9	74.9	81.8	0.446
Dyslipidemia, %	72.5	66.6	66.1	65.7	71.0	0.626	69.5	62.7	64.7	68.6	72.5	0.128	68.2	67.6	65.6	64.5	73.7	0.326
BMI, kg/m^2	29.9 ± 3.9	29.8 ± 3.7	29.7 ± 3.4	29.6 ± 3.2	29.2 ± 3.3	0.004	29.8 ± 4.0	29.9 ± 3.3	29.7 ± 3.3	29.3 ± 3.3	29.4 ± 3.4	0.021	30.0 ± 4.0	29.6 ± 3.4	29.7 ± 3.5	29.5 ± 3.2	29.3 ± 3.3	0.017
Waist, cm	99.5 ± 10.8	98.9 ± 10.3	98.7 ± 9.5	100 ± 9.4	99.7 ± 10.0	0.279	98.0 ± 10.7	99.5 ± 9.6	99.4 ± 9.9	98.9 ± 9.8	99.7 ± 9.4	0.020	98.9 ± 11.0	98.4 ± 9.6	99.1 ± 9.5	100 ± 9.5	99.9 ± 9.8	0.037
Physical activity, MET min/wk	1546 ± 1451	1825 ± 1729	1727 ± 1510	1990 ± 1883	2194 ± 1949	<0.001	1680 ± 1434	1787 ± 1745	1937 ± 1757	2060 ± 1844	2010 ± 1840	0.001	1617 ± 1428	1835 ± 1745	2047 ± 1756	1993 ± 1632	2158 ± 2023	<0.001
Alcohol, g/wk	4.8 (0–70)[3]	10.4 (0–73)	13.8 (0–83)	33.4 (0–161)	56.2 (5–190)	<0.001	4.8 (0–52)	10.4 (0–77)	30.7 (0–103)	35.5 (0–182)	46.6 (0–191)	<0.001	4.8 (0–49)	9.2 (0–73)	15.2 (0–89)	38.9 (0–182)	55.6 (5–190)	<0.001
Wine, g/wk	0.0 (0–30)	4.7 (0–55)	4.7 (0–70)	14.0 (0–79)	30.0 (0–175)	<0.001	0.0 (0–30)	4.7 (0–70)	10.0 (0–70)	14.3 (0–88)	30.0 (0–173)	<0.001	0.0 (0–30)	2.3 (0–56)	4.7 (0–70)	20.0 (0–85)	30.0 (0–175)	<0.001
Virgin olive oil, g/wk	55 (0–175)	70 (0–175)	70.0 (0–350)	70.0 (0–350)	175 (0–350)	0.007	50.1 (0–114)	51.4 (0–84)	69.8 (0–114)	95.4 (0–123)	107 (138)	<0.001	42.5 (0–175)	70.0 (0–350)	70.0 (0–350)	70 (0–175)	175 (0–350)	<0.001
Fruits, g/d	319 (222–448)	336 (235–452)	325 (214–460)	314 (235–443)	306 (206–438)	0.126	316 (218–450)	300 (217–425)	329 (232–454)	333 (225–473)	324 (208–450)	0.712	322 (221–449)	323 (216–471)	325 (231–447)	314 (225–460)	311 (206–438)	0.276
Vegetables, g/d	285 (215–371)	292 (222–384)	295 (230–395)	292 (222–387)	307 (232–409)	0.039	277 (214–366)	282 (221–364)	299 (232–398)	301 (229–395)	312 (232–418)	<0.001	285 (218–373)	276 (216–374)	301 (226–399)	293 (238–387)	309 (227–412)	0.011
Adherence to TMD[4]	8.6 ± 1.8	8.8 ± 1.9	8.7 ± 1.9	8.8 ± 1.9	8.8 ± 1.9	0.056	8.7 ± 1.8	8.5 ± 2.1	8.8 ± 1.9	8.8 ± 1.8	8.9 ± 2.0	0.051	8.6 ± 1.9	8.6 ± 1.8	8.8 ± 1.9	8.8 ± 1.9	8.8 ± 1.9	0.063

[1] Total hydroxytyrosol is the sum of hydroxytyrosol and *O*-methyl-hydroxytyrosol. *P*-trend across quintiles was based on linear regression for continuous variables and logistic regression for binary variables. MET, metabolic equivalent; Q, quartile; TMD, traditional Mediterranean diet.
[2] Mean ± SD (all such values).
[3] Median; IQR in parentheses (all such values).
[4] Calculated by the 14-point score.

TABLE 2
Prospective associations (HRs) of urinary hydroxytyrosol, homovanillyl alcohol, and total hydroxytyrosol with primary cardiovascular event among 1851 individuals at risk of CVD[1]

	Quintile							
	1	2	3	4	5	*P*-trend[2]	*n*	*P*-group effect and quintile interaction[3]
Hydroxytyrosol								
Participants, *n*	363	362	363	362	362			
Events, *n*	32	34	25	17	23			
mmol/L	27.6	58.1	97.9	166.8	430.5			
Age- and sex-adjusted	1 (ref)	1 (0.62–1.63)[4]	0.71 (0.42–1.2)	0.41 (0.23–0.75)	0.56 (0.32–0.96)	0.001	1812	0.526
Multivariate-adjusted[5]	1 (ref)	0.98 (0.6–1.6)	0.73 (0.43–1.23)	0.41 (0.23–0.75)	0.61 (0.36–1.06)	0.003	1783	0.247
Multivariate- + diet-adjusted[6]	1 (ref)	1.01 (0.62–1.65)	0.78 (0.46–1.34)	0.46 (0.25–0.84)	0.69 (0.4–1.21)	0.017	1779	0.457
O-Methyl-hydroxytyrosol								
Participants, *n*	354	354	354	354	353			
Events, *n*	41	36	20	19	17			
mmol/L	5.4	11.2	19.8	37.4	146.5			
Age- and sex-adjusted	1 (ref)	0.8 (0.51–1.25)	0.44 (0.25–0.74)	0.4 (0.23–0.69)	0.4 (0.22–0.71)	<0.001	1769	0.105
Multivariate-adjusted[5]	1 (ref)	0.78 (0.5–1.23)	0.43 (0.25–0.74)	0.41 (0.24–0.71)	0.41 (0.23–0.73)	<0.001	1745	0.139
Multivariate- + diet-adjusted[6]	1 (ref)	0.82 (0.52–1.29)	0.46 (0.27–0.8)	0.44 (0.25–0.77)	0.44 (0.25–0.8)	<0.001	1741	0.112
Total hydroxytyrosol[7]								
Participants, *n*	346	346	346	346	346			
Events, *n*	32	33	19	19	19			
mmol/L	0.3	0.5	0.8	1.5	3.7			
Age- and sex-adjusted	1 (ref)	1.04 (0.64–1.7)	0.56 (0.32–0.99)	0.51 (0.29–0.9)	0.52 (0.29–0.92)	0.002	1730	0.498
Multivariate-adjusted[5]	1 (ref)	1.09 (0.67–1.78)	0.61 (0.34–1.07)	0.52 (0.29–0.92)	0.59 (0.33–1.06)	0.005	1706	0.641
Multivariate- + diet-adjusted[6]	1 (ref)	1.11 (0.68–1.81)	0.64 (0.36–1.14)	0.58 (0.32–1.04)	0.68 (0.37–1.23)	0.028	1702	0.511

[1] All *P* values were determined with the use of Cox regression analysis. A cardiovascular event was defined as a composite of myocardial infarction, stroke, or death from cardiovascular causes. CVD, cardiovascular disease; ref, reference.
[2] *P* value corresponding to the interaction with the type of diet followed during the study. All models were stratified by the center.
[3] *P* value corresponding to the improvement of the model when including intervention group and its interaction with quintiles.
[4] Median; IQR in parentheses (all such values).
[5] Adjusted for age, sex, education, current smoking, waist circumference, physical activity, diabetes, and dyslipidemia.
[6] Further adjusted for virgin olive oil, wine, and vegetable consumption.
[7] Sum of hydroxytyrosol and *O*-methyl-hydroxytyrosol.

During 13,070 person-years of follow-up, 142 cardiovascular events and 123 deaths occurred. Across quintiles and after adjusting for demographic, cardiovascular, lifestyle, and dietary factors, concentrations of HVAL were associated with a lower incidence of cardiovascular events (myocardial infarction, stroke, or cardiovascular death) (**Table 2**). Participants in the third or higher quintile of HVAL (≥20 mmol/L) had a 56% lower risk (*P*-trend < 0.001) than those in the lowest quintile. There was a significant trend for a decreasing CVD risk across quintiles for all biomarkers ($P < 0.05$) (Table 2). Concerning total mortality (**Table 3**), no differences across quintiles of biomarker concentrations were found, but a decreasing trend across quintiles was observed for MOHTyr ($P = 0.017$) (Tables 2 and 3). Sensitivity analyses for the association of HVAL with CVD by group of intervention showed that, despite the same trend in all groups, MOHTyr achieved the greatest significance ($P < 0.001$) in the group consuming the Mediterranean diet enriched with VOO (Supplemental Table 1).

In semiparametric analyses, associations of urinary hydroxytyrosol, HVAL, and TOHTyr with primary cardiovascular events were significant in a linear manner (**Figure 1**), with a decrease of HRs from low to high hydroxytyrosol, HVAL, and TOHTyr concentrations ($P < 0.005$). Associations of the biomarkers with total mortality showed that the HR decreased linearly from low to high MOHTyr concentrations ($P = 0.024$) only in the case of HVAL (**Figure 2**).

From all biomarkers, only HVAL concentrations were significantly associated with gained years of life or years free of CVD (**Supplemental Figure 1**) after the age of 65 y. Individuals in the highest quintile of HVAL had a mean 9.5 y (95% CI: 3.5, 20.8 y) longer life after the age of 65 y. With regard to being free of a cardiovascular event, individuals aged >65 y in the highest quintile of HVAL had a mean 6.3 additional years free of CVD (95% CI: 2.32, 12.15 y) compared with individuals with lower concentrations of HVAL (Supplemental Figure 1). Findings were similar for both sexes separately.

The *COMT* genotype distribution [Val/Val (G/G), Val/Met (G/A), or Met/Met (A/A)] among individuals was in Hardy-Weinberg equilibrium. Waist circumference and vegetable consumption ($P < 0.05$) were lower in the rs4680AG genotype (**Supplemental Table 7**). Individuals with the rs4680GG genotype had higher concentrations of HVAL than those with other genotypes (**Table 4**). The distribution of the *COMT* rs4680 alleles was similar among survivors and those who died and among those free of a cardiovascular event or those who had suffered one (**Supplemental Table 8**). No association was obtained among *COMT* genotypes and all-cause mortality or CVD risk. No interaction between the *COMT* rs4680 genotype and HVAL was found (**Supplemental Table 9**). Individuals with low HVAL concentrations had an ∼2-fold greater risk of CVD and all-cause mortality than those with high HVAL concentrations independently of the *COMT* genotype, with multiplicative and additive interactions being nonsignificant ($P > 0.05$).

TABLE 3
Prospective associations (HRs) of urinary hydroxytyrosol, homovanillyl alcohol, and total hydroxytyrosol with total mortality among 1851 individuals at risk of CVD[1]

	Quintile							
	1	2	3	4	5	*P*-trend[2]	*n*	*P*-group effect and quintiles interaction[3]
Hydroxytyrosol								
Participants, *n*	363	362	363	362	362			
Events, *n*	23	28	26	14	27			
mmol/L	27.6	58.1	97.9	166.8	430.5			
Age- and sex-adjusted	1 (ref)	1.17 (0.67–2.04)[4]	1.03 (0.58–1.8)	0.47 (0.24–0.92)	0.93 (0.53–1.64)	0.165	1812	0.171
Multivariate-adjusted[5]	1 (ref)	1.14 (0.66–1.99)	1.04 (0.59–1.84)	0.46 (0.24–0.9)	0.98 (0.55–1.73)	0.205	1783	0.18
Multivariate- + diet-adjusted[6]	1 (ref)	1.15 (0.66–2)	1.02 (0.57–1.82)	0.45 (0.23–0.88)	0.98 (0.55–1.75)	0.195	1779	0.175
O-Methyl-hydroxytyrosol								
Participants, *n*	354	354	354	354	353			
Events, *n*	28	36	23	21	14			
mmol/L	5.4	11.2	19.8	37.4	146.5			
Age- and sex-adjusted	1 (ref)	1.23 (0.75–2.03)	0.79 (0.45–1.37)	0.7 (0.39–1.24)	0.55 (0.29–1.05)	0.011	1769	0.327
Multivariate-adjusted[5]	1 (ref)	1.25 (0.76–2.06)	0.76 (0.44–1.33)	0.71 (0.4–1.27)	0.56 (0.29–1.08)	0.014	1745	0.298
Multivariate- + diet-adjusted[6]	1 (ref)	1.24 (0.75–2.05)	0.74 (0.42–1.3)	0.7 (0.39–1.25)	0.57 (0.3–1.1)	0.017	1741	0.288
Total hydroxytyrosol[7]								
Participants, *n*	346	346	346	346	346			
Events, *n*	26	27	25	17	22			
mmol/L	0.3	0.5	0.8	1.5	3.7			
Age- and sex-adjusted	1 (ref)	1.09 (0.64–1.87)	0.94 (0.54–1.62)	0.57 (0.31–1.06)	0.78 (0.44–1.39)	0.098	1730	0.141
Multivariate-adjusted[5]	1 (ref)	1.11 (0.64–1.91)	0.98 (0.56–1.71)	0.56 (0.3–1.05)	0.84 (0.47–1.5)	0.134	1706	0.164
Multivariate- + diet-adjusted[6]	1 (ref)	1.1 (0.64–1.89)	0.94 (0.53–1.66)	0.54 (0.29–1.02)	0.83 (0.46–1.5)	0.12	1702	0.136

[1] All *P* values were determined with the use of Cox regression analysis. CVD, cardiovascular disease; ref, reference.
[2] *P* value corresponding to the interaction with the type of diet followed during the study. All models were stratified by the center.
[3] *P* value corresponding to the improvement of the model when including intervention group and its interaction with quintiles.
[4] Median; IQR in parentheses (all such values).
[5] Adjusted for age, sex, education, current smoking, waist circumference, physical activity, diabetes, and dyslipidemia.
[6] Further adjusted for virgin olive oil, wine, and vegetable consumption.
[7] Sum of hydroxytyrosol and *O*-methyl-hydroxytyrosol.

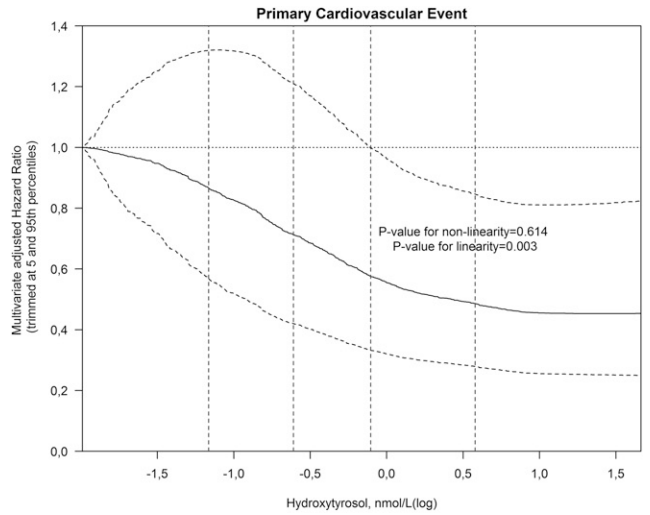
DISCUSSION

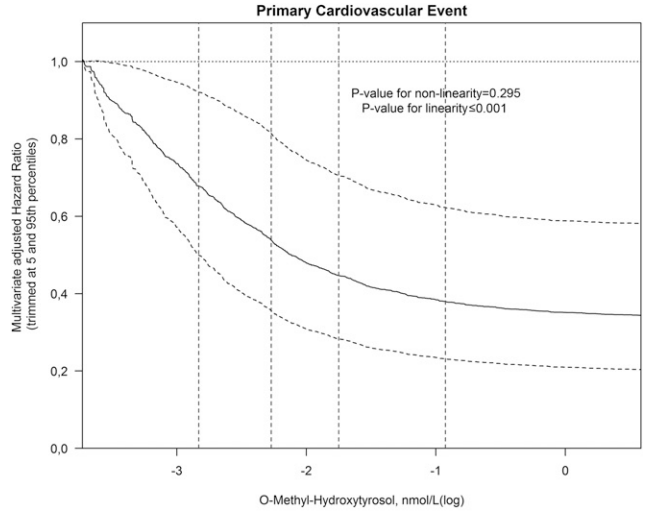
HVAL concentrations were associated with a 56% lower CVD risk across quintiles in individuals at high risk of CVD in this study. After the age of 65 y, our predictive model suggested that the gained years of life and the years free of CVD could be 9.5 and 6.3 y, respectively, among participants with higher urinary HVAL concentrations compared with lower ones. In addition, HVAL was associated with a lower total mortality and lower CVD risk.

Carriers of the *COMT* rs4680 GG genotype displayed higher HVAL concentrations. Neither an association between *COMT* genotypes and CVD or all-cause mortality nor an interaction between *COMT* genotypes and HVAL concentrations was found.

Both experimental and human studies show the benefits of hydroxytyrosol-related foods such as VOO and wine on CVD risk factors, such as: *1*) decreasing heart rate, blood pressure, LDL oxidation, inflammation, thrombotic markers, and lipoprotein particle atherogenic ratios; *2*) increasing HDL cholesterol and HDL lipoprotein functionality; and *3*) improving endothelial function (12, 16, 27). Polyphenols from VOO have also been shown to decrease the expression of atherosclerosis-related genes (27, 28). We have recently provided a mechanistic explanation for the combined protective effect of the simultaneous consumption of the 2 key components of the Mediterranean diet: VOO and wine (10). On the one hand, VOO provides tyrosol and hydroxytyrosol, whereas through the effect of alcohol on dopamine and tyramine metabolism, wine increases the endogenous generation of hydroxytyrosol and tyrosol in humans (10). On the other hand, alcohol from wine increases tyrosol bioavailability in humans, and an in vivo conversion of tyrosol to hydroxytyrosol occurs (10). Thus, a synergic effect of wine and VOO on increasing the human hydroxytyrosol pool in vivo is likely to occur. HVAL in vivo concentrations, however, are not only dependent on the hydroxytyrosol concentrations but also the individual metabolic capacity for promoting COMT-regulated hydroxytyrosol methylation. A substitution of Val (G) by Met (A) at codon 158 of the *COMT* gene affects the activity of the COMT enzyme. Individuals with the rs4680 GG genotype have a 3- to 4-fold higher activity than those with other genotypes (15). In agreement with this, in our study the GG genotype was associated with higher HVAL concentrations. This fact indicates the relevance of nondietary processes for having high concentrations of HVAL.

The hydroxytyrosol and HVAL concentrations observed herein could be referred to as steady-state concentrations. Despite their short half-life (13), hydroxytyrosol and HVAL accumulate in the body after the sustained consumption of hydroxytyrosol-rich foods such as VOO (11). From our data, protection from CVD seems to occur from HVAL urinary concentrations ≥20 mmol/L (Table 2). This value could be considered a protective threshold for the combined adherence of 2 key products of the Mediterranean Diet: VOO and wine. Similar concentrations of HVAL have been reached in the plasma of healthy individuals after a daily





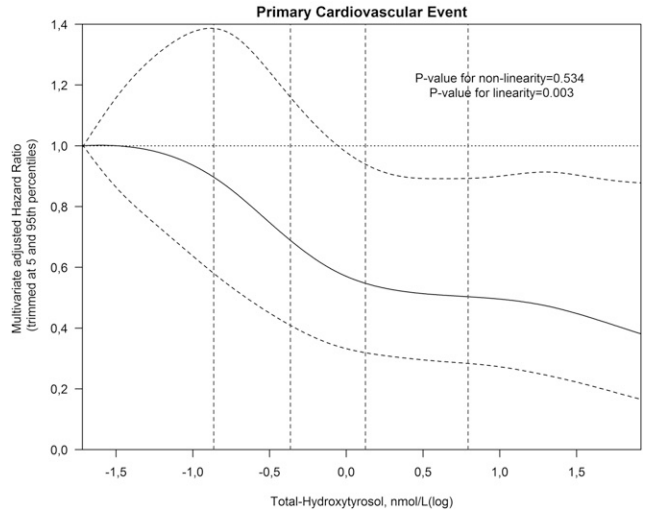
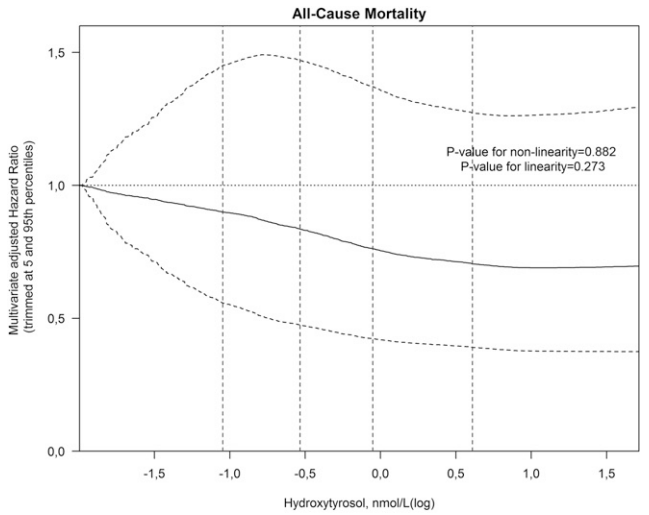
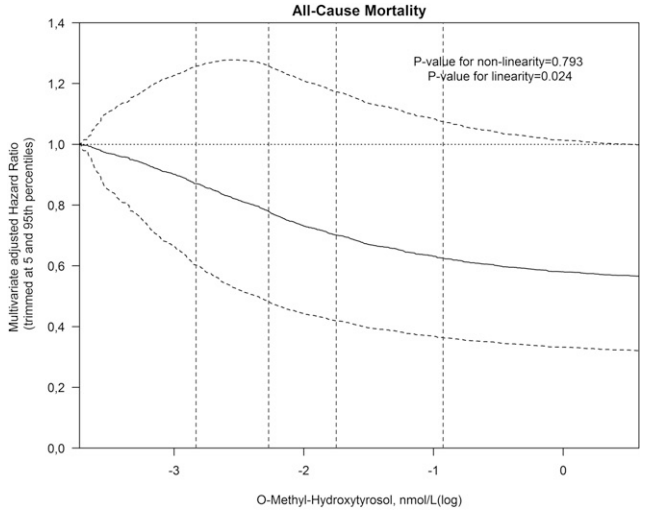


FIGURE 1 Multivariate-adjusted relation of hydroxytyrosol, homovanillyl alcohol, and total hydroxytyrosol (hydroxytyrosol + homovanillyl alcohol) with primary cardiovascular event. Associations were evaluated with the use of restricted cubic splines. The solid lines represent the central risk estimate, and the dotted lines represent the 95% CIs adjusted for age, sex, center, education, current smoking, waist circumference, physical activity, diabetes, and dyslipidemia and virgin olive oil, wine, and vegetable consumption.





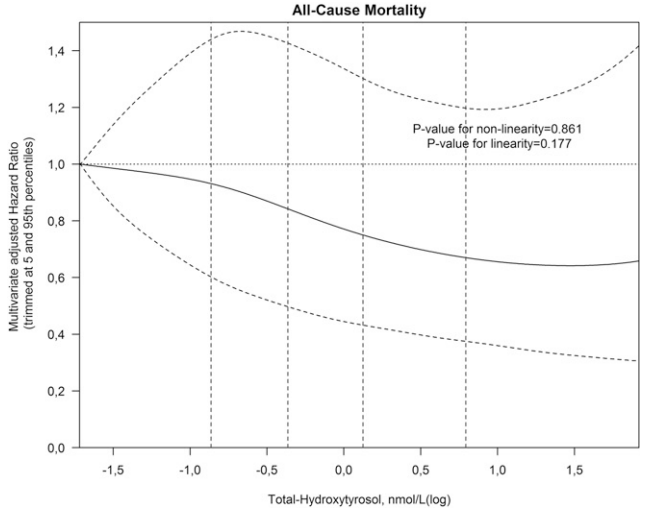


FIGURE 2 Multivariate-adjusted relation of hydroxytyrosol, homovanillyl alcohol, and total hydroxytyrosol (hydroxytyrosol + homovanillyl alcohol) with all-cause mortality. Associations were evaluated with the use of restricted cubic splines. The solid lines represent the central risk estimate, and the dotted lines represent the 95% CIs adjusted for age, sex, center, education, current smoking, waist circumference, physical activity, diabetes, and dyslipidemia and virgin olive oil, wine, and vegetable consumption.

TABLE 4
Relation between *COMT* genotypes and homovanillyl alcohol among 1851 individuals at risk of CVD[1]

COMT rs4680 genotype	*n* (%)	β Coefficients (95% CIs)	*P* values
GG	509 (28.1)	Reference	
AG	904 (49.8)	−0.01 (−0.24, 0.05)	0.19
AA	400 (22.1)	−0.12 (−0.24, 0.00)	0.05
AG + AA	1304 (71.9)	−0.11 (−0.22, 0.00)	0.05
GG + AG	1413 (77.9)	Reference	
AA	400 (22.1)	−0.02 (−0.14, 0.10)	0.74

[1] Adjusted by age and sex, center, and virgin olive oil and wine consumption. *P* values were determined with the use of linear regression analysis. *COMT*, catechol-O-methyltransferase.

sustained consumption during 4 d of 25 mL rich-hydroxytyrosol VOO (13), and 6-fold higher HVAL urinary concentrations were observed after moderate red wine ingestion (150 mL) (10).

The protective antioxidant activity of HVAL in experimental studies has been said to be greater than (29), similar to (30), and lower than (31) that of hydroxytyrosol according to the experimental model used. However, chemically, HVAL is a compound that is far more stable in biological fluids than hydroxytyrosol (32). This stability allows HVAL to exist for longer than hydroxytyrosol in biological fluids and intracellular spaces; thus, the former can exert benefits for longer. Further studies on the effect of HVAL on pathologic mechanisms, such as inflammation, endothelial function, and thrombosis, are warranted.

Contradictory data exist on the influence of *COMT* genotypes on CVD risk. The rs4680GG genotype has been associated with a high risk of hypertension (33) and CVD (34), whereas the rs4860AA genotype has been shown to be protective against myocardial infarction in hypertensive patients (35). In contrast, the low COMT activity of the rs4860AA genotype has been shown to be an independent risk factor for acute coronary events in Finnish men (36). In our study, however, we did not find this association. Differences between populations could explain the differences in the results obtained herein. In agreement with others (37), in this study we did not observe any association between *COMT* genotypes and total mortality. Taking into account the absence of a strong association between the *COMT* polymorphism and HVAL concentrations, as well as the high pleiotropy of the COMT enzyme, this polymorphism cannot be used as a proxy for Mendelian randomization (38). Therefore, the absence of associations between the *COMT* genotypes and CVD or total mortality cannot be interpreted as not causal.

Our study has strengths and limitations. All variables in the multicenter study were collected through well-established common protocols (39). The associations among biomarkers and CVD or all-cause mortality were adjusted by possible confounders. The biomarkers in this study were measured at baseline, however, and changes over time could influence the results and in some cases lead to misclassifications. In addition, the sample size could not allow enough power to detect small differences, particularly in the case of genetic data. In addition, this was an observational study and thus cannot demonstrate causality. Cardiovascular events and total mortality were adjudicated with the use of medical records that were examined by an endpoint adjudication committee. However, some misclassifications could occur. The fact that our participants were at a high risk of CVD limits the generalizability of the results to other populations.

In summary, we report herein for the first time to our knowledge an independent association between high urinary HVAL concentrations and a lower risk of CVD and total mortality in elderly individuals at a high risk of CVD. From our results, VOO and wine consumption and a high metabolic capacity of COMT-mediated methylation are key factors for achieving high HVAL concentrations. The association that stems from our results reinforces the benefits of consuming 2 key components of the Mediterranean diet.

The authors' responsibilities were as follows—RDlT, DC, MIC, and M Fitó: conceived and designed the study; RDlT, DC, OC, MIC, and M Fitó: acquired, analyzed, and interpreted the data; JV: conducted the statistical analysis; and all authors: drafted the manuscript, critically revised the manuscript for important intellectual content, and read and approved the final manuscript. ER and JS-S are consultants for the California Walnut Commission and International Nut Council, respectively. None of the other authors reported a conflict of interest related to the study.

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