p-Coumaric acid, a common dietary phenol, inhibits platelet activity *in vitro* and *in vivo*

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p-Coumaric acid (3-(4-hydroxyphenyl)-2-propenoic acid; 4CA), is a ubiquitous plant metabolite with antioxidant and anti-inflammatory properties. The antiplatelet activity of this compound was analysed both *ex vivo* and *in vitro*. 4-CA, administered to rabbits for 2 weeks at the dose of 5 mg/kg, mixed with food, inhibited ADP-induced platelet aggregation without affecting blood coagulation. This effect was associated with a marked increase in plasma antioxidant activity, measured as ferric reducing ability of plasma, and with the reduction of thromboxane B_2 production. The antiplatelet effect was confirmed by *in vitro* experiments on human blood: 4CA (500 μM and 1 mM) reduced ADP-induced platelet aggregation (55·2 (SE 4·01) % and 35·6 (SE 2·35) % relative to basal level, respectively). 4CA was able to modify platelet function, measured with PFA-100™, a shear-inducing device that simulates primary haemostasis. 4CA interfered also with arachidonic acid cascade, reducing thromboxane B_2 production and lipopolysaccharide-induced prostaglandin E_2 generation (IC_{50} 371 and 126 μM, respectively). The data show that 4CA is an antioxidant compound with good antiplatelet activity at doses that can be obtained with dietary intervention, suggesting possible applications for primary prevention of vascular disease.

Phenols: Platelet aggregation: Antioxidant activity

Epidemiological studies suggest that moderate red wine consumption may reduce the risk of CVD (de Gaetano & Cerletti, 2001) and this effect can be partially due to some naturally occurring constituents of this beverage. Wine contains a wide variety of polyphenols, including tannins, anthocyanins and phenolic flavonoids (including flavonols and flavones) (Rasmussen *et al.* 2005) that may contribute to the cardioprotective effects of grape products, as suggested by several studies (Duffy *et al.* 2001; da Luz & Coimbra, 2004; Vita, 2005).

One proposed mechanism of the apparent benefit of various sources of polyphenols is their favourable effect on platelet aggregation (Ruf, 2004; Cordova *et al.* 2005). Platelet aggregation plays a critical role in the pathogenesis of vascular disorders, and there is evidence that antiplatelet therapy reduces the risk of CVD (Youssef *et al.* 2005).

Polyphenols may inhibit platelet aggregation through a number of different mechanisms, including inhibition of cyclooxygenase (COX), lipoxygenase (Schubert *et al.* 1999; Hong *et al.* 2001) and phosphodiesterase activities (Dell'Agli *et al.* 2005). Inhibition of phosphodiesterase activity, increasing platelet levels of cAMP or cGMP, lowers platelet calcium levels and inhibits platelet activation both *in vitro* and *in vivo* (Demrow *et al.* 1995).

Other inhibitory effects of polyphenols on platelet aggregation include scavenging of reactive oxygen species such as superoxide anion (Frei & Higdon, 2003), decreasing phospholipase C (Pignatelli *et al.* 2000) and inhibition of lipid peroxidation (Aviram *et al.* 2002).

A relevant effect of polyphenols on the cardiovascular system could be due to their ability to interact with the L-arginine–nitric oxide pathway leading to the generation of NO from vascular endothelium (Andriambeloson *et al.* 1997), since this molecule has vasorelaxant and antiaggregation properties. Freedman *et al.* (2001) examined the effects of grape juice on platelet function, observing that the addition of grape juice to platelets *ex vivo* was associated with a decrease in the production of superoxide anion from platelets, a reduction in their aggregation and a significant increase in platelet NO production, effects related to reduced activation of protein kinase C. The findings were also reproduced *in vivo* in platelets isolated from healthy volunteers who had consumed grape juice (7 ml/kg per d) for 2 weeks (Freedman *et al.* 2001). Grape seed and grape skin, in combination, exhibited a high antiplatelet effect *in vitro* and *ex vivo*, in dog platelets at doses ranging between 2 and 20 mg/kg (Shanmuganayagam *et al.* 2002).

Results of studies on the effect of red or white wine consumption on platelet aggregation are contrasting (Pignatelli *et al.* 2002). Some studies have focused on the effect of single wine constituents such as resveratrol, a phytoalexin present at low concentration in grapes and some wines, which is able to reduce several platelet functions (Olas *et al.* 2002), and the flavonoid quercetin, which inhibits collagen-induced platelet aggregation and adhesion (Pignatelli *et al.* 2000; Kikura *et al.* 2004).

p-Coumaric acid (3-(4-hydroxyphenyl)-2-propenoic acid; 4CA) is another wine constituent, widely distributed in cereals, fruits and vegetables (being a ubiquitous plant metabolite), intermediate in the synthesis of many phenols (Clifford, 2000). Studies performed in our laboratory demonstrate that 4CA has marked antioxidant and anti-inflammatory properties in rat colonic mucosa (Guglielmi *et al.* 2003; Luceri *et al.* 2004).

The purpose of the present study was to evaluate the effects of 4CA on systemic oxidant–antioxidant status and on platelet function.

Materials and methods

Venous blood was sampled from rabbits and healthy human volunteers using a 21-gauge needle (Pentaven®; Pentaferte, Campli, Teramo, Italy) and collected in evacuated polypropylene tubes containing 3·8 % sodium citrate (9:1, v/v; Vacuette®; Greiner bio-one, Kremsmünster, Austria) or EDTA (Vacutest®; Vacutest Kima, Azzergrande, Padova, Italy).

4CA and arachidonic acid (AA) were purchased from the Sigma Chemical Co. (St Louis, MO, USA) and ADP was purchased from Mascia Brunelli (Milan, Italy). Thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2) enzyme immunoassay kits were obtained from the Cayman Chemical Co. (Ann Arbor, MI, USA).

Ex vivo *studies in rabbit blood*

All the procedures adopted were carried out in agreement with the European Union Regulations on the Care and Use of Laboratory Animals (OJ of ECL 358/1, 12/18/1986) and the experiments were conducted according to Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/1992). Blood samples from rabbits were obtained by puncture of an ear vein.

The first experiment was a dose–response study: twelve albino rabbits (2·5–3 kg) obtained from Charles River (Lecco, Italy) were randomly divided into three groups of four animals, each housed in a single cage: controls were fed a powdered standard lab chow purchased from Harlan (Milan, Italy), the 2030 Global Diet for rabbits, whereas the treated groups received the same diet enriched with 4CA; the proper amount of 4CA was mixed with the daily dietary mass (190 g) to a final dosage of 5 and 500 mg/kg, for 4 weeks.

The second experiment was a time-course study, performed using the lowest effective 4CA dose (5 mg/kg). Blood samples were obtained from five rabbits at time 0 and then after 2, 4, 6, 8 and 10 weeks of treatment.

Platelet aggregation. At the indicated time-points, blood samples were collected from rabbits into tubes containing 3·8 % trisodium citrate solution (9:1, v/v). Platelet-rich plasma (PRP) was obtained by centrifuging the blood samples (80 *g*, 10 min, 20°C). After PRP removal, the remaining plasma was centrifuged (900 *g*, 10 min, 20°C) and the supernatant was collected to obtain platelet-poor plasma, used to dilute autologous PRP and to equilibrate the aggregometer. Platelet aggregation was determined by a four-channel aggregometer (model PAP-4; Biodata Corporation, Horsham, PA, USA) according to the method of Born (1962). Aggregation was induced by the addition of ADP or AA (final concentrations 3 and 2·86 mM, respectively). Aggregation was measured over 5 min and the extent of aggregation was estimated by measuring the maximal curve height above the baseline level.

Determination of anticoagulant activity. An aliquot of venous blood samples collected into citrate-containing tubes was used to determine prothrombin time and activated partial thromboplastin time, using a coagulometer (Dade Behring, Newark, DE, USA).

Whole blood cell counts. Blood cell counts, Hb concentration, haematocrit value and mean platelet volume were measured in whole blood collected in EDTA-containing tubes, using a haematology analyser (Coulter Electronics, Hialeah, FL, USA).

Antioxidant activity. Plasma total antioxidant power (ferric reducing ability of plasma) measures the ability of the antioxidants contained in a sample to reduce ferric ions (Benzie & Strain, 1996). The method is based on the reduction of a ferric-tripyridyltriazine [Fe(III)–tripyridyltriazine] complex to the ferrous [Fe(II)] form, which has an absorption maximum at 593 nm. Fe(III) is used in excess so that the colour formation is linearly related to the amount of reductant (antioxidant) present. Data are expressed as μmol/l.

Thromboxane B_2 release from activated platelets. TXB_2, the stable metabolite of thromboxane A_2 released during aggregation, was used as an index of COX-1 activity (Patrignani *et al.* 1994). Immediately after platelet aggregation induced by ADP or AA, the tubes containing PRP were transferred to an ice bath and later centrifuged in an Eppendorf microcentrifuge for 5 min. The supernatant was collected and frozen at −20°C for the determination of TXB_2 concentration by enzyme immunoassay kit, according to the manufacturer's instructions.

In vitro *study in human blood*

The study protocol followed our institutional guidelines and an informed consent was obtained from each subject. Ten healthy, non-smoking volunteers (six women, four men) aged 23–39 years participated in the study. None of the participants had reported previous CVD, metabolic or systemic disease, or had taken any drug in the 2 weeks before the study. Women were premenopausal and were not taking oestrogens.

Venous blood was carefully drawn from an antecubital vein by trauma-free venepucture with rapid flow of blood. Within 10 min, whole blood was incubated for 60 min at room temperature with 4CA (dissolved in 2 % 1 M-NaOH) or the vehicle alone. 4CA final concentrations were 10, 100, 500 and 1000 μM.

Platelet aggregation. After 60 min incubation with 10, 100, 500 and 1000 μM-4CA, blood samples were centrifuged (160 g, 10 min, 20°C) and the supernatant separated as PRP. After PRP removal, the remaining plasma was centrifuged (2000 g, 10 min, 20°C) and the supernatant was separated to obtain platelet-poor plasma. PRP was diluted to $2-3 \times 10^{11}$ platelets/l with autologous platelet-poor plasma. Platelet aggregation was performed at 37°C using a four-channel photometric aggregometer (model PAP-4; Biodata Corporation) based on the method of Born (1962). Aggregation was induced by ADP (final concentration 3 μM). This concentration was chosen to ensure a high probability of aggregation based on previous experience. The aggregation was allowed to proceed for 5 min and the extent was estimated quantitatively by measuring the maximal curve height above baseline level.

Whole blood cell counts. Whole blood cell counts were measured as described earlier for rabbit samples.

PFA-100™ closure time. Citrated whole blood samples incubated at room temperature for 60 min with 500 μM or 1 mM 4CA were analysed by PFA-100™ (Dade Behring), a high-shear-inducing device that simulates primary haemostasis. We determined closure times on duplicate samples of 800 μl using cartridges containing collagen–epinephrine or collagen–ADP membranes.

Prostaglandin E_2 production. Plasma PGE_2 levels were determined as an index of whole blood COX-2 activity. Aliquots of citrated whole blood were incubated with 4CA (dissolved in 2% 1 M-NaOH) or the vehicle alone, in the presence of endotoxin (lipopolysaccharide) derived from *Escherichia coli* 026:B6 (Sigma Chemical Co.) at 37°C for 60 min. The contribution of platelet COX-1 was suppressed by the addition of 200 μM-aspirin (Patrignani *et al.* 1994). The final concentrations of 4CA were 10, 100, 500 μM and 1 mM. Aliquots of citrated whole blood were incubated with celecoxib (final concentration 1 μM) in the presence of 10 μg/ml lipopolysaccharide, as positive control. Plasma was separated by centrifugation (1000 g, 20 min, 20°C) and assayed for PGE_2 using an enzyme immunoassay kit according to the manufacturer's instructions.

Thromboxane B_2 release from human activated platelets. The determination of TXB_2 production was performed as described earlier for rabbit samples.

Statistical analysis

Experiments were performed at least in duplicate and the results were expressed as means and their staandard errors. Statistical analysis was conducted using a two-way ANOVA.

Results

Ex vivo *studies in rabbit blood*

Inhibitory effects of 4CA on platelet aggregation. The dose–response study was performed using two extreme doses: 5 and 500 mg/kg 4CA, chosen considering that the estimated human daily intake of 4CA is about 15 mg/kg. At both doses 4CA was able to inhibit AA-induced platelet aggregation in a not statistically significant dose-dependent way (Fig. 1).

In the second experiment we observed that ADP-induced platelet aggregation was inhibited by 4CA (5 mg/kg) in a
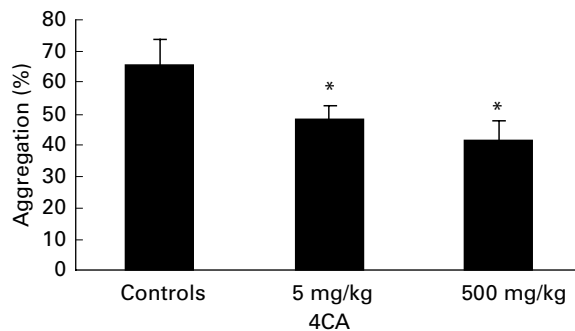

Fig. 1. Inhibition of platelet aggregation in blood samples from rabbits fed a standard diet (controls) or a diet enriched with *p*-coumaric acid (4CA; 5 and 500 mg/kg) for 4 weeks. For details of procedures, see p. 459. Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the controls: *$P < 0.05$.

time-dependent manner: the effect started after 2 weeks, reaching the maximum inhibitory effect after 8 weeks of treatment (Fig. 2).

Blood cell counts and coagulation tests. Treatments with 4CA did not modify blood cell counts, Hb concentration, haematocrit value, mean platelet volume, platelet count, prothrombin time or activated partial thromboplastin time (data not shown).

Inhibition of thromboxane B_2 production. TXB_2 release was measured in both dose–response and time-course experiments. In rabbits treated with 4CA (5 or 500 mg/kg for 4 weeks), TXB_2 release, measured in PRP after AA-induced aggregation, was significantly reduced and the differences between the two doses were not statistically significant (Fig. 3(A)).

In rabbits treated with 4CA (5 mg/kg) TXB_2 release during ADP-induced aggregation was significantly reduced after 2 weeks of treatment (Fig. 3(B)).

Ferric reducing ability of plasma assay. The administration of 4CA (5 mg/kg) significantly increased the plasma antioxidant activity from 160 (SE 15) μmol/l at time 0 to 310 (SE 30) μmol/l after 1 week ($P < 0.05$) and to 380 (SE 50) μmol/l after 3 weeks of feeding ($P < 0.05$).
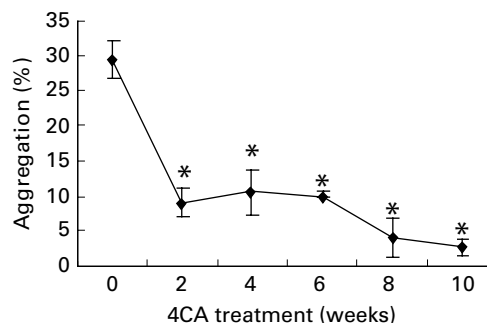

Fig. 2. Inhibition of platelet aggregation in blood samples from rabbits fed a standard diet enriched with 5 mg/kg *p*-coumaric acid (4CA). For details of procedures, see p. 459. Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those at time 0: *$P < 0.05$.
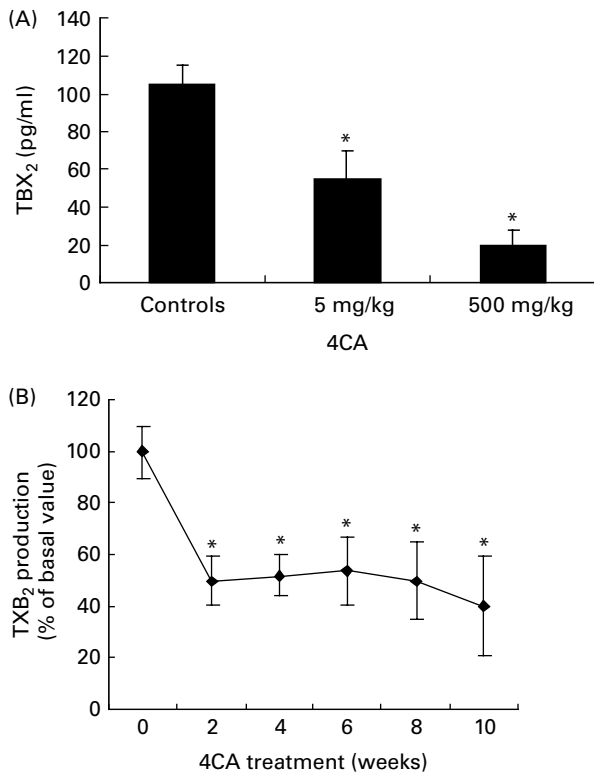
Fig. 3. A, Thromboxane B_2 (TXB2) production after arachidonic acid-induced platelet aggregation in blood samples from rabbits fed *p*-coumaric acid (4CA; 5 or 500 mg/kg) for 4 weeks. B, TXB_2 production after ADP-induced aggregation in blood samples from rabbits treated with 4CA (5 mg/kg). For details of procedures, see p. 459. Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the controls (A) or at time 0 (B): *$P < 0.05$.

Table 1. Effect of blood incubation with different *p*-coumaric acid (4CA) doses on platelet function†

(Mean values with their standard errors)

	Collagen–epinephrine closure time (s)		Collagen–ADP closure time (s)	
	Mean	SE	Mean	SE
Control	127·3	6·8	96·2	5·4
500 μM-4CA	178·9	29·5	166·0	32·5
1 mM-4CA	216·7	31·4*	178·4	31·0*

Mean values were significantly different from those of the control group: *$P < 0.05$.
† For details of procedures, see p. 459.

in marked induction of PGE_2 formation. The vehicle alone (2 % 1 M-NaOH) had no effect on PGE_2 production, whereas 4CA reduced lipopolysaccharide-induced PGE_2 generation with an IC_{50} of 126 μM.

Discussion

Platelets play a pivotal role in health and disease, given their central involvement in haemostasis and thrombosis. Treatment with drugs that specifically inhibit platelet function has proven to be beneficial in the prevention of thrombosis.

The relationship between a diet high in vegetable products and a decreased risk of thrombosis is well established, although the explanation for this is far from clear (Liu & Willett, 2002). A number of studies have focused on the effects of dietary component, such as flavonoids on platelet function. Quercetin (40–100 μM) and catechin (100–420 μM) inhibit platelet aggregation *in vitro*, inhibiting the release of platelet hydrogen peroxide elicited by collagen, with almost complete suppression when used in combination (Pignatelli *et al.* 2000).

Resveratrol is another wine compound with several health-promoting properties; the most widely accepted mechanism of resveratrol-mediated cardioprotection is its ability to inhibit platelet aggregation. At the concentration of 3·56 mg/l, it lowers platelet aggregation by 50 % in healthy volunteers (Bertelli *et al.* 1995). Moreover, resveratrol and quercetin inhibited thrombin-induced ADP and ATP secretion from platelets in a concentration-dependent manner (Kaneider *et al.* 2004).

In the present study we analysed the effect of another wine component, 4CA, which is also widely distributed in fruits, vegetables, cereals, tea and wine, mostly as conjugates. It is present as a free acid (about 22 mg/l) and as *p*-coumaroyltartaric acid (about 139 mg/l) in red wine (Ghiselli *et al.* 1998). Other abundant sources of 4CA are apples and berries (69–1700 mg/kg) and maize bran (2·9 g/kg) (Stich, 1991; Clifford, 2000; Zhao *et al.* 2005).

Administered *in vivo*, 4CA was able to reduce significantly platelet aggregation induced by AA and ADP after 2 weeks of treatment and at a dose comparable to the daily intake for cinnamates (Clifford, 2000). In addition, the TXB_2 production measurements were well correlated with platelet aggregation in both experiments.

In vitro, 4CA inhibited platelet aggregation without effects on prothrombin time, activated partial thromboplastin time, Hb concentration, haematocrit value, mean platelet volume and platelet count. In PFA-100™ analysis, which simulates

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Inhibitory effects of 4CA on platelet function. All volunteers had normal platelet function and the vehicle alone (2 % 1 M-NaOH) had no effect on platelet aggregation. At the doses of 500 μM and 1 mM, 4CA significantly reduced ADP-induced platelet aggregation (35·6 (SE 2·35) % and 55·18 (SE 4·01) % of the basal level, respectively, $P < 0.05$), while at lower concentrations (10 and 100 μM) it did not induce a significant effect (data not shown).

Platelet function measured as PFA-100™ closure times. All volunteers had normal values and the vehicle alone (2 % 1 M-NaOH) had no effect on closure time. Blood samples incubated for 60 min with 500 μM-4CA showed a prolonged closure time. When incubated with 1 mM-4CA the difference was statistically significant, using cartridges containing both collagen–epinephrine and collagen–ADP membranes (Table 1).

Inhibition of thromboxane B_2 production. At the doses of 500 μM and 1 mM, 4CA was able to reduce TXB_2 release after ADP-induced platelet aggregation in PRP from healthy volunteers, with an IC_{50} of 371 μM.

Inhibition of prostaglandin E_2 production. Incubation of blood samples from healthy donors with lipopolysaccharide, in the presence of aspirin to block COX-1 activity, resulted

the process of platelet adhesion and aggregation induced by vascular injury, we also observed a significant effect of 4CA at high concentrations, using ADP as well as epinephrine cartridges. *In vivo* and *in vitro* studies suggested that 4CA modulates platelet aggregation through both COX-dependent and independent mechanisms.

There are many platelet pathways which could be influenced by 4CA: polyphenols may inhibit platelet aggregation because of their antioxidant activity (Salvemini & Botting, 1993; Neiva *et al.* 1999). 4CA is a potent inhibitor of LDL oxidation *in vitro* (Satué-Gracia *et al.* 1999; Morton *et al.* 2000). Owen *et al.* (2000) reported the antioxidant activity of 4CA against reactive oxygen species produced by hypoxanthine/xanthine oxidase and Lodovici *et al.* (2001) described its ability to reduce oxidative DNA damage induced *in vitro* by iron and cumene hydroperoxide. The present results show that the administration of 4CA (5 mg/kg) significantly increases plasma antioxidant activity after 1 week of treatment and more markedly after 3 weeks, confirming our previous data on the antioxidant activity of this molecule *in vivo*, on rat colon mucosa (Guglielmi *et al.* 2003).

It has also been suggested that the effect of polyphenols on platelet aggregation is related to the inhibition of the COX pathway, reducing the formation of TXB_2, a potent aggregating and vasoconstricting agent. The anti-inflammatory effects of 4CA are controversial since it has anti-inflammatory activity if administered locally, but demonstrates no effects *in vivo* on carrageenan-induced paw edema when administered at 200 mg/kg per d, 1 h before carrageenan injection (Fernandez *et al.* 1998). Recently we demonstrated that an acute intestinal inflammation induced by dextran sulphate sodium can be inhibited by pretreatment with 4CA (50 mg/kg) and that this effect was associated with the suppression of COX-2 expression and activity (Luceri *et al.* 2004). In the present study we demonstrate that the reduction of platelet aggregation induced by 4CA is correlated with decreased TXB_2 production, associated with the inhibition of COX-1 and COX-2 activities.

In summary, 4CA, a dietary phenolic compound, acts as a potent systemic antioxidant and shows good antiaggregant properties at concentrations that can be obtained through regular consumption of vegetables, cereals, fruits or a moderate quantity of red wine. It has been estimated that the daily dietary intake for total cinnamates and hydroxybenzoic acid derivatives should be 1 g/d, corresponding to about 15 mg/kg per d in man (Clifford, 2000; Tomàs-Barberan & Clifford, 2000).

Healthy subjects with no dietary restrictions had a relatively low concentration of 4CA in faecal water, ranging from 0·3 to 1·44 μM (Jenner *et al.* 2005). However, in a recent paper by Garrait *et al.* (2006) the authors demonstrated that 4CA is absorbed by all the digestive tracts and, when orally administered, is mainly eliminated in the urine. Konishi *et al.* (2003), studying 4CA intestinal absorption, demonstrated that 4CA is actively transported by the colonic monocarboxylate transporter in Caco-2 cells; after an oral dose of 16·4 mg/kg, the serum C_{max} was 165·7 μM and the area under the curve was almost 3 mM in rats (Konishi *et al.* 2004).

Free phenolic acids are well absorbed but in foods they are mainly in bound form, esterified to cell wall polymers. It has been reported that IR irradiation or fermentation can liberate covalently bound phenolic compounds with antioxidant activities and that the irradiation of sesame seeds significantly increases the amount of free 4CA (Lee *et al.* 2005); these data suggest that some industrial processes can enrich the amount of free 4CA in food.

In conclusion, the present data suggest that dietary intervention with foods rich in 4CA may be a possible strategy for primary prevention of vascular diseases. Further studies on patients at risk could help to determine the potential beneficial effects of 4CA as an antiplatelet and systemic antioxidant agent for prevention and treatment of thrombotic diseases.

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