Wine constituents inhibit thrombosis but not atherogenesis in C57BL/6 apolipoprotein E-deficient mice

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Regular and moderate wine consumption is one of the explanations suggested for the lower incidence of cardiovascular events in France compared with other industrialized countries. We evaluated whether alcohol alone or combined with red wine polyphenols reduced plaque size and/or attenuated thrombotic reactivity at the site of advanced atherosclerotic lesions. Red wine extract, or purified (+)-catechin with alcohol, or alcohol alone, was added for 12 weeks to the drinking water of apoE-deficient (apoE$^{-/-}$) C57BL/6 mice and wild-type counterparts. In the apoE$^{-/-}$ mice, all alcohol-containing mixtures were associated with a larger size of aortic atherosclerotic lesions. On the other hand, red wine extract and (+)-catechin significantly inhibited blood thrombotic reactivity ($P < 0.05$) as assessed in a cylindrical perfusion chamber model of experimental thrombosis: area reductions in cross-sectional surface of the *ex vivo* thrombus were 64 % and 63 %, respectively. In the wild-type mice, red wine extract and (+)-catechin tended to reduce thrombogenicity, which was on the whole less marked than in the apoE$^{-/-}$ mice. These findings suggest that a moderate and regular consumption of red wine may protect against clinical cardiovascular events, mainly by attenuating the thrombogenic response rather than by reducing the development of atherosclerotic lesions. This antithrombogenic effect may include normalization of the abnormally high thrombogenic responsiveness in apoE$^{-/-}$ mice as well as a direct antithrombotic effect.

Atherosclerosis: Thrombosis: Polyphenols: Alcohol: Apolipoprotein E-deficient mouse

Atherothrombosis is the leading cause of death in industrialized countries (Murray & Lopez, 1997). The clinical manifestations of atherothrombosis are ischaemic events due to the evolution/complication of atherosclerotic plaques and the thrombotic reaction onto these complicated atherosclerotic lesions. The 'French paradox' hypothesizes that the reduced incidence of cardiovascular manifestations of atherothrombotic disease in France and neighbouring Mediterranean countries is due, at least in part, to their cultural dietary features, mainly a regular and moderate consumption of wine, in comparison to Anglo-Saxon populations whose incidence of cardiovascular events is much higher for the same level of 'regular' cardiovascular risks (hypercholesterolaemia, hypertension, diabetes, etc.). Numerous experimental studies have been conducted to try to give grounds to this hypothesis, but most of them have focused on the potential anti-atherosclerotic effects of wine-derived polyphenols, based mainly on the antioxidant properties of these compounds and their influence on lipid metabolism (Hayek *et al.* 1997; Maor *et al.* 1997; Moghadasian *et al.* 1999). The potential antioxidant/anti-atherosclerotic benefit of wine polyphenols has

been extended to phenols of other origins such as green tea (Crespy & Williamson, 2004; Sano *et al.* 2004; Vinson *et al.* 2004; Erba *et al.* 2005; Rah *et al.* 2005; Sung *et al.* 2005; Suzuki *et al.* 2005) and cocoa (Grassi *et al.* 2005; Keen *et al.* 2005; Kurosawa *et al.* 2005). Most of the animal studies have suspected a preventive effect of wine-derived phenols through their antioxidant activity, using very young animals before they had their first lesions and evaluating the beneficial effect as the prevention of lesion development (Hayek *et al.* 1997; Fuhrman *et al.* 2005). In man, intervention trials with antioxidant dietary compounds, mainly vitamins E and C, have never sustained a protective cardiovascular effect of such a strategy. The main explanation evoked is that the animal studies focus strictly on prevention while human trials deal with already blown-up atherosclerosis. This specific issue was addressed using models of aged animals with evolved atherosclerosis (Bentzon *et al.* 2001), which did not find any beneficial effect, while most animal models using young animals with evolving pathology show a preventive effect of phenols (from wine and other origins). The animal model used in most of these experimental studies is based

on mice genetically engineered to develop hypercholesterolae-mia and atherosclerotic lesions mimicking some aspects of human arterial pathology, mainly mice whose genes coding apoE and/or LDL receptor have been knocked out (Zhang *et al.* 1992).

By contrast, much less attention has been focused on the potential role of phenols in the thrombotic reaction occurring in complicated arterial lesions (Pace-Asciak *et al.* 1995; Wollny *et al.* 1999; Booyse & Parks, 2001). This phenomenon is the rationale we followed to test the effects of several red wine components on both atherosclerotic lesion development and arterial thrombotic reactivity in apoE-deficient (apoE$^{-/-}$) mice. These apoE$^{-/-}$ mice have spontaneously high plasma cholesterol levels and develop aortic athero-sclerotic lesions when fed a regular low-fat/low-cholesterol rodent diet (Plump *et al.* 1992; Reddick *et al.* 1994). We com-paratively tested alcohol, a red wine extract (RWE) containing a complex polyphenol mixture and a representative phenol of this mixture, i.e. (+)-catechin. These components were added to drinking water in order to simulate the consumption of wine. The control animals were mice with an identical genetic background (C57BL/6) but with normal plasma cholesterol levels and no spontaneous atherosclerotic lesions.

Material and methods

Animals

Ninety-six male C57BL/6J mice and ninety-six male C57BL/6J-Apoe tm1Unc (apoE$^{-/-}$) mice aged 3·5 weeks, purchased from Charles River Laboratories (L'Arbresle, France), were included in the present study.

Wine polyphenols

Preparation of the red wine extract. The RWE was prepared from a French red wine (Origin, Corbières AOC 1999). Briefly, the wine was run through a preparative column at a flow rate of 1 l/h. After washing with water, the phenolic com-pounds were desorbed selectively with methanol. The extract was gently evaporated and the concentrated residue was freeze-dried and finely sprayed into a dry powder. One litre of red wine yielded 3 g RWE, which was stored at −80°C. HPLC analysis as described by Carando *et al.* (1999) showed that 1 g RWE contained 471 mg phenolic compounds, expressed as gallic acid equivalents; these compounds included 8·6 mg (+)-catechin, 8·7 mg (−)-epicatechin, several dimers (B1, 6·9 mg; B2, 8·0 mg; B3, 20·7 mg; B4, 0·7 mg), anthocyanins (malvidin-3-glucoside, 11·7 mg; peonidin-3-glu-coside, 0·66 mg; cyanidin-3-glucoside, 0·06 mg) and phenolic acids (gallic acid, 5·1 mg; caffeic acid, 2·5 mg; caftaric acid, 12·5 mg).

(+)-Catechin purification. We further purified 95 % pure (+)-catechin (Sigma-Aldrich, L'Isle d'Abeau, France) by dissolving the product in ultrafiltered water heated to 120°C, filtering the hot solution through a no. 4 glass filter under vacuum, and allowing the filtrate to crystallize overnight at 4°C. The filtrate containing crystallized (+)-catechin was fil-tered through a no. 4 glass filter under vacuum, the crystals were then rinsed with cold water and dried in a desiccator

containing P_2O_5. The final purity of (+)-catechin was 99 %, as ascertained by MS−HPLC.

Diet and drinks

All mice were fed on a semi-synthetic diet free of polyphenols (Table 1; SAFE-UAR, Augy, France). Food pellets were packed in double-vacuum bags and sterilized by γ-irradiation at 45 kGy (IBA Mediris, Fleurus, Belgium). Depending on their treatment allocation, mice received one of the following drinks. (i) RWE−alcohol: RWE 3 g/l, dissolved in acidified (pH 3·3) water containing 100 ml alcohol/l, to replicate the alcohol concentration and pH of the wine used to prepare the RWE; (ii) CAT−alcohol: purified (+)-catechin 160 mg/l, dissolved in acidified water containing 100 ml alcohol/l, to replicate the total flavan-3-ol concentration in the wine used to prepare the RWE; (iii) alcohol: acidified water containing 100 ml alcohol/l; or (iv) water: acidified water. Mice were watered only on their experimental drink, which implies that those receiving an alcohol-containing drink had alcohol con-sumption above the moderate consumption level rec-ommended in man. Drinks were freshly prepared every 2 d and RWE−alcohol and CAT−alcohol drinks were protected from light to avoid phenol oxidation by UV radiation.

Experimental design and sample collection

After 1-week acclimatization, the mice (18 ± 1 g) were ran-domly assigned to the four experimental drinks (twenty-four mice per drink in each strain). They were housed in groups of six animals per cage with free access to food and drink for the 12-week study period. The animal room was main-tained at a temperature of 21 ± 1°C and a relative humidity of 50 ± 5 % with a 12 h light/12 h dark cycle. Drink intake

Table 1. Composition of the diet* given to the wild-type and apoE-deficient mice

Ingredient	g/kg
Mashed potato	290·00
Maize starch	289·85
Saccharose	50·00
Casein	50·00
Soya isolate†	120·00
Maize oil	30·00
Lard	30·00
Cholesterol	0·15
Cellulose	60·00
Mineral additive‡	70·00
Vitamin additive§	10·00

* Analytical compounds of dry matter were: crude proteins (N × 6·25) 18 wt %, crude fat 8 wt %, ash 6 wt %, carbohydrate 68 wt %. Energy 19·33 MJ/kg of dry matter (Eurofins Scientific Analytics, Nantes, France).
† Nurish 1500 (DuPont Protein Technologies, Paris, France).
‡ The mineral additive provided (g/kg diet): Ca, 2·11; P, 5·46; Na, 2·74; K, 3·67; Mg, 1·02; Fe, 0·10; Cu, 0·09; Mn, 0·55; Zn, 0·31; I, 4·3 × 10^{-3}; Co, 0·7 × 10^{-3}.
§ The vitamin additive provided (units/kg diet): retinol, 20 000 IU; cholecalciferol, 2500 IU; tocopherol, 175 IU; menadione, 17·6 mg; thiamine, 20 mg; riboflavin, 15 mg; nicotinic acid, 100 mg; pantothenic acid, 6·9 mg; pyridoxine, 10 mg; folic acid, 5 mg; biotin, 0·3 mg; cyanocobalamine, 0·05 mg; L-ascorbic acid, 0·8 mg; choline, 1·36 g; myoinositol, 150 mg.

was measured every 2 d and body weight gain and food intake once a week.

Two weeks before the end of the experiment, six of the twenty-four mice in each group were placed in individual metabolism cages for daily individual collection of urine and faeces, which were stored at $-20°C$ until assayed. To protect the urine samples from bacterial contamination, 100 μl of 0·4 % sodium azide solution v/v was added every day to the urine collection receptacles.

At the end of the experiment, the mice were killed by intra-peritoneal injection of pentobarbital at a dose of 60 mg/kg body weight (Sanofi-Synthelabo, Le Plessis Robinson, France). After dissection, blood was drawn from the vena cava through a 25-gauge butterfly infusion set (Becton Dickinson, Le Pont de Claix, France) connected to an elasto-mer tube (Dow Corning, Midland, MI, USA) and a syringe pump (Harvard type 22; Ealing France, Les Ulis, France). The volume of blood that could be collected from a single mouse ranged from 500 to 1000 μl, which was not sufficient to allow determination of all the study variables in all animals. Therefore, in each treatment group, thrombotic reactivity and plasma LDL susceptibility to oxidation were each tested in six mice; plasma (+)-catechin concentration and antioxidant capacity were assayed in another six mice; and blood from the remaining six mice was used for platelet count and blood chemistry (plasma levels of cholesterol and triacylgly-cerols, activity of aspartate and alanine aminotransferases). Depending on the assay to be performed, the elastomer tube was filled with an anticoagulant agent (10 μl of 0·4 M-EDTA or 50 μl of a heparin solution, 500 IU/ml) prior to the blood collection. Platelets were counted in EDTA-anticoagulated blood using an MS4-CellDyn Analyser (Melet-Schloesing Laboratoires, Cergy-Pontoise, France); the blood was sub-sequently centrifuged (1500 *g*, room temperature, 10 min) and the plasma was stored at $-80°C$ until chemistry assays. The heparin-anticoagulated blood collected for analysis of (+)-catechin and antioxidant status was centrifuged under the same conditions; plasma was then bubbled with N_2 to avoid oxidation and stored at $-80°C$.

All procedures were carried out in accordance with Euro-pean guidelines for the care and use of laboratory animals.

Blood chemistry assays

Plasma triacylglycerol and total cholesterol concentrations were determined using enzymatic methods on an AU800 ana-lyser (Olympus, Rungis, France). The activities of aspartate aminotransferase and alanine aminotransferase were measured using standard methods to check that long-term consumption of alcohol did not induce liver damage.

Analysis of (+)-catechin in plasma, urine and faeces

Within each treatment group, the plasma samples from the six-mice subgroup intended for (+)-catechin analysis were pooled. After protein precipitation, (+)-catechin was analysed by HPLC using fluorescence detection as described by Car-ando *et al.* (1998).

For each of these mice, the faecal samples daily collected over the 2-week period in metabolism cages were pooled, freeze-dried, weighed and finely ground in an electric coffee

grinder; extraction of (+)-catechin was carried out by thoroughly mixing 1 g faecal powder with 8 ml sodium acetate buffer (0·02 M, pH 4·0); suspensions were subsequently centri-fuged (8000 *g*, 4°C, 15 min) and supernatants were collected. Following thawing, urine samples were also pooled, then centrifuged (1000 *g*, 4°C, 15 min), and the supernatants were acidified with one-fifth (v/v) of sodium acetate buffer. (+)-Catechin was selectively extracted from urine supernatants and faecal extracts as described by Franke & Custer (1994) using Sep Pak Plus C18 cartridges (Waters, Saint-Quentin-en-Yvelines, France); extracts were concentrated to dryness by rotary evaporation and dissolved in 2 ml sodium acetate buffer. Preliminary assays with urine and faeces spiked with 6·8 mM-(+)-catechin showed that recovery was 97 %. Half of each extract was stored at $-20°C$; to allow measurement of glucuronidated and/or sulfated catechin, the other half was hydrolysed with β-glucuronidase and sulfatase from *Helix pomatia* type HP-2S (Sigma-Aldrich) as described by Franke & Custer (1994).

Native and hydrolysed extracts were subsequently analysed for (+)-catechin content by HPLC (Waters). Samples (100 μl) were injected with an autosampler 2690 onto a C18 reverse-phase column (LiChrospher® 100 RP-18e, 250 mm × 4 mm, 5 μm; Merck, Paris, France) fitted with a LiChroCART® car-tridge (4 mm × 4 mm, 5 μm; Merck). Separation was achieved at a flow rate of 1·0 ml/min by gradient elution, using ultrafil-tered water with 0·025 % trifluoroacetic acid added as solvent A and methanol with 0·025 % trifluoroacetic acid v/v added as solvent B. The gradient programme was: 10 to 15 % B, 0 to 30 min; 15 % B, 30 to 40 min; 15 to 30 % B, 40 to 110 min; 30 % B, 110 to 120 min; 30 to 10 % B, 120 to 125 min. (+)-Catechin was monitored at 280 nm using a photodiode array detector 996. Data were collected and peaks integrated using the Millenium® software (version 4.0; Waters); quantification was achieved by comparing the peak areas with those of auth-entic standards spiked from 10 to 1000 μM.

Antioxidant status

Plasma antioxidant capacity. Plasma antioxidant capacity was measured using the total antioxidant status (TAS) method of Randox (Randox Laboratories, Crumlin, UK).

LDL susceptibility to oxidation. Within each treatment group, the plasma samples from the six-mice subgroup intended for analysis of LDL oxidation were pooled. Plasma LDL was separated by sequential density ultracentrifugation as previously described (Aviram, 1983). The LDL fraction was then dialysed for 24 h against PBS (without EDTA) to remove heparin. The protein content was determined using the method of Lowry *et al.* (1951) and adjusted to 100 μg pro-tein/ml with PBS. LDL oxidation was induced by adding $CuSO_4$ at a final concentration of 5 μmol/l. Conjugated diene formation was monitored at 234 nm using a DU640 spectrophotometer (Beckman, Gagny, France). Absorbance at the beginning of the reaction was set to zero. Lag times were determined graphically as the time points at which the tangent to the curve at the propagation phase peak intercepted the time axis.

Nitrate:nitrite ratio. NO production was assessed indirectly by determination of the nitrate:nitrite ratio in the

urine of six mice per treatment group, using the method of Giovannoni *et al.* (1997).

Ex vivo *thrombogenic reactivity*

Ex vivo thrombogenic reactivity was assessed with the perfusion chamber model used in our laboratory (Andre *et al.* 1996) and adapted to studies in mice. A 25-gauge butterfly set (Becton Dickinson) inserted into the vena cava was connected to a syringe pump that was used to draw blood through the cylindrical perfusion chamber consisting of a calibrated glass tube (length 32 mm, inner diameter 0·35 mm) whose inner surface was uniformly coated with human type III collagen (final density 2·5 μg/cm^2; Sigma-Aldrich). The uniformity of the collagen deposit and its resistance to disruption by blood flow were checked before the experiment. By modifying the flow rate of the pump, this set-up can reproduce the shear rate characteristics of various normal and diseased blood vessels; shear rate is the haemorheological parameter with the greatest influence on thrombus formation. In the present experiment, the shear rate was set to 1000 s^{-1}, which is characteristic of arteries with moderate stenosis. After 2 min of perfusion of native blood through the chamber, the thrombotic deposit was rinsed, fixed, and embedded in Epon epoxy resin (Sigma-Aldrich, Saint-Quentin Fallavier, France). A microtome (Leica, Rueil-Malmaison, France) was used to cut 4-μm sections perpendicularly to the direction of blood flow, 5 mm from the proximal part of the perfusion chamber.

Aortic tissue processing

After blood collection from the vena cava, the aortic tree was fixed by infusion with 2·5 % glutaraldehyde v/v in 0·1 M-sodium cacodylate buffer, pH 7·4, using a 5-ml syringe inserted into the left ventricle. In apoE$^{-/-}$ mice, the aortic arch region, with the valves and branch points of the two main arterial trees, is the site most susceptible to atherosclerosis (Tangirala *et al.* 1995b; Johnson & Jackson, 2001). Therefore, for comparative histomorphometric studies of atherosclerotic lesion development, atherosclerosis was quantified at three predetermined locations of the aortic arch: (i) at the proximal aorta and (ii) above and (iii) below the start of the brachiocephalic trunk. The aortic arch was dissected free from the surrounding fatty tissue, then fixed and embedded as for thrombotic deposits (Andre *et al.* 1996). At each of the three locations, the maximal lesion was identified under microscopic examination. At each of the three sites of maximal lesion, fifteen transverse sections 1 μm in thickness were sequentially cut on a microtome (Leica, Rueil-Malmaison, France) and stained with alkaline toluidine blue and fuchsin red to provide optimal resolution.

Computer-assisted morphometry

Thrombotic deposit after ex vivo *perfusion.* After staining of semi-thin cross-sections of embedded thrombotic deposits, thrombogenesis was evaluated using an optical microscope (10 × magnification; Zeiss, Le Pecq, France) and the image analysis software Atherose™ (Microvision Instruments, Evry, France). The main criterion was the area (μm^2) of the thrombus cross-sectional surface, which is representative of the thrombus size.

Atherosclerotic lesions. Plaques were measured using the same computer-assisted image analysis system. Size criteria were thickness, expressed as the ratio of plaque thickness over media thickness, and circumference, expressed as the percentage of cross-sectional circumference covered by the plaque. For each mouse and for the three aortic arch locations, the individual values are the mean of the fifteen serial sections. The values at the three sites for each treatment mice are the mean of twenty-four mice.

Statistical analyses

Data are expressed as means and their standard errors (*n* 6 to 24, depending on the parameter). To test the effect of apoE gene deletion, we compared the wild-type mice drinking water with their apoE$^{-/-}$ counterparts consuming the same drink, using Student's *t* test. The effect of the experimental drinks was analysed separately in each mouse strain, using a one-way ANOVA. When ANOVA indicated significant differences, treatments were compared using the Newman–Keuls multiple comparison test. *P* < 0·05 was considered statistically significant. Calculations were performed using the Statview® software (version 5.0; SAS Institute, Cary, NC, USA).

Results

Effect of genetic status and drink composition on body weight gain and food and drink intakes

Significant body growth was observed during the 12-week study period that was similar in both strains and independent of the drink they consumed. The overall weight gain averaged 8 (SE 0·7) g. However, mice receiving alcohol, CAT–alcohol and RWE–alcohol ate and drank less than control mice given water. The overall food intake was 5·9 kg in the groups of wild-type and apoE$^{-/-}$ mice drinking water, *v*. 5·1, 5·2 and 5·3 kg in the counterpart groups drinking alcohol, CAT–alcohol and RWE–alcohol. In the wild-type mice, the overall drink consumption was 8·1 litres in the water group *v*. 7·5 litres in the alcohol and CAT–alcohol groups and 6·5 litres in the RWE–alcohol group. In a similar fashion, whereas water consumption in apoE$^{-/-}$ mice reached 9·0 litres, it was only 8·4 litres in the alcohol and CAT–alcohol groups and 7·2 litres in the RWE–alcohol group. On the whole, reduced energy intake from food was counterbalanced by the energy supplied by alcohol (23·2 MJ/l).

Effect of genetic status and drink composition on blood haematology and biochemistry

Platelet counts. Wild-type and apoE$^{-/-}$ mice drinking water had similar platelet counts (Table 2). In wild-type mice, consumption of the alcohol drink was associated with a lower platelet count compared with the group drinking water. In apoE$^{-/-}$ mice, those drinking RWE–alcohol had significantly more platelets than those drinking water, alcohol or CAT–alcohol.

Aminotransferase activities. Alanine aminotransferase and aspartate aminotransferase levels remained normal (data not

Table 2. Effect of genetic status (wild-type and apoE-deficient, apoE$^{-/-}$) and drink composition (water, alcohol, (+)-catechin (CAT)−alcohol and red wine extract (RWE)−alcohol) on blood haematology and biochemistry and plasma antioxidant capacity of the mice

(Mean values with their standard errors*)

	Platelets (10^9/l)			Total cholesterol (mM)			Triacylglycerols (mM)		TAS (TEAC, mM)		LDL oxidation (lag time, min)	Nitrate: nitrite ratio (μmol/mmol creatinine)		
	Mean	P	SEM	Mean	P	SEM	Mean	SEM	Mean	SEM		Mean	P	SEM
Wild-type mice														
Water	1079^a		84	2·7^a		0·1	0·71	0·10	0·45	0·06	ND	497^a		53
Alcohol	683^b	0·0054	87	2·2^b	0·0092	0·2	0·58	0·14	0·58	0·03	ND	841^b	0·0057	86
CAT−alcohol	871^{a,b}		97	2·6^{a,b}		0·1	0·56	0·04	0·67	0·05	ND	781^b	0·0187	86
RWE−alcohol	816^{a,b}		96	2·4^{a,b}		0·1	0·73	0·05	0·62	0·06	ND	718^{a,b}		84
ApoE$^{-/-}$ mice														
Water	947^a		62	12·4†		1·1	0·90	0·14	0·64†	0·04	48·7	434^a		56
Alcohol	749^a		98	12·9		1·4	1·09	0·17	0·69	0·08	53·6	1013^b	0·0001	104
CAT−alcohol	722^a		121	16·1		2·6	0·92	0·12	0·88	0·07	42·5	867^b	0·0008	80
RWE−alcohol	1295^b		117	16·7		1·0	0·87	0·11	0·72	0·11	53·9	837^b	0·0016	61

TAS, total antioxidant status; TEAC, Trolox® equivalent antioxidant capacity; ND, not determined.
^{a,b} Mean values within each strain group with unlike superscript letters were significantly different (ANOVA plus Newman−Keuls test): $P < 0.05$.
* n 5 to 6, except for the analysis of LDL oxidation for which the plasma samples from the six mice were pooled (for details, see Material and methods, p. 291).
Mean values were significantly different from those of the wild-type mice drinking water (effect of genetic status; Student's t test): †$P < 0.05$.

shown), indicating that the alcohol intake did not induce any noticeable lysis of hepatocytes.

Plasma cholesterol and triacylglycerol levels. As reported consistently with apoE$^{-/-}$ mice (Plump *et al.* 1992), a severe hypercholesterolaemia occurred in this strain compared with its wild-type counterpart (Table 2). Among wild-type mice, those given the alcohol drink had a lower plasma cholesterol level than those given water only, whereas no difference was found for the groups given CAT−alcohol or RWE−alcohol. Among apoE$^{-/-}$ mice, drink composition had no influence on plasma cholesterol levels. With regard to plasma triacylglycerols, a trend towards higher concentrations was observed in apoE$^{-/-}$ mice compared with their wild-type counterparts. On the other hand, within each mouse strain, plasma triacylglycerol levels were similar across treatment groups.

(+)-Catechin concentrations in plasma, urine and faeces

As expected, no (+)-catechin was detected in either the plasma or the urinary and faecal excreta of wild-type and apoE$^{-/-}$ mice given the water and alcohol drinks. In both strains, plasma (+)-catechin levels were similar in the groups given CAT−alcohol and RWE−alcohol: 114·4 and 96·4 μg/l in the wild-type mice and 94·4 and 102·8 μg/l in the apoE$^{-/-}$ mice, respectively. However, neither free nor glucuronidated/sulfated (+)-catechin could be detected in the urine and faeces of mice given CAT−alcohol or RWE−alcohol, regardless of the strain.

Effect of genetic status and drink composition on antioxidant status

Plasma antioxidant capacity. As previously reported, plasma TAS was low in both mouse strains compared with values found in human subjects (Diaz *et al.* 1998). Among mice drinking water, the apoE$^{-/-}$ animals had higher TAS levels

than their wild-type counterparts (Table 2). In neither strain did the alcohol-containing drinks affect plasma TAS levels.

LDL susceptibility to oxidation. No noticeable differences were observed between the LDL oxidation lag times measured in the apoE$^{-/-}$ mice, whether they drank water or alcohol-containing beverages (Table 2).

Nitrate:nitrite ratio. The nitrate:nitrite ratio was similar in the apoE$^{-/-}$ and wild-type mice drinking water (Table 2). In both strains, consumption of alcohol-containing drinks was associated with significantly higher nitrate:nitrite ratios compared with water consumption, although this difference was only a trend in the wild-type mice given the RWE−alcohol drink.

Effect of genetic status and drink composition on thrombogenic reactivity

The thrombus size, as assessed by the area of the thrombus cross-sectional surface, tended to be greater in apoE$^{-/-}$ mice drinking water compared with their wild-type counterparts consuming the same drink. Among wild-type mice, the thrombus size was similar across treatment groups. In contrast, among apoE$^{-/-}$ mice, the CAT−alcohol and RWE−alcohol drinks significantly reduced thrombus size, while the alcohol drink remained ineffective (Fig. 1).

Effect of genetic status and drink composition on atherosclerotic lesions

As expected, no atherosclerotic lesions were found in the wild-type mice. Among the apoE$^{-/-}$ mice, the thickness and extent of the aortic arch atherosclerotic lesions, observed above the root of the brachiocephalic trunk, were significantly greater in animals consuming the alcohol-containing drinks than in those consuming water only (Fig. 2); this increase in lesion size was not significantly affected by phenolic compounds. In the proximal aorta and below the root of the brachiocephalic
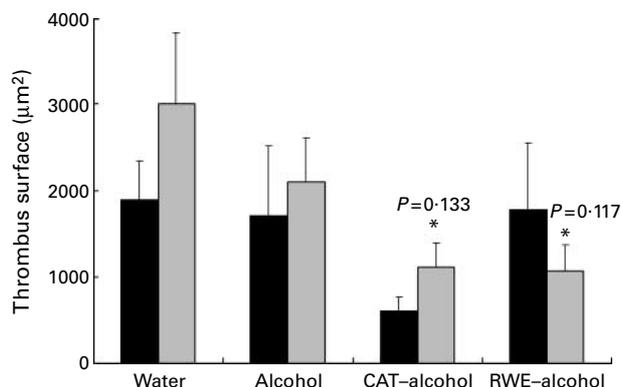
Fig. 1. Effect of genetic status (■, C57BL/6J wild-type mice; ▨, C57BL/6J apoE-deficient mice) and drinking composition (water, alcohol, (+)-catechin (CAT)−alcohol, red wine extract (RWE)−alcohol) on *ex vivo* thrombus reactivity in a perfusion chamber assessed by the cross-sectional surface area of the thrombus, which reflects thrombus size. Values are means with their standard errors shown by vertical bars for six mice per group. Mean values were significantly different from those of the same-strain group given water only (ANOVA): *$P < 0.05$.

trunk, plaque thickness and extent were similar across treatment groups. At the site of the aorta where lesions were observed, administration of alcohol, CAT−alcohol or RWE−alcohol had no effect on vessel media thickness and inner circumference (Table 3).

Discussion

Our findings indicate that adding either purified (+)-catechin or red wine extract to the drinking water of genetically hypercholesterolaemic mice failed to alleviate the atherosclerotic plaque burden. These results are at variance with a substantial body of evidence from other models in mice (Emeson *et al.* 1995) and other species (Goto *et al.* 1974; Klurfeld & Kritchevsky, 1981; da Luz *et al.* 1999; Yamakoshi *et al.* 1999), but agree with a study by Munday *et al.* (1999) as well as with work by Bentzon *et al.* (2001) in the same mouse model. In the present study, alcohol-containing drinks increased the extent of the atherosclerotic lesions, regardless of their phenol content. The potential pro-atherogenic effect of alcohol observed in our mice is consistent with data from diet-induced atherosclerosis in rabbits (Shaish *et al.* 1997). Atherosclerosis is usually associated with oxidative stress characterized by a reduction in endogenous antioxidant capacity (Oemar *et al.* 1998). In models that showed chemoprevention by wine polyphenols against atherosclerosis, this effect was related to variously measured biomarkers of antioxidant status (Hayek *et al.* 1997). In the present study, neither phenols nor alcohol had any *in vivo* antioxidant effects detectable by the TAS assay or the LDL oxidation assay. Apart from wine phenols, various antioxidants are reported to protect against atherosclerosis (Meagher & Rader, 2001) although the effects are not consistent with the various types of antioxidant. Thus, vitamin E (Pratico *et al.* 1998) or *N*,*N'*-diphenyl-1,4-phenylenediamine (Tangirala *et al.* 1995*a*) reduced atherosclerotic lesions in apoE$^{-/-}$ mice, whereas probucol, another compound with antioxidant effects in the same animal model, was pro-atherogenic (Zhang *et al.* 1997). Thus, it remains unclear whether the effects of these agents
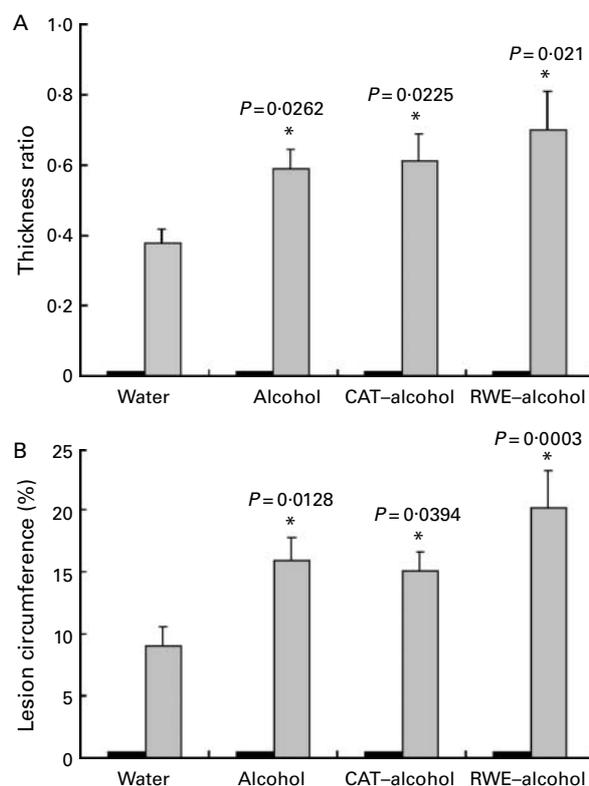

Fig. 2. Effect of genetic status (■, C57BL/6J wild-type mice; ▨, C57BL/6J apoE-deficient mice) and drinking composition (water, alcohol, (+)-catechin (CAT)−alcohol, red wine extract (RWE)−alcohol) on atherosclerotic lesions in the aortic arch at the root of the brachiocephalic trunk, as assessed by (A) thickness ratio and (B) inner circumference (%). Values are means with their standard errors shown by vertical bars for fifteen serial sections from each of six mice per group. Mean values were significantly different from those of the same-strain group given water only (ANOVA): *$P < 0.05$.

on atherosclerotic lesions are ascribable to their antioxidant properties.

NO production is usually believed to exert beneficial effects on cardiovascular physiology through its vasodilating, antihypertensive and antithrombotic effects. Reactive oxygen species would reverse this effect by interacting with NO to produce peroxynitrates, which can cause oxidative damage to the endothelium (Lopez-Jaramillo & Casas, 2002). The antioxidant effect of wine phenols could prevent this potentially deleterious effect. Additionally, Andriambeloson *et al.* (1998) reported that wine polyphenols induced a direct vessel relaxation, but this was shown in an *in vitro* experiment in which relaxation of the rat aorta precontracted by noradrenaline was measured when various concentrations of polyphenols were added to the organ bath. These data were confirmed in a more recent study, in which endothelial cells exposed to red wine polyphenolic extract released NO in a concentration-dependent manner (Leikert *et al.* 2002). In the present *in vivo* study, measurement of the nitrate:nitrite ratio indicated an increase of endogenous NO production in both mice strains following the consumption of alcohol-containing drinks. However, this effect was more probably related to alcohol rather than phenols, as most of the effect occurred with alcohol alone. Despite the effect on

Table 3. Effect of drink composition (water, alcohol, (+)-catechin (CAT)−alcohol and red wine extract (RWE)−alcohol) on the inner circumference and media thickness at the origin of the brachiocephalic trunk from the aortic arch in apoE-deficient mice

(Mean values with their standard errors*)

	Water		Alcohol		CAT−alcohol		RWE−alcohol	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Inner circumference (μm)	2986	66	3088	68	3037	83	3000	59
Media thickness (μm)	67·2	2·4	66·3	1·5	63·3	2·8	67·6	2·1

* n 13 to 17.
No significant differences were observed across treatment groups.

NO production, addition of alcohol and phenols to drinking water failed to modify parameters reflecting vessel contraction and relaxation, such as arterial vessel diameter or vessel wall thickness.

Two strategies of chemoprotection against atherosclerosis can be investigated in the apoE$^{-/-}$ model: treatment early in life to prevent the development of atherosclerotic lesions, or curative treatment started later in life of animals that already have developed atherosclerosis. In this latter strategy, treatment effects are inferred from changes in the atherosclerotic lesions present at baseline. Overall, the data reported in this field suggest that wine polyphenols may be effective in preventing the development of atherosclerosis but not in alleviating the burden of established atherosclerotic lesions (Bentzon *et al.* 2001). In the present experiments, the aortic arch specimens taken above the root of the brachiocephalic trunk after a 12-week consumption of water, alcohol or alcohol combined with polyphenols showed atherosclerotic lesions in all the apoE$^{-/-}$ mice. Compared with lesions found in human subjects, the murine lesions resembled more fatty streaks than mature atherosclerotic plaques, whose characteristic features are fibrous tissue proliferation and a lipid core rich in cholesterol crystals. Maximal atherosclerotic lesion size was quantified at three sites of the aortic arch known to develop the maximal lesions in apoE$^{-/-}$ mice (Tangirala *et al.* 1995*b*; Johnson & Jackson, 2001). This quantification of the maximal lesion unveiled an increased lesion size with alcohol intake at only one of these three aortic sites, namely the one above the root of the brachiocephalic trunk. Lesion size at the other two aortic sites was not affected by the experimental drinks. The structure of the lesions seemed similar across the three sites and not affected by the experimental drinks. The discrepancy between our results and those describing a phenol-induced reduction of atherosclerotic lesions in apoE$^{-/-}$ mice (Hayek *et al.* 1997; Fuhrman *et al.* 2005) could be due to their expression of the results as the mean of all the lesions, without distinction between each of the individual locations of the aortic arch developing lesions, and our expression of lesion size focusing on maximal size.

The 'French paradox' is a way to express the unexpectedly lower rate of clinical cardiovascular events in France compared with populations with similar cardiovascular risk factors in Northern European and Anglo-Saxon industrialized countries. One possible explanation is that cultural differences in diet and wine consumption may influence the ischaemic cardiovascular event rate via an effect on atherogenesis and/or the thrombotic response to atherosclerotic lesions. To reflect the possible contribution of these two factors, the term 'atherothrombosis' is better adapted – from a pathogenic point of view – to designate the cardiovascular lesions responsible for ischaemic events. So far, the potential effect of polyphenols and alcohol on thrombus formation has received far less attention than effects on atherogenesis, and few reports come from studies focusing on arterial thrombosis (Demrow *et al.* 1995; de Gaetano *et al.* 2002). In most of them, polyphenols reduced the thrombotic response via an effect on platelets (Osman *et al.* 1998; Ruf, 1999; Russo *et al.* 2001). Abundant experimental and clinical evidence indicates that alcohol affects platelet functions (Lacoste *et al.* 2001). Our results open up the important possibility that the ability of wine intake to attenuate thrombotic reactivity may be related not mainly to alcohol, but primarily to phenolic compounds such as (+)-catechin and/or others contained in the red wine extract. Indeed, alcohol alone had no significant antithrombotic effect in our model. Nevertheless, we cannot exclude an adjuvant influence of alcohol on the effects of phenolic compounds.

Platelet counts were moderately reduced in mice of both strains consuming alcohol-containing drinks. This effect is well documented for excessive alcohol consumption (Ballard, 1989). Except in apoE$^{-/-}$ mice given RWE−alcohol, which had moderately increased platelet counts, the addition of phenols did not influence the alcohol-reducing effect. Thus, the antithrombotic effect associated with phenol intake is probably ascribable to an action on platelet functions, rather than on platelet counts.

(+)-Catechin was measurable in plasma from the wild-type and apoE$^{-/-}$ mice given CAT−alcohol or RWE−alcohol, proving that (+)-catechin was absorbed through the intestinal barrier and was available for exerting biological activities in blood and tissues. Plasma (+)-catechin levels were similar in the CAT−alcohol and RWE−alcohol groups, although the CAT−alcohol drink provided five times more (+)-catechin than did the RWE−alcohol. This is consistent with a saturable absorption mechanism and/or tissue storage. Kim *et al.* (2000) have shown that substantial amounts of (−)-epicatechin were recovered in tissues from rats given green tea. In our animals, tissue storage of (+)-catechin may have occurred and was perhaps more marked in the groups given the highest (+)-catechin concentration, i.e. the CAT−alcohol group. Alternatively, the proanthocyanidin dimers contained in RWE may have split into monomers in the gut mucosa. After perfusion of isolated rat small intestine with procyanidin dimers B2 (epicatechin-(4β-8)-epicatechin) or B5

(epicatechin-(4β-6)-epicatechin), large amounts of (−)-epicatechin monomers are detected on the serosal side (Spencer *et al.* 2001). These *ex vivo* results were confirmed by an *in vivo* study (Baba *et al.* 2002) showing (−)-epicatechin in plasma from rats previously given oral procyanidin B2.

In conclusion, the present data from apoE$^{-/-}$ mice indicate that wine polyphenols are absorbed following ingestion via an alcohol-containing drink that reproduces the alcohol concentration of natural wine. Mice supported by this sole type of beverage developed similar or slightly increased atherosclerotic lesions compared with controls mice while they expressed a decreased thrombotic reaction. The antithrombotic effect appears to depend on wine phenols and not on alcohol, and its occurrence in a model of arterial thrombosis suggests that it may be mediated, at least partly, by platelet inhibition, in keeping with data showing an anti-aggregating effect of wine intake on platelets from human volunteers (Ruf, 1999; de Lorgeril & Salen, 1999; de Gaetano *et al.* 2002).

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