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Red wine consumption does not affect oxidizability of low-density lipoproteins in volunteers¹⁻⁴

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ABSTRACT Phenolic compounds in red wine may protect low-density lipoproteins (LDL) against oxidative modification, thereby reducing the risk of cardiovascular morbidity. However, in vivo data are scarce. We gave 13 healthy volunteers 550 mL red wine and another 11 volunteers white wine for 4 wk in a randomized double-blind trial. Interference by alcoholic components of wine was eliminated by reducing the alcohol content to 3.5%. Red wine did not affect the susceptibility of LDL to Cu²⁺-mediated oxidative modification [lag time before and after red wine drinking: ($\bar{x} \pm$ SD) 61.8 \pm 7.7 and 62.7 \pm 11.8 min, respectively; lag time before and after white wine drinking: 64.5 \pm 10.4 and 63.3 \pm 10.8 min, respectively]. Concentrations of the antioxidants urate, vitamin C, and glutathione in plasma and of vitamin E and ubiquinol-10 in LDL were also unchanged after either red or white wine consumption. The results of this study do not show a beneficial effect of red wine consumption on LDL oxidation. *Am J Clin Nutr* 1996;63:329-34.

KEY WORDS Red wine, low-density lipoprotein, oxidation, antioxidants

INTRODUCTION

Populations with a high intake of cholesterol and saturated fat have a high mortality from coronary heart disease (CHD) (1). France is an exception, the CHD mortality is low despite a diet high in saturated fat (2, 3). The high consumption of alcohol-containing beverages, such as red wine, may, at least in part, explain this so-called French paradox. Alcohol consumption increases plasma concentrations of high-density-lipoprotein (HDL) cholesterol (4, 5). Moderate red wine consumption was also found to be associated with a favorable change in hemostatic factors, such as blood platelet aggregation (3). The question is whether the supposed cardioprotective effect of red wine can be explained solely by the alcohol component.

In the Zutphen Elderly Study and the Seven Countries Study, the risk of mortality from CHD was negatively correlated with intake of certain flavonoids (6, 7). Flavonoids consist of two phenylbenzene (chromanol) rings linked through a pyran ring. They are present in fruit, vegetables, and beverages such as tea and wine. The most important water-soluble polyphenols with antioxidative capacities in red wine are the flavonols quercetin and myricetin, and the 3-flavanols catechin and epigallocatechin. These are derived from the skin of the grape and are

therefore not present in white wine. Flavonoids are mainly responsible for the astringency, flavor, and color of red wine.

Frankel et al (8) reported that flavonoids extracted from Californian red wine protected low-density lipoprotein (LDL) against oxidation when added in vitro. The oxidative modification of LDL is thought to play a key role in the development of early atherosclerotic lesions (9). Red wine flavonoids could inhibit LDL oxidation (8, 10-13).

Data in vivo are contradictory. Fuhrman et al (14) found that LDL oxidation was inhibited in subjects who had drunk red wine, whereas Sharpe et al (15) did not find such an effect. We focused on the effect of the nonalcoholic components of red wine by reducing the alcohol content. Thus, we assessed whether the consumption of low-alcohol red wine affects the oxidizability of LDL and the concentrations of antioxidants in plasma.

SUBJECTS AND METHODS

Study design

This study was approved by the ethical committee of the Academic Hospital, University of Nijmegen, and all subjects gave their informed consent before participation. Twenty-four healthy nonsmoking, normolipidemic volunteers (aged 22-63 y; 19 males, 5 females) participated. Subjects were not using vitamin or mineral supplements. Before study entry subjects' average total alcohol consumption was 0-240 g alcohol/wk. Eleven subjects were moderate red wine drinkers (24-120 g alcohol/wk). All subjects consumed white wine for a 2-wk baseline period (Figure 1). They were then randomly assigned to consume white or red wine for another 4 wk (test period), with stratification for age, sex, and plasma cholesterol concentration. The distribution of the moderate red wine drinkers into

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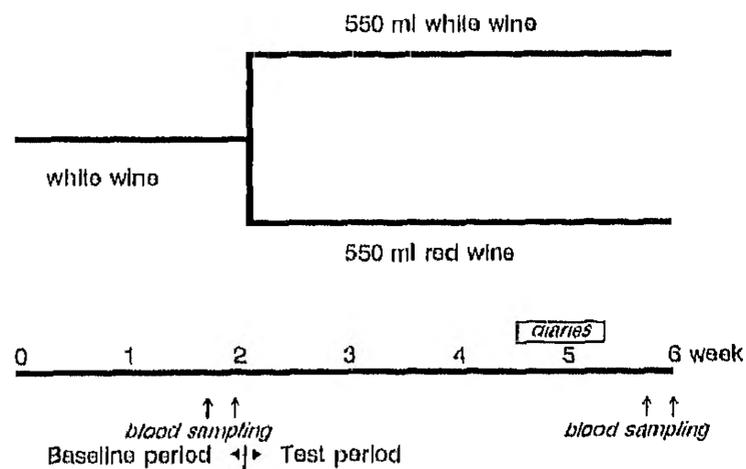


FIGURE 1. Study design. All subjects consumed low-alcohol white wine for 2 wk before being randomly assigned to consume low-alcohol red or white wine for an additional 4 wk.

the red wine and the white wine groups was seven and four, respectively. Ten men and three women aged 38.2 ± 9.8 y [with a plasma total cholesterol concentration of 5.25 ± 0.71 mmol/L and a body mass index (in kg/m^2) of 22.8 ± 2.2] received red wine and nine men and two women aged 36.4 ± 11.8 y (with a plasma total cholesterol concentration of 5.37 ± 0.82 mmol/L and a body mass index of 22.7 ± 2.1) continued to consume white wine.

Participants consumed 550 mL wine (four to five glasses, ≈ 12 g alcohol/d per person) in the evening. Wine was delivered daily to the volunteers. All subjects followed a low-flavonoid diet and abstained from tea and red wine. Food intake was checked by 5-d written records. Intake of quercetin, kaempferol, myricetin, apigenin, and luteolin was estimated by using data from Hertog et al (16).

Preparation of wines

We used white wine from the Loire region in France (Baron Charles, 1993) and red wine from Italy (Chianti Classico, 1991) because this red wine tends to have higher quercetin contents than French wines (17). Alcohol was removed by evaporation at 35°C under a pressure of 2 cm Hg. Total volume loss was $< 15\%$. Final alcohol concentrations ($\bar{x} \pm \text{SEM}$) were $3.5 \pm 0.2\%$ for white wine and $3.4 \pm 0.3\%$ for red wine. The alcohol content was determined by using a Boehringer Mannheim test kit (no. 176290; Mannheim, Germany). Nonpolyphenolic pigments (E102, 4-(4'-sulfo-1'-phenylazo)-1-(4'-sulfophenyl)-5-hydroxypyrazolon-3-carbonic acid; E110, chinophthalon-disulfate; E122 2-(4'-sulfo-1'-naphthylazo)-1-naphthol-4-sulfate; E132, indigotin-5'-disulfate; and E151, Brilliant Black; Jacob Hooi, Limmen, Netherlands) and red beet (root) juice (37.5 mL/L; Loverendale, Netherlands) were added to both wines to make them indistinguishable. In addition, the wines were coded with random codes. As a result, subjects were unable to distinguish white from red wine. Quercetin and myricetin concentrations were determined essentially as described by Hertog et al (16). Catechin, epicatechin, and epigallocatechin were determined essentially as described by Bailey and Nursten (18). Before removal of the alcohol the red wine contained (wet wt) 6.2 ± 0.2 mg quercetin/kg, 3.4 ± 0.2 mg myricetin/kg, 27 mg catechin/kg, 20 mg epicatechin/kg, and 116 mg epigallocatechin/kg. The white wine contained < 0.1 mg quercetin/kg, 1.1 mg myricetin/kg, and 2 mg gallate/kg. The low-alcohol red wine contained 6.1 ± 0.1 mg quercetin/kg, 3.3 ± 0.1 mg myricetin/kg, 12 mg catechin/kg, 19 mg

epicatechin/kg, and 107 mg epigallocatechin/kg. The low-alcohol white wine contained < 0.1 mg quercetin/kg, 1 mg myricetin/kg, and 2 mg gallate/kg.

In vitro measurement of antioxidant capacity of wine

To determine the antioxidant activity of the two wines in vitro, plasma (containing 1 mmol EDTA/L) from a normolipidemic donor was dialyzed against two changes of 1 L chelex 100-treated phosphate-buffered saline at 4°C to remove contaminating transition metal ions (19). Before the incubation, 1 mL plasma was preincubated with 10–50 μL white or red wine for 5 min at 37°C , followed by addition of 94 mmol/L 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) (200 μL 625 mmol AAPH/L in 0.154 mol NaCl/L) (Polysciences, Inc, Warrington, FL). AAPH generates water-soluble peroxy radicals at a constant rate (19). After 3 h of incubation, the oxidation was stopped by refrigeration and the degree of lipid peroxidation was assessed by measuring the amount of thiobarbituric acid-reactive substances (TBARS), expressed as μmol malondialdehyde (MDA) equivalents/L plasma (20, a modification of 21).

Blood measurements

Two fasting blood samples were collected 10–12 h after wine consumption into evacuated tubes on separate days at the end of both the baseline and test periods. Blood was collected into tubes containing $\text{K}_3\text{-EDTA}$ (1 g/L) for cholesterol, α -tocopherol, ubiquinol-10, and glutathione determinations and for the oxidation assay. Additive-free tubes were used for urate and lithium-heparin-containing tubes for vitamin C. Blood was placed immediately on ice and plasma was separated by centrifugation at $3200 \times g$ for 8 min at 4°C within 1 h of sampling. Plasma aliquots were rapidly frozed in nitrogen and stored at -80°C after the addition of saccharose (6 g/L) to stabilize the lipoproteins. Butylated hydroxytoluene (final concentration 250 mg/L), a lipophilic antioxidant, was added to the plasma samples used for the measurement of α -tocopherol and ubiquinol-10 concentrations and TBARS.

Cholesterol concentrations were determined with an enzymatic method with reagent from Boehringer (CHOD-PAP reagent no. 1442350; Boehringer Mannheim, Mannheim, Germany) on a Hitachi 747 analyzer (Boehringer Mannheim). HDL cholesterol was quantified in plasma after precipitation of LDL and very-low-density lipoprotein/intermediate-density lipoprotein with the polyethylene glycol 6000 method (22). For the oxidation assay, the protein content of LDL was measured by the method of Lowry et al (23), with chloroform extraction to remove turbidity, and using bovine serum albumin as a standard. The interassay CVs were $< 2\%$ for both cholesterol and triacylglycerol determinations.

Samples of the same subjects were always analyzed in the same run. In each run, samples obtained from subjects who received red wine were analyzed together with samples obtained from subjects who received white wine. Ascorbic acid and ubiquinol-10 were analyzed within 30 min after thawing.

Determination of antioxidant concentrations

Glutathione was determined in whole blood by an enzymatic method according to Griffith (24). The oxidized form of glutathione (GSSG) was determined within 6 h of blood sampling.

The reduced form of glutathione (GSH) was determined within 4 wk in the acid, protein-free supernate. Urate in serum was determined within 3 h with a Hitachi 747 analyzer (25). Vitamin C (sum of L-ascorbic and dehydro-L-ascorbic acid) in whole blood was determined by HPLC with fluorometric detection (26).

α -Tocopherol and ubiquinol-10 were determined within 2 wk of storage by HPLC coupled to an electrochemical detector (Decade; Antec, Leiden, Netherlands) (27). To determine antioxidant concentrations in LDL, LDL was isolated by a short-run nonequilibrium density-gradient ultracentrifugation ($120\,000 \times g$ for 4 h at 4°C) using a Kontron TFF 45.6 fixed-angle rotor (Kontron AG, Zurich, Switzerland) (28). After isolation, LDL preparations were immediately assayed for antioxidants as described above. To prevent oxidation of ubiquinol-10, LDL was not dialyzed. The concentration was expressed per mmol LDL cholesterol because of potential contamination with trace amounts of albumin.

Oxidizability of LDLs

Oxidation experiments were performed within 2 mo of storage. Plasma samples of participants were thawed rapidly and LDL was isolated by density ultracentrifugation ($285\,000 \times g$ for 18 h at 4°C) using a swingout Beckman SW40 rotor in a Beckman L55 ultracentrifuge (Beckman, Palo Alto, CA). To protect the LDL against oxidative modification during isolation, $10\ \mu\text{mol}$ EDTA/L was added to each density solution. LDL was isolated at a density of 1.019–1.063 kg/L. LDL was not dialyzed. The time between isolation and the oxidation experiment was always < 90 min. The susceptibility of LDL to in vitro Cu^{2+} -mediated oxidation was determined as described by Esterbauer et al (29) as modified by Princen et al (30). After isolation, LDL was diluted immediately with NaCl:EDTA (1.18 mol/L:10 μmol /L) to a final concentration of 114 mg protein/L, and sodium phosphate (pH 7.4) was added to a final concentration of 10 mmol/L. Oxidation was initiated by adding CuSO_4 to a final concentration of 38 μmol /L at 37°C . The kinetics of the oxidation of LDL were determined by monitoring the change of the 234-nm diene absorption in a thermostat-controlled ultraviolet spectrophotometer (Lambda 12; Perkin Elmer, Gouda, Netherlands), equipped with a nine-position automatic sample changer. Absorbance curves of four LDL preparations from one subject, two before and two after the test period, were analyzed in parallel. In this way small differences in the oxidation parameters between the LDL preparations isolated from the plasma before and after the test period could be detected. Each LDL preparation was oxidized in two oxidation runs on the same day. The oxidation assay was validated by analyzing one reference LDL, prepared from pooled plasma stored at -80°C , in every oxidation run. For the reference LDL, the interassay CV for lag time, oxidation rate, and the total amount of conjugated dienes formed per milligram of LDL protein were $< 3\%$.

Statistical methods

For each subject the change of each variable was calculated as the mean of the two values obtained at the end of the test period minus the mean at the end of the baseline period. The null hypothesis was that the mean of the changes in the red wine group equalled those in the white wine group. This

hypothesis was tested by a *t* test. Repeated-measures analysis of variance (ANOVA) was used to assess the effect of the baseline versus the test period and of white versus red wine. Where *F* test results were significant, the results of unequal variances with separate degrees of freedom were used. Where *F* test results were not significant, results from the pooled degrees of freedom were used. All data analyses were performed by using SPSS/PC software (SPSS Inc, Chicago).

RESULTS

The daily average intake of the sum of quercetin, kaempferol, myricetin, apigenin, and luteolin from food was 8.1 ± 4.6 mg in the white wine group and 10.0 ± 6.0 mg in the red wine group. The daily additional intake of quercetin plus myricetin was 5.3 mg from red wine and 0.6 mg from white wine. Beside these two flavonoids, high concentrations of catechin-like flavonoids are present in red wine; but in this study the additional dietary intake of the latter could not be calculated.

Wine inhibited AAPH-induced lipid peroxidation when added in vitro to plasma (Figure 2). At 5%, native red Chianti wine inhibited lipid peroxidation by 61%, as measured with the TBARS assay, whereas no effect was found with white wine even when it was added at a final concentration of 10%. The low-alcohol red wine plus colorants as given to the participants exerted the same protective effect on plasma oxidation in vitro as did the native red wine. Likewise, the low-alcohol white wine plus added colorants failed to inhibit the AAPH-induced lipid peroxidation when added to plasma in vitro (Figure 2).

In contrast with the in vitro findings, consumption of either wine for 4 wk did not affect LDL oxidizability, measured either as lag times, oxidation rate, or final amount of diene formation. Lag times, reflecting the resistance of isolated LDL against oxidative modification, were similar in both wine groups and were not prolonged after consumption of 550 mL red wine/d for 4 wk (Table 1). The nonsignificant differences between changes in the red and white wine groups were in fact in the opposite direction of what would be expected if red wine

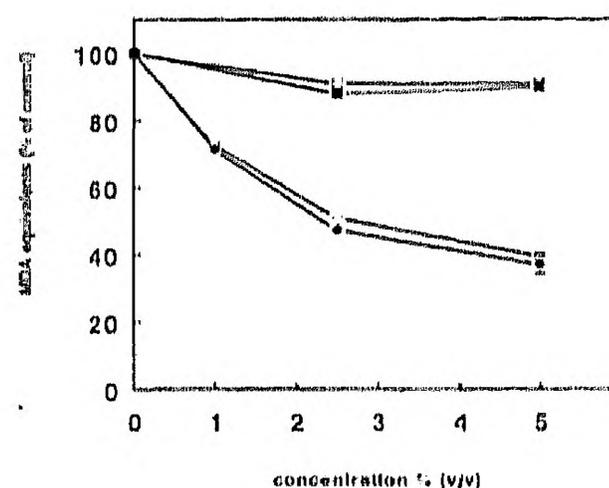


FIGURE 2. The effect of in vitro addition of white or red wine on the susceptibility of plasma to lipid peroxidation before and after reduction of the alcohol content. Plasma isolated from a normolipidemic donor was incubated with 2,2'-azobis(2-amidinopropane)(AAPH, 100 mmol/L) in the presence of the indicated concentrations of either white or red wine for 3 h at 37°C . (○), native white wine; (■), native red wine; (△), low-alcohol white wine plus colorants; (●), low-alcohol red wine plus colorants. Values are expressed as percentages of maximal lipid peroxidation ($n = 2-3$). The maximal AAPH-induced lipid peroxidation, 18.9 μmol malondialdehyde (MDA) equivalents/L, in plasma was measured in the absence of wine.

TABLE 1

Oxidation kinetics of LDL isolated from the plasma of subjects at the end of the 2-wk baseline period (white wine) and at the end of the 4-wk test period (red or white wine)

	White wine ¹ (n = 11)	Red wine ¹ (n = 13)	Difference between changes ²
Lag time (min)			
Baseline (white wine only)	64.5 ± 10.4	61.8 ± 7.7	
Test period	63.3 ± 10.8	62.7 ± 11.8	1.4 (-3.1, 5.9)
Oxidation rate (nmol dienes · g LDL protein ⁻¹ · min ⁻¹)			
Baseline (white wine only)	14.1 ± 3.1	13.9 ± 2.1	
Test period	13.3 ± 2.4	14.6 ± 2.3	1.5 (-0.5, 3.5)
Dienes _{max} (nmol/g LDL protein)			
Baseline (white wine only)	514.3 ± 18.2	525.9 ± 34.8	
Test period	502.1 ± 21.6	532.0 ± 31.8	18.4 (0, 36.7)

¹ $\bar{x} \pm$ SD.

² \bar{x} ; 95% CI in parentheses.

protected against oxidation. In three subjects with a basal flavonoid intake > 15 mg, lag times after red wine consumption were also not different from baseline values. The rate of diene formation and the maximal amount of dienes formed after red wine consumption were not different from baseline values (Table 1). Antioxidant concentrations were not affected either; the mean value for α -tocopherol was 4.1 ± 1.1 μ mol/mmol LDL cholesterol at baseline and 4.1 ± 1.1 after red wine drinking, and the content of ubiquinol-10 was 86 ± 51 nmol/mmol LDL cholesterol and changed to 91 ± 69 (NS). The concentrations of aqueous and lipophilic antioxidants in plasma were also unchanged (Table 2) as were TBARS (baseline and test values: 1.08 ± 0.27 and 0.99 ± 0.13 μ mol/L, respectively). Total cholesterol, LDL cholesterol, HDL cholesterol, and total triacylglycerol concentrations in plasma did not change significantly in either group (Table 3).

DISCUSSION

In our double-blind study consumption of four to five glasses of red wine per day for 4 wk did not affect copper-mediated

LDL oxidation in healthy volunteers. α -Tocopherol and ubiquinol-10 concentrations of LDL after red wine consumption were similar to baseline values.

Interest in red wine flavonoids has been stimulated by the growing evidence that free radical-mediated events are involved in the early formation of fatty streaks in coronary arteries (9). The beneficial effect of red wine consumption on the prevention of CHD (31) might be due to the HDL-raising effect of ethanol rather than of flavonoids. We therefore used wine from which most of the alcohol had been removed. This was evidently successful because no effect on plasma HDL-cholesterol concentrations was seen. The lack of change in plasma lipids also prevented compositional changes in LDL, which can influence LDL oxidizability (32, 33).

Our data conflict with those of Fuhrman et al (14), who observed a fourfold increase in the lag phase before oxidation of LDL in volunteers who had consumed 500 mL red wine compared with white wine. The effect was seen after 2 wk of treatment, but no difference was seen after 1 wk. The extent of the effect that Fuhrman et al saw is surprising: high doses of vitamin E (30, 34–36) decreased LDL oxidizability only 1.3- to

TABLE 2

Concentrations of antioxidants in plasma or blood at the end of the 2-wk baseline period (white wine) and after the 4-wk test period (red or white wine)

	White wine ¹ (n = 11)	Red wine ¹ (n = 13)	Difference between changes ²
α -Tocopherol (μ mol/L plasma)			
Baseline	23.5 ± 6.3	22.8 ± 6.6	
Test period	23.7 ± 5.6	22.9 ± 5.0	-0.22 (-2.5, 2.1)
Ubiquinol-10 (nmol/L plasma)			
Baseline	598 ± 176	553 ± 163	
Test period	530 ± 152	613 ± 301	108 (-88, 303)
Vitamin C (μ mol/L blood)			
Baseline	42.3 ± 8.7	48.4 ± 9.2	
Test period	46.3 ± 9.7	48.2 ± 10.6	0.12 (-10.9, 2.7)
Total glutathione (mmol/L blood)			
Baseline	0.79 ± 0.15	0.73 ± 0.16	
Test period	0.58 ± 0.15	0.63 ± 0.16	-4.12 (-0.05, 0.28)
Urate (mmol/L serum)			
Baseline	0.33 ± 0.07	0.33 ± 0.06	
Test period	0.33 ± 0.07	0.33 ± 0.07	-0.01 (-0.03, 0.01)

¹ $\bar{x} \pm$ SD.

² \bar{x} ; 95% CI in parentheses.

TABLE 3

Concentrations of total cholesterol, HDL cholesterol, and triacylglycerol in plasma at the end of the 2-wk baseline period (white wine) and after the 4-wk test period (red or white wine)¹

	White wine ¹ (n = 11)	Red wine ¹ (n = 13)	Difference between changes ²
<i>mmol/L</i>			
Cholesterol			
Baseline	5.37 ± 0.82	5.25 ± 0.71	
Test period	5.21 ± 0.75	5.13 ± 0.60	0.05 (-0.20, 0.30)
HDL cholesterol			
Baseline	1.29 ± 0.42	1.42 ± 0.33	
Test period	1.31 ± 0.38	1.49 ± 0.33	0.06 (-0.04, 0.16)
Triacylglycerol			
Baseline	1.16 ± 0.44	0.98 ± 0.35	
Test period	1.20 ± 0.47	1.00 ± 0.31	-0.02 (-0.19, 0.15)

¹ $\bar{x} \pm SD$.

² \bar{x} ; 95% CI in parentheses.

2.5-fold, and even long-term treatment with the most efficient antioxidant known, probucol (250 mg/d for 4 mo), prolonged the lag time by no more than threefold (37). The fourfold increase in lag time reported by Fuhrman et al (14) for a relatively modest intervention is thus unprecedented and difficult to explain.

The design of our study differed from that of Fuhrman et al (14) by having a baseline period of 2 wk with white wine, followed by a 4-wk treatment. The effect of red wine consumption on LDL oxidizability was expected to be greater after 4 than after 2 wk. In contrast with the study of Fuhrman et al, all subjects followed a low-flavonoid diet during the study. There were also essential differences in the methods used by both research groups for measuring LDL oxidizability, ie, the omission of the dialysis step in our procedure to prevent early oxidative modification of LDL. The red Italian wine we used showed the same antioxidative capacity *in vitro* as that found by Fuhrman et al (14) with French wine. This antioxidative capacity was not influenced by reduction of the alcohol content or addition of pigments and beet juice used for blinding. Flavonoid concentrations of the red wine were similar to those measured in French wines (38, 39), and were not changed by these manipulations. In addition, flavonoid concentrations in plasma are not influenced by storage of plasma at -80 °C for as long as 4 mo (PCH Hollman, unpublished observations, 1995).

Red wine has been reported to increase the antioxidative capacity of plasma (14, 40). This effect was also ascribed to red wine flavonoids. Because of the close interaction between the hydrophilic and lipophilic antioxidant mechanisms, an increase in the antioxidant capacity of plasma could decrease LDL oxidizability *in vivo* and probably also *in vitro*. Fuhrman et al (14) assessed total plasma lipid peroxidation *in vitro* by measuring TBARS after the addition of the free radical generator AAPH whereas we estimated potential plasma lipid peroxidation by measuring a spectrum of relevant protective antioxidants. It has been reported that urate, ascorbic acid, thiol proteins, α -tocopherol, and bilirubin are the main contributors to total plasma antioxidant capacity (41). Our data showed that red or white wine did not influence the concentration of either

lipophilic or hydrophilic antioxidants; it is therefore not surprising that LDL oxidizability was also not influenced.

A recent study by Ruf et al (42) in rats reported that oral administration of low-alcohol red wine resulted in decreased alcohol-induced lipid peroxidation in plasma and reduced platelet aggregation *in vivo*. Ruf et al (42) suggested that alcohol promotes absorption of polyphenolics in the intestine. Hitherto, few data were available on the absorption and metabolism of individual flavonoids after consumption. Our knowledge of the absorption of flavonoids from foods is limited and studies of this kind are needed. The amounts absorbed may be insufficient to exert antioxidative activity *in vitro* either in total plasma or in isolated LDL.

In conclusion, consumption of a fairly large dose of red wine did not affect *in vitro* LDL oxidizability in volunteers. The French paradox, if it exists at all, may be due to factors other than the effect of wine on LDL oxidation. ‡

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