

## De-alcoholised white and red wines decrease inflammatory markers and NF- $\kappa$ B in atheroma plaques in apoE-deficient mice

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### Abstract

**Aims of the study** Wine polyphenols attenuate the development of atherosclerosis, which involves an inflammatory process. We studied the beneficial effect of de-alcoholised white and red wines (DWW and DRW, respectively) on the development of atheroma plaques and on the expression of biomarkers.

**Methods** We administered control or de-alcoholised wine-rich diets to apoE-deficient mice for 12 or 20 weeks. We then used optical microscopy or immunofluorescence to examine atherosclerotic lesion development in the thoracic aorta and aortic root and assessed the presence of cytokines and adhesion molecules by qPCR and immunofluorescence in total aorta and aortic root, respectively.

**Results** Atherosclerotic lesions in thoracic aorta were significantly decreased in mice supplemented with DWW (30 %) and DRW (62 %) for 20 weeks. In addition, the expressions of interferon- $\gamma$ , interleukin-1 $\beta$ , the monocyte chemoattractant protein-1 and CD68 were reduced by

DRW. The adhesion molecule P-selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 were decreased by 52, 76 and 45 %, respectively, in mice fed DRW for 12 weeks, whereas DWW reduced these parameters in a minor extent. The NF- $\kappa$ B expression in total aorta was significantly decreased in the mice treated with de-alcoholised wines for 12 weeks.

**Conclusions** DRW is shown to be more effective than DWW on cytokines and adhesion molecule expression, in the early stages of the inflammatory events associated with atherosclerosis development, probably due to the high phenolic content of red wine. Downregulation of NF- $\kappa$ B expression may be involved in the mechanism by which de-alcoholised wines modulate atherosclerosis.

**Keywords** Atherosclerosis · Wine polyphenols · Adhesion molecules · Cytokines · Nuclear factor-kappa B · Inflammation

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### Introduction

Inflammation is recognised as a key chronic process in the atherogenesis present in events ranging from the emergence of fatty streaks and endothelial activation to remodelling of the internal vascular environment and vessel occlusion. The cells involved in atherosclerosis—namely macrophages, T-lymphocytes, natural killer cells, dendritic cells and smooth muscle cells—secrete cytokines, which are crucial for the initiation and development of this inflammatory response.

Proinflammatory cytokines, such as interferon-gamma (IFN- $\gamma$ ) and interleukin-1 beta (IL-1 $\beta$ ), and chemokines such as the monocyte chemoattractant protein-1 (MCP-1) are involved in vascular inflammation by enhancing the

expression of adhesion molecules such as selectins, the vascular cell adhesion molecule-1 (VCAM-1) and the intercellular adhesion molecule-1 (ICAM-1) [1]. In addition to attracting monocytes, MCP-1 facilitates the sub-endothelial migration of these cells by engagement with its receptor (CCR2) [2]. Selectins bind sialylated and fucosylated carbohydrate ligands and mediate capture, tethering and rolling along the endothelium [3]. Endothelial VCAM-1 and ICAM-1 are involved in firm binding to the activated integrins of monocytes and favour monocyte internalisation, the first step in the development of atherosclerosis [4]. A common aspect of these inflammatory markers is their regulation by nuclear factor-kappa B (NF- $\kappa$ B), a redox-sensitive transcription factor [5].

Polyphenols are secondary metabolites of plants that show antioxidant activity [6]. Wine polyphenols normalise the oxidative stress associated with ageing in rats [7], exert protective effects against atherosclerosis in apoE-deficient mice [8], and decrease the morbidity and mortality rate of cardiovascular diseases [9, 10]. Studies on the benefits of polyphenols against inflammatory processes are scarce. The existing studies have examined the activity of these compounds *in vitro* [11, 12] and *in vivo* [13]. The administration of a mix of some of the pure polyphenols present in wines may modulate cell signalling by reducing the endothelial transmigration of monocytes and thereby contributing to the regulation of inflammation [14].

Although wine is rich in polyphenols, it also contains alcohol, which can slow down atherosclerosis [15]. However, the mechanisms involved after the administration of de-alcoholised wine have been scarcely studied. Thus, in the current paper, we study the effects of de-alcoholised white and red wines (DWW and DRW, respectively) on the progression of atherosclerosis in apoE-deficient mice, which spontaneously develop this disease [16]. We focused this research on the effects of the de-alcoholised wines on cytokines-chemokines, adhesion molecules, CD68 and NF- $\kappa$ B expression in the vascular wall at the early stages of atherosclerosis to explain the anti-inflammatory/antiatherosclerotic activities of wine polyphenols as a novel mechanism of action.

## Materials and methods

### Wine analyses

Liquid chromatography–mass spectrometry analysis of Spanish commercial white wine (WW) and red wine (RW) were performed as described in a previous paper [13]. Wines were de-alcoholised in a rotary evaporator [17], and a mass spectrometer (Fisons MD800, Thermo Finnigan, Ringoes, NJ, USA) connected to a headspace gas chromatograph (GC8000-Top, Carlo Erba, Milan, Italy) was

used to evaluate the residual ethanol in the de-alcoholised wines, using 2-methyl-1-propanol as standard.

Total phenols in 1/10 diluted wines were measured by the Folin–Ciocalteu colorimetric method [18] using gallic acid as standard. The results are expressed as mg/l gallic acid equivalents. The reducing power of the beverages was determined in 1/20 diluted wines using quercetin as standard [19]. The results are expressed as mg/l quercetin equivalents.

### Animals and diets

Male C57BL/6 J apoE-deficient mice (Charles River, L'Arbresle, France) were housed in temperature (21–23 °C) and humidity (40–60 %) controlled rooms and exposed to 12 h:12 h light–dark cycles. At 4 weeks of age, the mice were randomly distributed into three groups of fourteen mice and fed one of the following semi-purified diets for 12 (mice were aged 16 weeks) or 20 (mice were aged 24 weeks) weeks: a control diet, a DWW-rich diet or a DRW-rich diet. The solid components of the diet were mixed with 125 ml/kg of water (control diet) or with 125 ml/kg of DWW/DRW, which replaced the water in treated mice [13]. The semi-purified diets were prepared weekly, air-dried for 18 h and stored at –20 °C to prevent oxidation and loss of antioxidants. Mice ingested a dose of 25 ml of de-alcoholised wine/kg body weight/day, which was included in the diet. This is approximately equivalent to 300 ml of daily consumption for humans, as mice have a higher surface area per unit of weight than humans [20]. All diets also contained 0.15 % of cholesterol to accelerate the development of atherosclerosis. The quantity of food was restricted to 5–6 g/day to avoid differences in de-alcoholised wine and cholesterol ingestion. Food was provided and removed daily. All the mice were weighed weekly and examined after fasting overnight.

At the end of the treatment periods, mice were anaesthetised with 150 mg/kg ketamin:10 mg/kg xylazine and were exsanguinated by left ventricle puncture. Eight mice were used to evaluate the total plasma cholesterol and the atherosclerotic lesions and the other six mice were used to measure mRNA expression of cytokines and adhesion molecules by qPCR. The cytokines, adhesion molecules and CD68 expression were also determined by immunofluorescence. All experimental protocols were reviewed and approved by the University of Barcelona's Ethics Committee for Animal Experimentation, following European Union guidelines.

### Plasma analyses

Plasma was obtained by blood centrifugation at 1,770×g for 10 min at 4 °C and stored at –80 °C until the analysis at 12 and 20 weeks after dietary treatments.

Total cholesterol concentration in plasma was evaluated enzymatically at 500 nm in a spectrophotometer (iEMS Reader MF, Labsystems, Helsinki, Finland) using a commercial kit (Randox, Crumlin, UK). Results are expressed as mmol/l plasma.

#### Atherosclerotic lesions

The lesions in the aortic root were evaluated in serial 10- $\mu$ m cross-sections of the aortic root after 20 weeks of dietary treatments obtained from the left ventricle, where the aortic valves were first visible, up to where the valve cups disappeared [21].

The thoracic aorta (including the ascending aorta, the arch fraction and the descending aorta) was isolated after perfusion with 4 % paraformaldehyde in phosphate-buffered saline pH 7.4. It was then cleaned of attached connective tissue, opened longitudinally and fixed in 4 % paraformaldehyde overnight. The atherosclerotic lesions were visible and clearly distinguishable from the lesion-free areas on the luminal surface of the vessels without staining. Images were taken with a digital camera (Olympus BX-40, Hamburg, Germany) connected to a microscope (Olympus BH2-UMA) and recorded in 24-bit true image format. The atherosclerotic lesions of each mouse were quantified with the Analysis-Soft Imaging System (Olympus Soft Imaging Solutions GmbH, Münster, Germany) and expressed as a percentage of the total luminal surface.

#### qPCR assays

The total aorta (thoracic plus abdominal) was isolated, and RNA extraction in homogenised tissue was carried out using the Nucleospin Macherey–Nagel kit (Düren, Germany). The quality of the extraction was checked in a 0.8 % agarose gel, and it was quantified spectrophotometrically (Genesis UV, Thermo Scientific, Waltham, MA, USA).

The reverse transcription was performed with approximately 1  $\mu$ g of RNA using the Roche Diagnostics kit

(Mannheim, Germany). We designed the following primer sequences with Primer 3 input v. 0.4.0 and tested their presence in several exon sequences using the Ensembl Genome Browser (Table 1). The qPCR was carried out with a MyiQ Single-Color Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA), and the process was performed at a number of annealing temperatures (53 °C for VCAM-1 and ICAM-1; 55 °C for P-selectin, MCP-1 and NF- $\kappa$ B; 57 °C for IFN- $\gamma$  and IL-1 $\beta$ ). The hypoxanthine–guanine phosphoribosyltransferase gene was used as internal standard in the reaction for normalisation.

#### Immunofluorescence of MCP-1, adhesion molecules and CD68

Serial cross-sections of the aortic root after 12 and 20 weeks of dietary treatments were used for immunofluorescence studies, as previously described [21]. The following primary antibodies were used: goat anti-mouse MCP-1 (2  $\mu$ g/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-mouse P-selectin (5  $\mu$ g/ml, Chemicon Immuno-stains, Hampshire, UK), rat anti-mouse VCAM-1 (20  $\mu$ g/ml, eBioscience, San Diego, CA, USA), goat anti-mouse ICAM-1 (2  $\mu$ g/ml, R&D Systems, Abingdon, UK) and rat anti-mouse CD68 (1  $\mu$ g/ml, Abcam Inc. Cambridge, MA, USA). The corresponding secondary antibodies (4  $\mu$ g/ml) were donkey anti-rabbit 647, donkey anti-rat 488, donkey anti-goat 546 and goat anti-rat 488 from Molecular Probes (Poort Gebouw, The Netherlands). The images were captured on a confocal inverted fluorescence microscope Olympus Fluoview 500 (IX-70), and the fluorescence was quantified in lesion areas by MetaMorph analysis software (Universal Imaging Corporation, San Diego, CA, USA).

#### Statistical analyses

Data are expressed as mean and standard error (SE). The total phenols and reducing power of WW/DWW and RW/DRW were compared by Hotelling's T-square statistical

**Table 1** Sequences of primers for qPCR

Gene	Accession number	Sense primer	Anti-sense primer
MCP-1	NM 011333.2	AGGTCCTGTGTCATGCTTCTG	TCTGGACCCATTCTTCTTG
IFN- $\gamma$	NM 008337	GCTTTGCGACTCTTCTCAT	GTCACCATCCTTTTGCCAGT
IL-1 $\beta$	NM 008361	CAGGCAGGCAGTATCACTCA	AGCTCATATGGGTCCGACAG
P-selectin	NM 011347	GCTCACGGAGAGTTTGGTGT	AAGTGGTGTTCGGACCAAAG
VCAM-1	NM 011693	ATTTTCTGGGGCAGGAAGTT	ACGTCAGAACAACCGAATCC
ICAM-1	NM 010493	TCACACTGAATGCCAGCTC	GTCTGCTGAGACCCCTCTTG
NF- $\kappa$ B	NM 019408	TGACTGTGGAGCTGAAGTGG	GGTGTGTTCCAGCAAAGGT
hprt	NM 013556	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAACTT

MCP-1 monocyte chemoattractant protein-1; IFN- $\gamma$  interferon- $\gamma$ ; IL-1 $\beta$  interleukin-1 $\beta$ ; VCAM-1 vascular cell adhesion molecule-1; ICAM-1 intercellular adhesion molecule-1; NF- $\kappa$ B nuclear factor- $\kappa$ B; hprt hypoxanthine–guanine phosphoribosyl transferase

analysis. Samples were independently drawn from two independent multivariate normal distributions of two dimensions. Comparisons between alcoholised and de-alcoholised wines were carried out by the Student's *t* test for paired samples. The differences between groups of mice treated with the control diet and diets supplemented with de-alcoholised wines were analysed by one-way ANOVA and the LSD multiple comparison test for post hoc analysis. The differences between 12 and 20 weeks in the same treatment group were analysed by the Student's *t* test. Differences were considered significant at  $p < 0.05$ . All statistical analyses were performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA).

## Results

### Wine analyses

RW has a higher concentration of phenolic compounds such as gallic acid, catechin, epicatechin, quercetin and resveratrol than WW (Table 2). RW had higher total phenol content and reducing power than WW and

de-alcoholisation did not alter these characteristics (Table 2). The gas chromatography analysis showed that DWW and DRW can be considered non-alcoholic beverages (Table 2).

De-alcoholised wine-rich diets did not change body weight and total plasma cholesterol

Weight gain was approximately the same for all the groups throughout the study. Control apoE-deficient mice weighed  $11.6 \text{ g} \pm 0.39$  and  $14.9 \text{ g} \pm 0.54$  after 12 and 20 weeks of treatment, respectively. The weight of the mice fed the diets rich in DWW ( $9.9 \text{ g} \pm 1.56$  and  $16.7 \text{ g} \pm 1.94$  after 12 and 20 weeks, respectively) and DRW ( $10.0 \text{ g} \pm 0.76$  and  $12.7 \text{ g} \pm 0.94$  after 12 and 20 weeks, respectively) showed that there were no significant differences between the three groups at a fixed age.

No differences in plasma concentration of total cholesterol between the three groups of mice were observed after 12 and 20 weeks of DWW and DRW intake ( $19.9 \text{ mmol/l} \pm 3.2$  and  $17.5 \text{ mmol/l} \pm 2.5$  for the control group after 12 and 20 weeks of treatment, respectively;  $24.0 \text{ mmol/l} \pm 2.3$  and  $23.3 \text{ mmol/l} \pm 2.0$  for the mice fed the DWW-rich

**Table 2** Phenolic compounds, total phenols, reducing power and alcohol degree in white wine (WW), red wine (RW), dealcoholized white wine (DWW) and dealcoholized red wine (DRW)

	WW	RW	DWW	DRW
Phenolic compounds (mg/l)				
Phenolic acids				
Gallic acid	$3.2 \pm 0.03$	$26.7 \pm 0.30^*$		
p-Coumaric acid	$0.8 \pm 0.04$	$6.7 \pm 0.05^*$		
Caffeic acid	$0.4 \pm 0.01$	$2.5 \pm 0.02^*$		
Other phenolic acid	$4.9 \pm 0.06$	$54.0 \pm 0.40^*$		
Flavanols				
Catechin	$5.3 \pm 0.12^*$	$29.8 \pm 0.10^*$		
Epicatechin	$1.9 \pm 0.03$	$17.7 \pm 0.20^*$		
Flavonols				
Miricetin	ND	$1.8 \pm 0.03$		
Quercetin	ND	$51.4 \pm 0.60$		
Anthocyanins				
Pelargonidin-3-glucoside	ND	$3.7 \pm 0.02$		
Cyanidin-3-glucoside	ND	$0.5 \pm 0.01$		
Petunidin-3-glucoside	ND	$4.3 \pm 0.04$		
Peonidin-3-glucoside	ND	$6.1 \pm 0.07$		
Malvidin-3-glucoside	ND	$29.1 \pm 0.15$		
Cyanidin-3-glucoside-malonyl	ND	$1.4 \pm 0.00$		
Peonidin-3-glucoside-p-coumaroyl + malvidin-3-glucoside-p-coumaroyl	ND	$2.1 \pm 0.00$		
Trans-resveratrol	ND	$3.6 \pm 0.11$		
Reducing power (mg/l quercetin equivalents)	$135 \pm 12$	$1558 \pm 58^*$	$94 \pm 7$	$1744 \pm 137^*$
Total phenols (mg/l gallic acid equivalents)	$257 \pm 18$	$1434 \pm 89^*$	$269 \pm 26$	$1633 \pm 61^*$
Alcohol degree (%)	11.2	11.2	<0.6	<0.2

Data were expressed as mean (SE), ( $n = 3$ ) for phenolic compounds (from three different bottles, ( $n = 7$ ) separate evaluations for total phenols and reducing power. ND not detected

\*Mean values were significantly different comparing to the WW or DWW ( $p < 0.05$ )

diet for 12 and 20 weeks, respectively;  $21.0 \text{ mmol/l} \pm 2.3$  and  $20.1 \text{ mmol/l} \pm 1.6$  for the mice fed the DRW-rich diet for 12 and 20 weeks, respectively).

De-alcoholised wine-rich diets reduced the atherosclerotic lesions

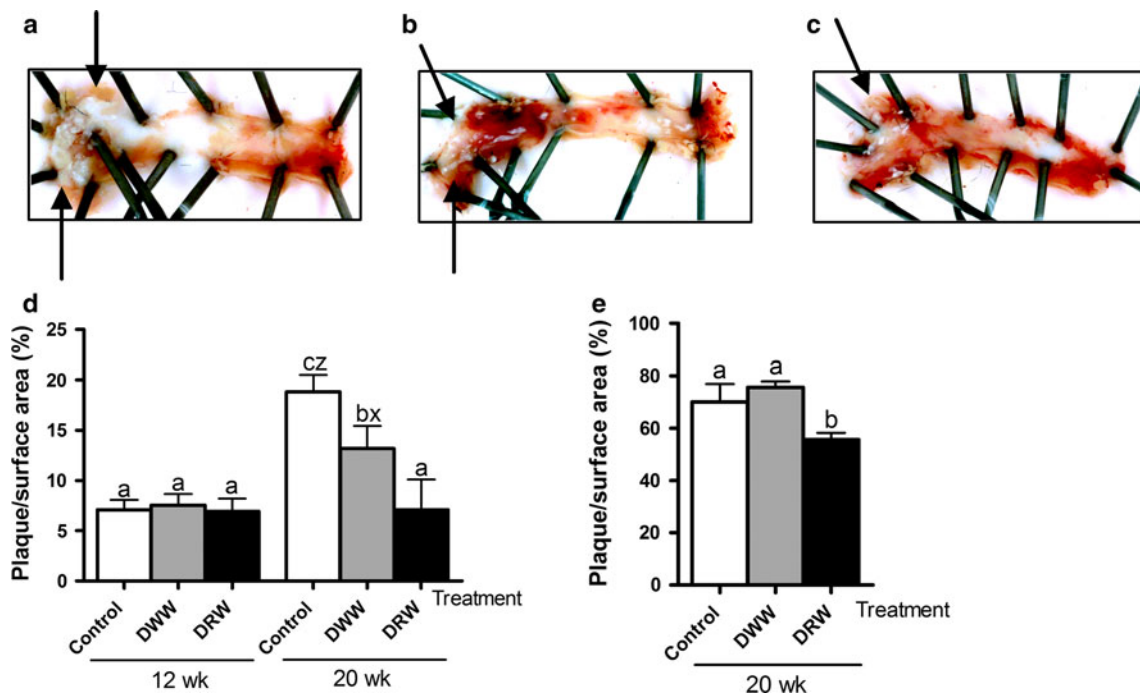
Morphometric analysis showed that atheroma plaques in the thoracic aorta were present mainly in the aortic arch of apoE-deficient mice, and only very small lesions were observed in the descending aorta. The mean percentage of atheroma plaque area with respect to the total thoracic aorta area was similar in the three groups of mice after 12 weeks of treatment. The lesions progressed with time in the control and the DWW-treated mice but not in the DRW-treated group, which suggests that DRW had a greater protective effect. Thus, the atherosclerotic lesions in the thoracic aorta were reduced in the DWW- and DRW-fed mice after 20 weeks (by 30 and 62 %, respectively,  $p < 0.05$ ) (Fig. 1a–d). Interestingly, DRW was able to significantly reduce the aortic root lesions by about 16 % ( $p < 0.05$ ) after 20 weeks while DWW had no effect (Fig. 1e). We also observed a 27 % ( $p < 0.05$ ) reduction of

the presence of macrophages in DRW after 20 weeks of dietary treatment by using CD68 (Fig. 2).

De-alcoholised wine-rich diets modulate biomarkers of inflammation

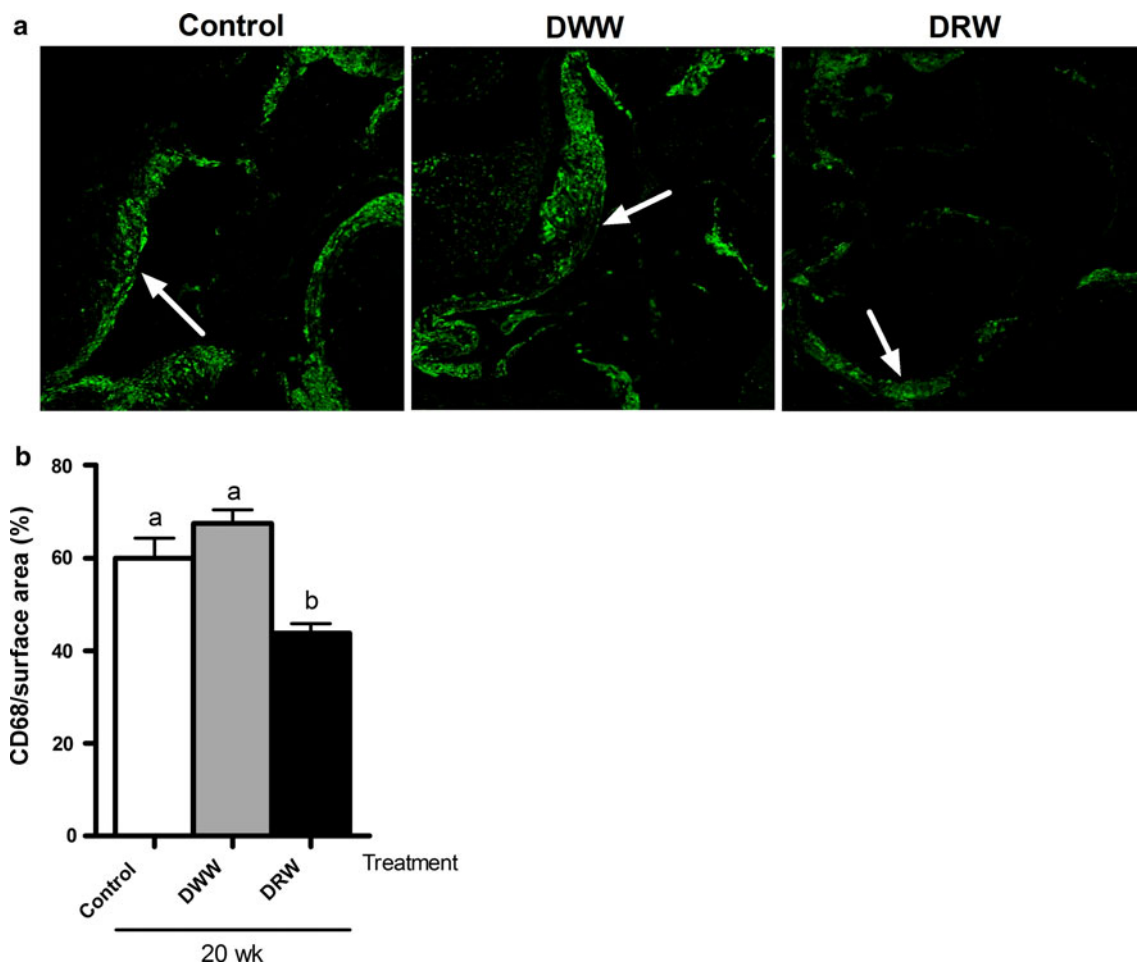
To obtain some insights into the mechanisms by which DWW and especially DRW may delay atherosclerosis development in apoE-deficient mice, we investigated whether de-alcoholised wines could decrease the inflammatory process associated with atherosclerosis by evaluating the mRNA expression of some cytokines and chemokines before atherosclerotic lesions were well established. The mRNA expression of IFN- $\gamma$ , IL-1 $\beta$  and MCP-1 at 12 weeks of treatment in total aorta, calculated as the percentage with respect to the atherosclerotic control group, was significantly decreased (77, 75 and 56 %, respectively) in the DRW group. IFN- $\gamma$  was also significantly reduced (52 %) in the DWW group at 12 weeks of treatment (Fig. 3).

We also assessed and compared the effect of the two de-alcoholised wines on endothelial signal activation, including the enhancement of adhesion molecule expression.



**Fig. 1** Effect of dietary supplementation with de-alcoholised white wine (DWW) and de-alcoholised red wine (DRW) for 12 and 20 weeks on atheroma plaque development in the thoracic aorta and aortic root of apoE-deficient mice. Representative images of the thoracic aorta opened longitudinally in the control (a), DWW-treated (b), and DRW-treated (c) apoE-deficient mice for 20 weeks. The arrows indicate the atheroma plaques, which were mainly located in the aortic arch. The atherosclerotic lesions were expressed as a

percentage of the total thoracic aorta luminal surface (d) and as a percentage of the surface area of the aortic root section (e). Data are expressed as mean (SE) ( $n = 8$  and  $n = 5$  for thoracic aorta and aortic root, respectively). Mean values within the three groups that do not share a common letter (a, b, c) were significantly different ( $p < 0.05$ ). Differences between duration of treatment were evaluated by the Student's *t* test ( $x$ ,  $p < 0.05$ ;  $z$ ,  $p < 0.001$ )



**Fig. 2** Effect of dietary supplementation with de-alcoholised white wine (DWW) and de-alcoholised red wine (DRW) for 20 weeks on CD68 expression in cross-sections of aortic root. Representative images of immunofluorescence. The arrows indicate the fluorescence signal of CD68 (a). The atherosclerotic lesions were expressed as a

percentage of the surface area of aortic root section (b). Data are expressed as mean (SE) ( $n = 5$ ). Mean values within the three groups that do not share a common letter (a, b) were significantly different ( $p < 0.05$ )

Thus, P-selectin, VCAM-1 and ICAM-1 were expressed in the total aorta of control apoE-deficient mice after 12 weeks of treatment. Although the mRNA expression of P-selectin showed a 52 % decrease in the group treated with DRW, no significant difference was observed with respect to the control atherosclerotic group (Fig. 4a). In the group treated with DRW, we found a greater reduction for VCAM-1 (by 76 %) than for ICAM-1 (by 45 %) mRNA expressions (Fig. 4b, c), while DWW only appreciably decreased VCAM-1 mRNA expression (Fig. 4b).

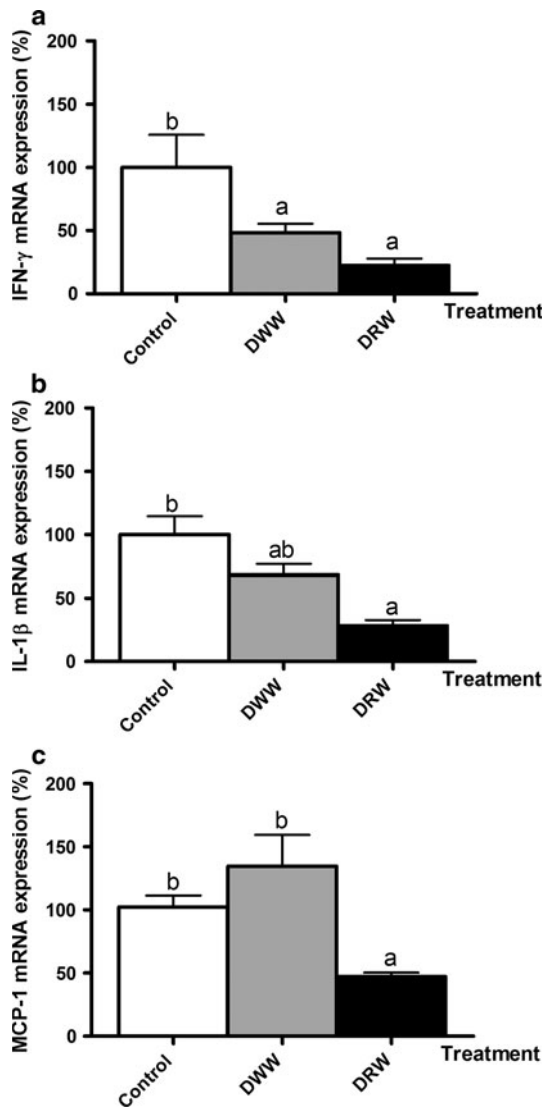
In agreement with the results for the mRNA expression of MCP-1 in total aorta, the immunofluorescence images of the aortic root of apoE-deficient mice treated with DRW for 12 weeks showed decreased MCP-1 protein expression compared to the non-treated group (Fig. 5a). Figure 5b–d presents P-selectin, VCAM-1 and ICAM-1 expression by immunofluorescence in the endothelial layer of the atheroma plaques of the aortic root and that ICAM-1 was less

expressed than VCAM-1 in the control group. The images also agree with the results obtained by the qPCR in total aorta, essentially for VCAM-1, and they show decreasing expression of the chemokine and adhesion molecules in the aorta of mice fed the DRW-rich diet for 12 weeks.

As NF- $\kappa$ B is a transcriptional factor that regulates cytokines and endothelial adhesion molecule expression, it was interesting to quantify its mRNA expression in total aorta of control apoE-deficient mice. The results indicate that mRNA expression was lower (about 60 %) in mice fed the DWW and DRW for 12 weeks than in the control group (Fig. 6).

## Discussion

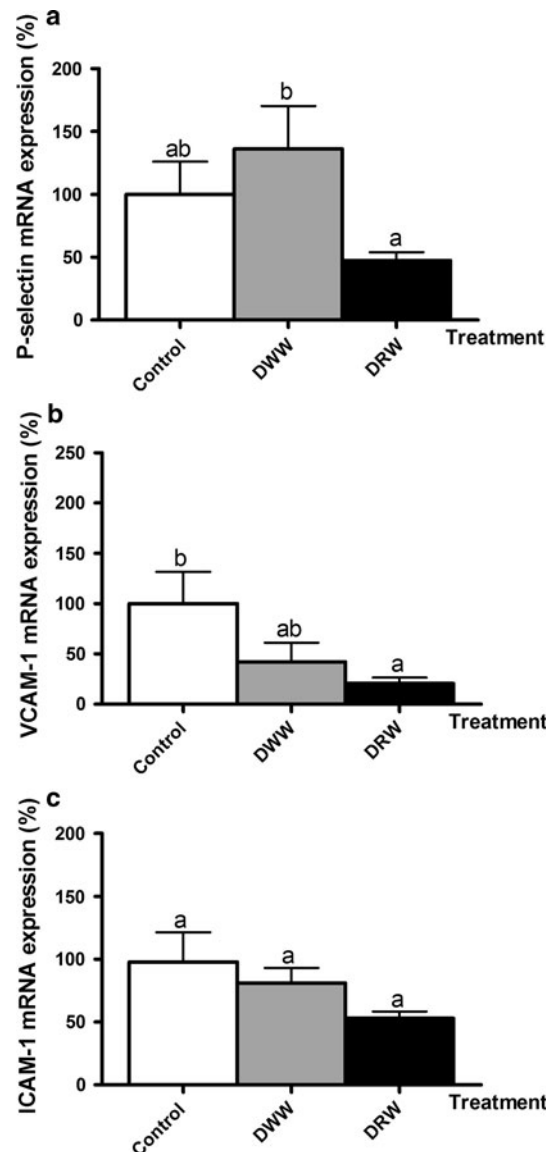
Atherosclerosis is a multifactorial disease associated with high levels of plasma cholesterol and oxidative stress.



**Fig. 3** Effect of dietary supplementation with de-alcoholised white wine (DWW) and de-alcoholised red wine (DRW) for 12 weeks on the mRNA expression of interferon-gamma (IFN- $\gamma$ ) (a), interleukin-1 beta (IL-1 $\beta$ ) (b), and monocyte chemoattractant protein-1 (MCP-1) (c) in total aorta of apoE-deficient mice. Percentage of mRNA expression relative to the control group. Data were expressed as mean (SE) ( $n = 6$ ). Mean values within the three groups that do not share a common letter ( $a, b$ ) were significantly different ( $p < 0.05$ )

ApoE is an apolipoprotein that directs the transport and metabolism of cholesterol, and an apoE deficiency favours the formation of atheroma plaques [22]. Thus, apoE-deficient mice are a highly suitable model to study the modulation of atherosclerosis by diet [16].

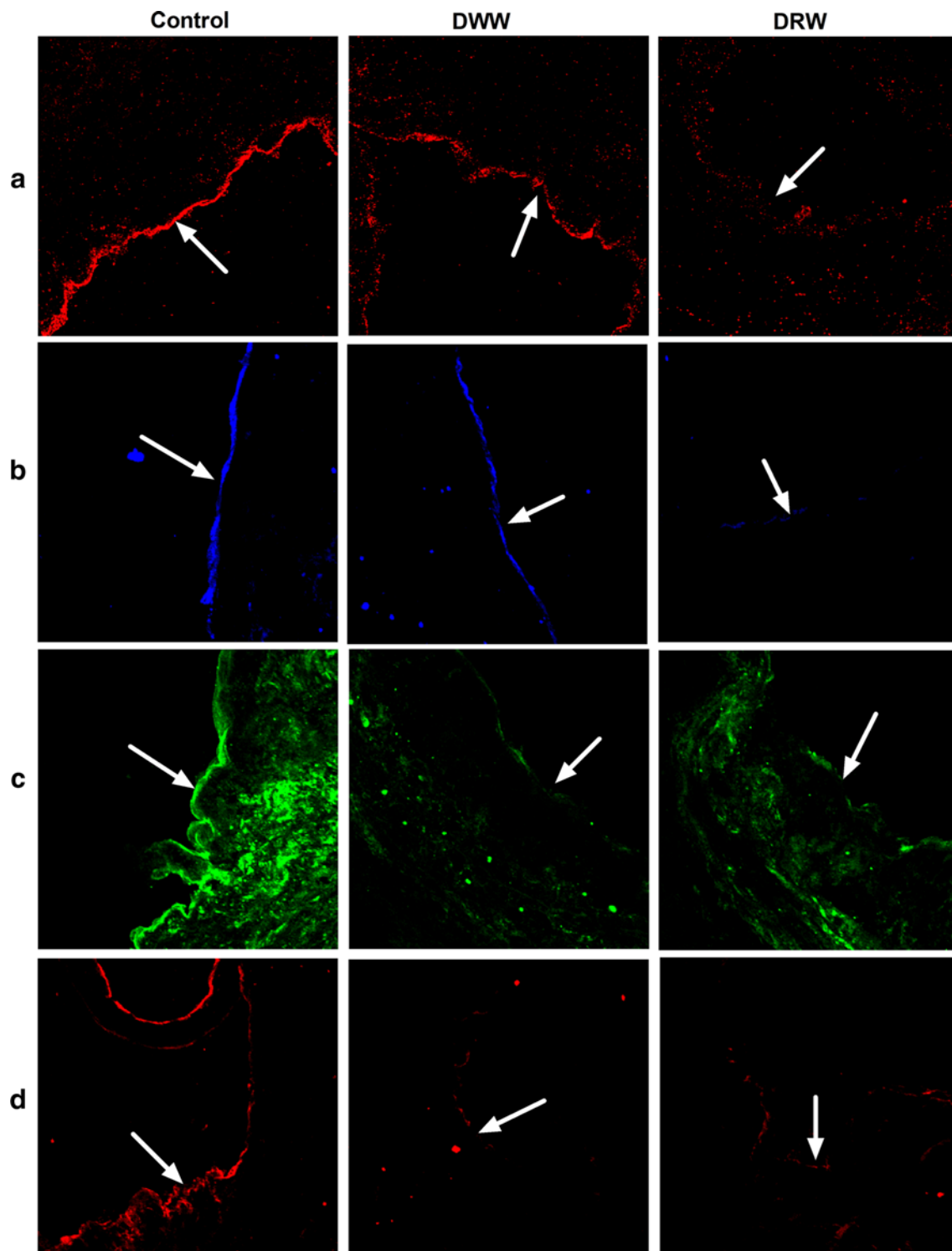
Wine contains several compounds such as polyphenols that have potent antioxidant capacity and afford protection against cardiovascular disease [8, 10, 17, 23]. Here, we provide evidence that the daily dietary administration of DWW or DRW to apoE-deficient mice, in a dose equivalent to two cups of wine a day for humans, reduces the



**Fig. 4** Effect of dietary supplementation with de-alcoholised white wine (DWW) and de-alcoholised red wine (DRW) for 12 weeks on the mRNA expression of P-selectin (a), vascular cell adhesion molecule-1 (VCAM-1) (b), and intercellular adhesion molecule-1 (ICAM-1) (c) in total aorta of apoE-deficient mice. Percentage of mRNA expression relative to the control group. Data are expressed as mean (SE) ( $n = 6$ ). Mean values within the three groups that do not share a common letter ( $a, b$ ) were significantly different ( $p < 0.05$ )

development of atherosclerotic lesions and the associated inflammation, whereas high levels of total cholesterol in the plasma of these mice were not reduced by the de-alcoholised wines. This finding is consistent with the results of other studies on apoE-deficient mice fed DRW-rich diets [8] or wine polyphenols [23].

Atherosclerotic lesions occupied more than 50 % of the aortic root and 20 % of the thoracic aorta at 20 weeks of dietary treatments, and lesions in the thoracic aorta were present mainly in the aortic arch. This confirms our



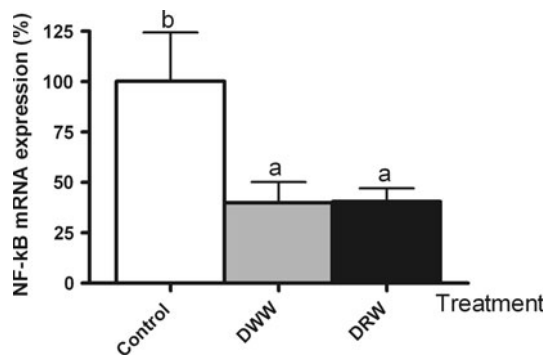
**Fig. 5** Representative images of immunofluorescence of cross-sections of aortic root of monocyte chemoattractant protein-1 (*MCP-1*) (**a**), P-selectin (**b**), vascular cell adhesion molecule-1 (*VCAM-1*) (**c**) and intercellular adhesion molecule-1 (*ICAM-1*)

(**d**) in control, de-alcoholised white wine (*DWW*) and de-alcoholised red wine (*DRW*)-treated apoE-deficient mice for 12 weeks. The *arrows* indicate the fluorescence signal of the adhesion molecules, which were mainly located in the endothelium

previous observation [21] that the development of atherosclerosis initiates at the aortic root and progresses caudally. The results are in agreement with those of Frangos et al.

[24] who proposed that atherosclerotic lesions appeared where blood flow is disturbed as a result of haemodynamic forces. The decreased surface area in the thoracic aorta





**Fig. 6** Effect of dietary supplementation with de-alcoholised white wine (DWW) and de-alcoholised red wine (DRW) for 12 weeks on the mRNA expression of nuclear factor-kappa B ( $NF-\kappa B$ ) in total aorta of apoE-deficient mice. Percentage of mRNA expression relative to the control group. Data are expressed as mean (SE) ( $n = 6$ ). Mean values within the three groups that do not share a common letter (*a*, *b*) were significantly different ( $p < 0.05$ )

affected by atherosclerosis in the apoE-deficient mice fed the DWW- and DRW-rich diets for 20 weeks suggests a delay in atherosclerotic lesion formation, which was greater in DRW-fed mice. Phenolic and non-phenolic compounds, such as ascorbic acid, sulphites and fructose, contribute to the reducing power of wines [25, 26]. The WW had several times less reducing power than the RW. This observation could be attributed to the low phenol and fructose content of the WW, as ascorbic acid and sulphites are present in similar concentrations in both wines [13]. Fructose is metabolised to uric acid in humans, which is considered a relevant antioxidant [25]. However, rodents have uricase and their plasma concentration of uric acid is very low. Thus, we can rule out its contribution in the current paper. Therefore, the distinct protective capacity shown by the two de-alcoholised wines must be attributed to their qualitative and quantitative differences in phenolic composition, which could explain the higher antiatherogenic effect of the DRW diet with its high polyphenol content. Loke et al. [27] reported a beneficial effect on thoracic aorta lesions in apoE-deficient mice after quercetin treatment with 64 mg/kg body mass/day.

Atherosclerosis is associated with an inflammatory process, and consequently, cytokines and adhesion molecules play a crucial role in the initiation/development of atherosclerosis in response to an atherogenic diet. Also, the attraction and adhesion of leucocytes to the vascular wall is essential for atheroma plaque formation and is controlled by chemokines and adhesion molecules. The expression of adhesion molecules is stimulated by early pro-inflammatory cytokines, such as  $IFN-\gamma$  and  $IL-1\beta$  [1, 2]. However, the regulation of these events by polyphenols during atherosclerosis remains poorly understood. Our findings show that the mRNA expression of  $IFN-\gamma$  and  $IL-1\beta$  in the total aorta of apoE-deficient mice was reduced after 12 weeks of

dietary supplementation with de-alcoholised wines. The differences in the expression of these cytokines in the total aorta of mice fed DWW and DRW may be attributable to the polyphenols ingested and to the metabolites generated by the mice. Here, we demonstrate that DRW reduced MCP-1 in aortic tissue of atherosclerotic mice, which is induced by  $IL-1\beta$  and inhibited by resveratrol [28]. The beneficial effect of wines may be, at least in part, attributed to polyphenols [29] and to the synergistic effect between polyphenols, as recently proposed in in vitro studies [30].

Leucocyte adhesion to endothelial cells and their subsequent infiltration into subendothelial spaces are mediated by chemokines such as MCP-1, and by various adhesion molecules such as P-selectin and VCAM-1 [31]. Sacanella et al. [32] observed that DWW and DRW were able to reduce the expression of adhesion molecules and monocyte adhesion to endothelial cells after intake of both types of wine in healthy volunteers. We observed that P-selectin and VCAM-1 mRNA expression in total aorta of apoE-deficient mice on the DRW-rich diet was considerably lower than that found in the control and in mice on the DWW-rich diet. The greater effect of DRW on VCAM-1 mRNA expression than on ICAM-1 is clinically relevant if we consider the major implication of VCAM-1 in early atherosclerosis [33] and the fact that it is an inducible molecule expressed mainly in predisposed lesion areas [34]. We have to take into account that for qPCR assays we used the total aorta (thoracic plus abdominal), and for the immunofluorescence assays, we used the aortic root, and although the results are not the same, they follow the same tendency. The absence of lesions in abdominal aorta, together with the above observation, may explain the absence of statistical significance for ICAM-1 mRNA expression, in spite of the 40 % decrease observed. From our results, we can suggest that wine compounds, probably polyphenols, may modulate cytokines expression, which in turn will downregulate the expression of adhesion molecules as was reported by resveratrol [35]. In addition, the downregulation of MCP-1 and adhesion molecules may lead to less monocytes/macrophages in the atherosclerotic lesions and thus could be responsible for the reduction in the plaque progression. This hypothesis is confirmed by the reduced monocyte/macrophages infiltration to the vascular wall after DRW intake observed in our experimental model.

$NF-\kappa B$  is a redox-sensitive transcription factor that plays a pivotal role in the development of chronic inflammatory processes by regulating the expression of cytokines and adhesion molecules [36]. Recently, Canali et al. [37] reported that red wine components modulate  $NF-\kappa B$  in endothelial cells. The present paper is the first to report the effects of de-alcoholised-wine consumption on the  $NF-\kappa B$  reduction associated with the inflammatory/atherosclerosis

response in aortic tissue, in conditions that reproduce the pathophysiological environment found *in vivo*. This result is in agreement with a study in humans that described an acute decrease in NF- $\kappa$ B after ingestion of red wine, which was attributed to an increased intake of phenolic compounds [38]. Thus, the protective effect of wine polyphenols in our experimental model for atherosclerosis may be mediated, at least in part, by downregulation of the NF- $\kappa$ B signalling pathway. However, considering that DWW reduces the expression of NF- $\kappa$ B to the same extent as DRW, other additional pathways, such as PI3K or MEK activation, may therefore be associated with the more effective action of DRW against the inflammatory process, as observed by administering wine polyphenols on smooth muscle cell proliferation and migration [39, 40]. Moreover, we must consider that DWW and DRW can have a different effect on NF- $\kappa$ B activation, and even directly be involved in cytokine and adhesion molecule expression.

In conclusion, this is the first time that the impairment of cytokine, chemokine and adhesion molecule expression in aortic tissue by DRW and, to a lesser extent, DWW has been associated with the attenuation of atherosclerotic lesion formation.

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## References

- Libby P, Sukhova G, Lee RT, Galis ZS (1995) Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. *J Cardiovasc Pharmacol* 25(Suppl 2):S9–S12
- Girn HR, Orsi NM, Homer-Vanniasinkam S (2007) An overview of cytokine interactions in atherosclerosis and implications for peripheral arterial disease. *Vasc Med* 12(4):299–309
- McEver RP (2002) Selectins: lectins that initiate cell adhesion under flow. *Curr Opin Cell Biol* 14(5):581–586
- Adams DH, Shaw S (1994) Leucocyte-endothelial interactions and regulation of leucocyte migration. *Lancet* 343(8901):831–836
- Barnes PJ, Karin M (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 336(15):1066–1071
- Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad Biol Med* 20(7):933–956
- Dal-Ros S, Zoll J, Lang AL, Auger C, Keller N, Bronner C, Geny B, Schini-Kerth VB (2011) Chronic intake of red wine polyphenols by young rats prevents aging-induced endothelial dysfunction and decline in physical performance: role of NADPH oxidase. *Biochem Biophys Res Commun* 404(2):743–749
- Stocker R, O'Halloran RA (2004) Dealcoholized red wine decreases atherosclerosis in apolipoprotein E gene-deficient mice independently of inhibition of lipid peroxidation in the artery wall. *Am J Clin Nutr* 79(1):123–130
- Renaud S, de Lorgeril M (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* 339(8808):1523–1526
- Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S et al (1995) Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch Intern Med* 155(4):381–386
- Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, Visioli F, Distante A, De Caterina R (2003) Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscl Thromb Vasc Biol* 23(4):622–629
- Ludwig A, Lorenz M, Grimbo N, Steinle F, Meiners S, Bartsch C, Stangl K, Baumann G, Stangl V (2004) The tea flavonoid epigallocatechin-3-gallate reduces cytokine-induced VCAM-1 expression and monocyte adhesion to endothelial cells. *Biochem Biophys Res Commun* 316(3):659–665
- Lopez D, Pavelkova M, Gallova L, Simonetti P, Gardana C, Lojek A, Loaiza R, Mitjavila MT (2007) Dealcoholized red and white wines decrease oxidative stress associated with inflammation in rats. *Br J Nutr* 98(3):611–619
- Norata GD, Marchesi P, Passamonti S, Pirillo A, Violi F, Catapano AL (2007) Anti-inflammatory and anti-atherogenic effects of catechin, caffeic acid and trans-resveratrol in apolipoprotein E deficient mice. *Atherosclerosis* 191(2):265–271
- Emeson EE, Manaves V, Singer T, Tabesh M (1995) Chronic alcohol feeding inhibits atherogenesis in C57BL/6 hyperlipidemic mice. *Am J Pathol* 147(6):1749–1758
- Jawien J, Nastalek P, Korbut R (2004) Mouse models of experimental atherosclerosis. *J Physiol Pharmacol* 55(3):503–517
- Benito S, Lopez D, Saiz MP, Buxaderas S, Sanchez J, Puig-Parellada P, Mitjavila MT (2002) A flavonoid-rich diet increases nitric oxide production in rat aorta. *Br J Pharmacol* 135(4):910–916
- Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Viticult* 16:144–158
- Osawa T (1999) Protective role of dietary polyphenols in oxidative stress. *Mech Ageing Dev* 111(2–3):133–139
- Pinkel D (1958) The use of body surface area as a criterion of drug dosage in cancer chemotherapy. *Cancer Res* 18(7):853–856
- Casos K, Saiz MP, Ruiz-Sanz JI, Mitjavila MT (2008) Atherosclerosis prevention by a fish oil-rich diet in apoE(–/–) mice is associated with a reduction of endothelial adhesion molecules. *Atherosclerosis* 201(2):306–317
- Mahley RW, Rall SC Jr (2000) Apolipoprotein E: far more than a lipid transport protein. *Ann Rev Genom Hum Genetics* 1:507–537
- Waddington E, Puddey IB, Croft KD (2004) Red wine polyphenolic compounds inhibit atherosclerosis in apolipoprotein E-deficient mice independently of effects on lipid peroxidation. *Am J Clin Nutr* 79(1):54–61
- Frangos SG, Gahtan V, Sumpio B (1999) Localization of atherosclerosis: role of hemodynamics. *Arch Surg* 134(10):1142–1149
- Mitsuhashi H, Ikeuchi H, Nojima Y (2001) Is sulfite an antiatherogenic compound in wine? *Clin Chem* 47(10):1872–1873
- Lotito SB, Frei B (2004) The increase in human plasma antioxidant capacity after apple consumption is due to the metabolic effect of fructose on urate, not apple-derived antioxidant flavonoids. *Free Rad Biol Med* 37(2):251–258
- Loke WM, Proudfoot JM, Hodgson JM, McKinley AJ, Hime N, Magat M, Stocker R, Croft KD (2010) Specific dietary

- polyphenols attenuate atherosclerosis in apolipoprotein E-knock-out mice by alleviating inflammation and endothelial dysfunction. *Arterioscl Thromb Vasc Biol* 30(4):749–757
28. Cullen JP, Morrow D, Jin Y, Curley B, Robinson A, Sitzmann JV, Cahill PA, Redmond EM (2007) Resveratrol, a polyphenolic phytoestrogen, inhibits endothelial monocyte chemoattractant protein-1 synthesis and secretion. *J Vasc Res* 44(1):75–84
  29. Garcia-Alonso M, Minihane AM, Rimbach G, Rivas-Gonzalo JC, de Pascual-Teresa S (2009) Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. *J Nutr Biochem* 20(7):521–529
  30. Vivancos M, Moreno JJ (2008) Effect of resveratrol, tyrosol and beta-sitosterol on oxidised low-density lipoprotein-stimulated oxidative stress, arachidonic acid release and prostaglandin E2 synthesis by RAW 264.7 macrophages. *Br J Nutr* 99(6):1199–1207
  31. Libby P (2002) Inflammation in atherosclerosis. *Nature* 420(6917):868–874
  32. Sacanella E, Vazquez-Agell M, Mena MP, Antunez E, Fernandez-Sola J, Nicolas JM, Lamuela-Raventos RM, Ros E, Estruch R (2007) Down-regulation of adhesion molecules and other inflammatory biomarkers after moderate wine consumption in healthy women: a randomized trial. *Am J Clin Nutr* 86(5):1463–1469
  33. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS (2001) A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest* 107(10):1255–1262
  34. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI (1999) Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circulation Res* 85(2):199–207
  35. Wung BS, Hsu MC, Wu CC, Hsieh CW (2005) Resveratrol suppresses IL-6-induced ICAM-1 gene expression in endothelial cells: effects on the inhibition of STAT3 phosphorylation. *Life Sci* 78(4):389–397
  36. Bharti AC, Aggarwal BB (2002) Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem Pharmacol* 64(5–6):883–888
  37. Canali R, Comitato R, Ambra R, Virgili F (2010) Red wine metabolites modulate NF-kappaB, activator protein-1 and cAMP response element-binding proteins in human endothelial cells. *Br J Nutr* 103(6):807–814
  38. Blanco-Colio LM, Valderrama M, Alvarez-Sala LA, Bustos C, Ortego M, Hernandez-Presa MA, Cancelas P, Gomez-Gerique J, Millan J, Egido J (2000) Red wine intake prevents nuclear factor-kappaB activation in peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia. *Circulation* 102(9):1020–1026
  39. Choi KH, Kim JE, Song NR, Son JE, Hwang MK, Byun S, Kim JH, Lee KW, Lee HJ (2010) Phosphoinositide 3-kinase is a novel target of piceatannol for inhibiting PDGF-BB-induced proliferation and migration in human aortic smooth muscle cells. *Cardiovasc Res* 85(4):836–844
  40. Iijima K, Yoshizumi M, Hashimoto M, Akishita M, Kozaki K, Ako J, Watanabe T, Ohike Y, Son B, Yu J, Nakahara K, Ouchi Y (2002) Red wine polyphenols inhibit vascular smooth muscle cell migration through two distinct signaling pathways. *Circulation* 105(20):2404–2410