

Effects of antiplatelet components of tomato extract on platelet function in vitro and ex vivo: a time-course cannulation study in healthy humans¹⁻³

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ABSTRACT

Background: Natural antithrombotic agents that influence platelet function are of potential interest for primary prevention of cardiovascular disease. Previous reports showed that tomato extracts inhibit platelet aggregation in vitro, but little is known of the active components, their mode of action, or their efficacy in vivo.

Objective: The objectives of the study were to examine the antiplatelet activity of specific tomato components by in vitro experimentation and to establish their ex vivo efficacy in healthy humans.

Design: The mechanisms of action of antiplatelet components isolated from tomato extracts were examined in vitro. A 7-h time-course study was carried out in cannulated human subjects ($n = 23$) to determine the ex vivo efficacy of a supplement drink containing tomato extract and the onset and duration of antiplatelet effects.

Results: The inhibition of ADP-, collagen-, thrombin-, and arachidonate-mediated platelet aggregation by tomato extract components appears to be linked to the inhibition of glycoprotein IIb/IIIa and platelet secretory mechanisms. We found a significant inhibition of baseline platelet function, from $2.9 \pm 1.4\%$ (optimal ADP concentrations; $P = 0.03$) to $20.0 \pm 4.9\%$ (suboptimal ADP concentrations; $P < 0.001$), 3 h after supplementation with a dose of tomato extract equivalent to 6 tomatoes. The observed effects persisted for >12 h. Coagulation variables were not affected.

Conclusions: The ingestion of tomato components with in vitro antiplatelet activity significantly affects ex vivo platelet function. The reported cardioprotective effects of tomatoes are potentially linked to a modulation of platelet function. *Am J Clin Nutr* 2006;84:570-9.

KEY WORDS Tomato, platelet, natural antiplatelet agents, thrombosis, cardiovascular disease

INTRODUCTION

The prevalence of atherosclerosis and coronary artery disease in industrialized countries (1) and the need to slow the progression of these diseases have focused attention on the influence of diet on the cardiovascular system (2-5). It is recognized that some dietary components have the potential to reduce levels of specific risk factors for cardiovascular disease (CVD) (6-11). In 2003, a review of the literature suggested that there was compelling evidence for tomatoes to be considered as cardiovascular-protective foods (12). Many nutrients present in the tomato are associated with theoretical or proven effects on the cardiovascular system (12, 13), and much literature dealing specifically with

the possible cardioprotective effects of lycopene has been published (14-17). Studies have suggested a link between tomato consumption and the lower incidence of CVD in Mediterranean countries (18, 19). Some studies that support this link have found higher plasma lycopene concentrations in their populations (20, 21). However, other studies have reported that, despite an inverse association between CVD risk and high intakes of tomato-based products, dietary lycopene is not strongly associated with the risk of CVD (18, 22). This finding suggests that other, unidentified compounds in tomatoes may have cardioprotective effects.

We previously identified potent antiplatelet factors in tomato juice that inhibit platelet aggregation in response to ADP, collagen, and thrombin (23). Platelets play a crucial role not only in hemostasis but also in the development of CVD (24). Evidence is increasing that acute clinical manifestations of coronary atherosclerotic disease are caused by plaque disruption and subsequent platelet-thrombus formation (25). Platelet activity can influence the progression of disease as well as the stability of atherosclerotic plaques (26, 27). We hypothesize that observed cardiovascular benefits attributed to the tomato could be linked to antiplatelet activity and thus to the suppression of platelet function in vivo. This type of natural antithrombotic agent could have an application in primary prevention of CVD.

This report presents further investigations into the antiplatelet activity of tomato juice. The compounds responsible for the observed activity were isolated as a mixture and subfractionated. The ability of these subfractions to modify the platelet response to stimulation by different agonists was evaluated by examining the changes in platelet aggregation response and expression of platelet activation markers on the platelet surface. In addition, the ex vivo efficacy of the tomato extract was evaluated in a study designed to examine the acute effect of supplementation with tomato extract, which was given in an orange juice matrix.

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² Supported by Proxavis plc.

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Received December 13, 2005.

Accepted for publication May 8, 2006.

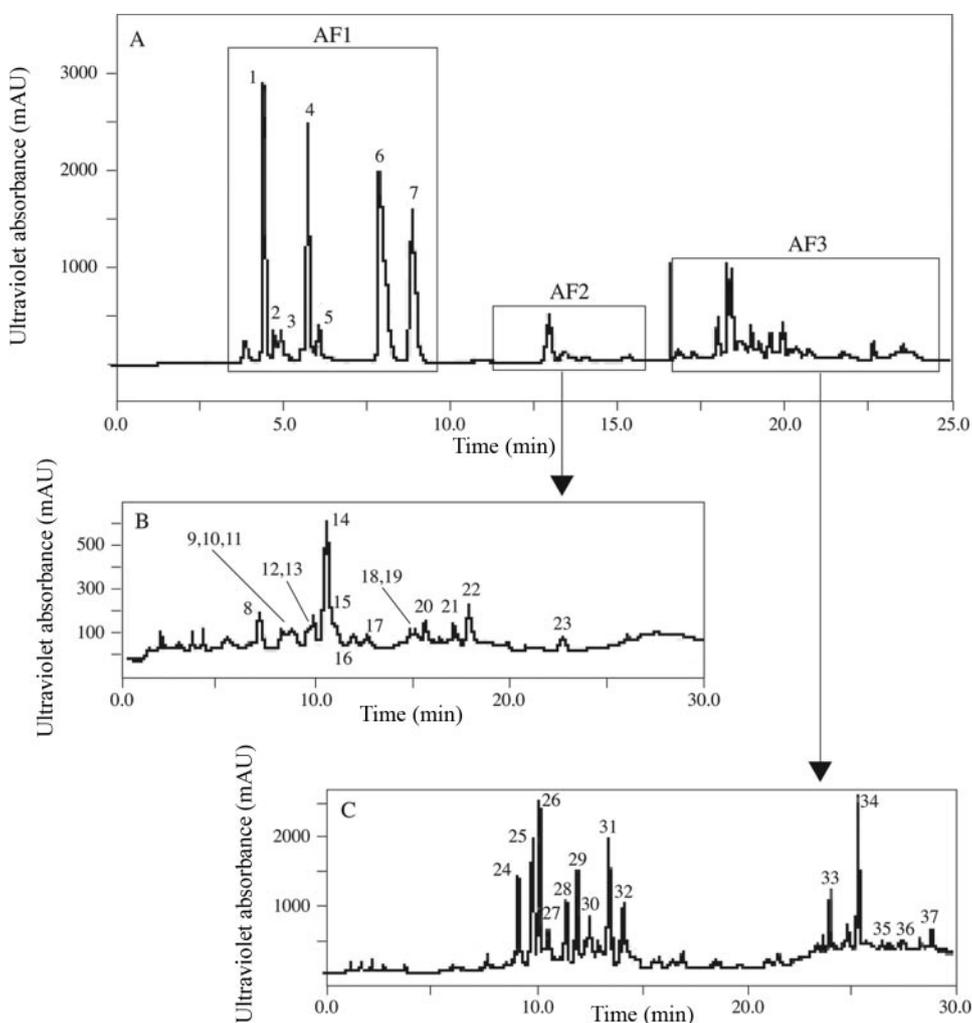


FIGURE 1. A: HPLC chromatogram of total active fraction (tAF), showing the 3 subfractions (AF1, AF2, and AF3), which are monitored by ultraviolet absorbance at 254 nm. Components within these subfractions that have antiplatelet activity *in vitro* (data not presented here) are numbered on the chromatogram. B: HPLC chromatogram of AF2, which was obtained under altered chromatographic conditions suitable for the separation of these components. C: HPLC chromatogram of AF3, which was obtained under altered chromatographic conditions to obtain increased component separation.

SUBJECTS AND METHODS

Preparation of tomato extract and subfractions for *in vitro* experiments

An aqueous extract from ripe tomato fruit was prepared by homogenization of fresh tomatoes (*Lycopersicon esculentum*, obtained locally), centrifugation at 3500 RPM for 15 min at 25 °C, and clarification of the resulting straw-colored liquid by ultrafiltration (ultrafiltration membrane, MW cutoff 1000 Da; Millipore Ltd, Watford, United Kingdom). Analysis showed that the aqueous tomato extract consisted largely of soluble sugars (85–90% of dry matter), which showed no *in vitro* antiplatelet activity. These inactive constituents were removed by using solid-phase extraction with styrene divinylbenzene (SDVB) cartridges (JT Baker, Mallinckrodt Baker BV, Deventer, Netherlands) at pH 2.5. Nonsugar components were retained on the cartridges and eluted in methanol. The nonsugar material that was isolated [total active fraction (tAF)] accounted for ≈4% of the aqueous extract dry matter and showed strong inhibition of platelet aggregation *in vitro*. Semipreparative HPLC [Synergy Polar-RP column (4 μ, 250 × 10 mm) and Luna C18(2) column

(3 μ, 250 × 10 mm); Phenomenex, Macclesfield, United Kingdom] was used to subfractionate tAF components into 3 broad groups by using a mixture of 0.05% trifluoroacetic acid and acetonitrile in gradient elution. These 3 groups, labeled AF1, AF2, and AF3 in decreasing order of polarity, are shown on the HPLC chromatogram (Figure 1). The isolated tAF and subfractions AF1–AF3 were reconstituted to known concentrations in phosphate-buffered saline (PBS; Sigma-Aldrich, Poole, United Kingdom), and the solution's pH was adjusted to 7.4 before use in *in vitro* experiments.

In vitro platelet aggregation studies

Blood for *in vitro* studies was collected from drug-free, healthy human volunteers, both male and female, aged 18–60 y who had normal platelet function. Blood was collected through siliconized needles into plastic syringes and transferred into citrated blood collection tubes (final sodium citrate concentration, 13 mmol/L). Platelet-rich plasma (PRP) was obtained by centrifugation of citrated blood for 15 min at 200 × *g* and 37 °C and used within 2 h for studies in which platelets were stimulated with

ADP, collagen, or arachidonic acid (Helena Biosciences, Sunderland, United Kingdom). When thrombin (Sigma-Aldrich) was used as an agonist, studies were carried out in washed platelet suspensions, which were prepared as described previously (28). The extent of aggregation was monitored on a platelet aggregometer (PACKS 4; Helena Biosciences) as described previously (28).

For *in vitro* experiments, the effects on platelet aggregation observed after incubating PRP or washed platelet suspension with test solutions are expressed as the percentage change in area under the aggregation curve or the percentage change in collagen lag time, as compared with control values.

In vitro platelet activation studies

To test the effects of tAF and its subfractions on platelet activation variables *in vitro*, freshly drawn whole blood diluted 1:10 with HEPES-Mg buffer, pH 7.4 (450 μ L), was incubated with the test substances of interest (50 μ L) or with HEPES-Mg buffer for 10 min. Aliquots of these mixtures (40 μ L) were then incubated with or without ADP (final concentration, 10 or 3 μ mol/L) or phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich; final concentration, 1 μ mol/L) in Falcon polystyrene tubes (BD Biosciences, Cowley, United Kingdom) for 5 min at room temperature. PMA was used to set the positive control marker on the flow cytometer. A saturating concentration (10 μ L) of fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies—anti-CD61-FITC (DakoCytomation, Ely, United Kingdom; 0.1 μ g), anti-CD62P-PE (Beckman Coulter, High Wycombe, United Kingdom; 0.03 μ g), and anti-fibrinogen-FITC (DakoCytomation; 0.9 μ g)—was added to the appropriate incubation tubes. FITC- and PE-labeled mouse immunoglobulin G antibodies (BD Biosciences; 0.1 μ g) were used as isotype controls. Incubation proceeded for 20 min in the dark at room temperature. Ice-cold PBS (2 mL) was then added and the samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Oxford, United Kingdom) with CellQuest software (version 2; BD Biosciences). Activated platelets were defined as the percentage of CD61-positive events co-expressing the CD62P receptor or fibrinogen binding.

Ex vivo cannulation study in healthy humans

Preparation of treatment and control supplement drinks for use in human study

A designated food production facility (Moorepark Technology Ltd, Teagasc, Fermoy, Ireland), was used to produce a standardized food-grade tomato extract that retained the bioactive components of interest. A compositional analysis of the concentrated extract was carried out, and the information obtained was used to produce a relative risk assessment of defined repeated oral exposure (Toxicology Advice and Consulting Ltd, Sutton, United Kingdom). Microbiological safety was verified in the Grampian Public Health Laboratory (Aberdeen, United Kingdom). For administration during the human volunteer study described, the extract was added to an orange juice matrix (Tesco freshly squeezed orange juice; Tesco, Aberdeen, United Kingdom). Two different extract-supplemented treatment drinks were prepared in 50- and 200-mL volumes. Each treatment drink contained 18 g tomato extract syrup, which is equivalent to the quantity of tAF found in 6 fresh tomatoes (total fresh weight, \approx 500 g). Equivalent control drinks were also prepared at each

volume. Control and treatment (tomato extract) supplements were prepared by mixing set quantities of ingredients in capped opaque containers immediately before use.

Subjects

Twenty-seven healthy adults of both sexes were recruited into the study. Subjects were aged 40–65 y and had no history of serious disease or hemostatic disorders. Suitability for inclusion into the study was assessed by using diet and lifestyle questionnaires and by medical screening, during which platelet function was assessed. Subjects were selected on the basis of high platelet function, as determined by the platelet aggregation response to 3 μ mol ADP/L. Subjects with low hematologic counts or low platelet function were not included in the study. Subjects habitually consuming dietary supplements (eg, fish oils or evening primrose oil) were asked to suspend these supplements for a minimum of 1 mo before participating in the study. Subjects were instructed to abstain from consuming drugs known to affect platelet function for a 10-d period before participation.

Written informed consent was obtained from all subjects. The study was approved by the Grampian Research Ethics Committee.

Phlebotomy

Subjects enrolled in the study underwent cannulation with a siliconized 21-gauge butterfly needle, to cause minimum disruption to the vein while taking multiple blood samples. To minimize activation of the hemostatic system, a maximum of 3 venipunctures was specified. The cannula remained in place over the entire 7-h period, and venous blood samples of \approx 20 mL were drawn at each sampling timepoint, and the first 2 mL was discarded on each occasion. After blood sample collection, the cannula was flushed with saline to prevent blockage. For measurements of platelet function and clotting time, blood was collected into plastic syringes and transferred into citrated blood collection tubes (final sodium citrate concentration, 13 mmol/L). For measurement of C-reactive protein (CRP), a single baseline blood sample (5 mL) was drawn into tubes containing EDTA anticoagulant (final concentration, 1.6 g/L). For measurement of fibrinopeptide A at each timepoint, 4.5 mL blood was collected into 0.5 mL of a mixed anticoagulant containing EDTA, trasylol, and chloromethylketone. Blood samples were incubated at 37 °C in a portable incubator for transfer to the laboratory. Any blood samples showing evidence of activation, defined as a fibrinopeptide A concentration >6 μ g/L, were discarded (30% of data points; *see* Results). Any volunteers showing evidence of an elevated inflammatory response, ie, a baseline CRP concentration >6 mg/L, were withdrawn from the study temporarily, and the scheduled intervention was undertaken at a later date.

Study design

A single-blinded study design was followed. Fasted subjects underwent cannulation, and 2 baseline samples were taken between 0700 and 0800. Immediately after the collection of the second baseline sample, subjects consumed either a tomato extract or a control supplement drink. Further blood samples were then withdrawn from the cannula at 1.5, 3, and 6 h. Subjects were offered small volumes (25 mL) of water after each sampling timepoint to avoid dehydration. A light snack (Alpen breakfast bar; Weetabix Ltd, Kettering, United Kingdom; nutritional



breakdown per 28-g bar: energy, 465 kJ or 110 kcal; protein, 1.8 g; carbohydrate, 19.9 g; and fat, 2.6 g) was given after the blood sample drawn at 3 h. Of the 23 subjects completing the study, 9 were involved in 2 interventions (ie, both the control and tomato extract drinks were consumed by these subjects, with the interventions timed ≥ 1 wk apart), and the remainder undertook one intervention only (control or tomato extract drink).

To measure the extent of the antiplatelet effect at 12, 18, and 24 h after consumption of the tomato extract supplement drink, an additional, small study was conducted after completion of the main study described above. Fifteen subjects were recruited (separately from the main study) and randomly assigned to 1 of 3 groups. All 3 groups consumed the tomato extract supplement drink and provided a total of 3 blood samples, taken over 3 different timecourses by single venipuncture. A baseline blood sample was obtained at $t = 0$ ($n = 15$), and a further sample was taken 3 h after supplementation ($n = 15$); a final sample was then taken either 12 ($n = 5$), 18 ($n = 5$), or 24 ($n = 5$) h after supplementation. The effects of the treatment supplement on ex vivo platelet function were assessed by using whole-blood aggregometry by single platelet counting (29).

Ex vivo platelet aggregation studies

Measurement of the extent of ADP-induced platelet aggregation in PRP was carried out at each timepoint. During prestudy screening, the optimal concentration of ADP agonist for each subject was determined. This subject-specific optimal concentration (between 7 and 8.5 $\mu\text{mol ADP/L}$) was used in the subsequent interventions. The platelet response to suboptimal ADP concentrations was also of interest; under these conditions, a biphasic aggregation response may be observed, which provides further information about the nature of the platelet response. Different ADP agonist concentrations may be used to approximate different physiologic conditions. To collect data under conditions of suboptimal platelet stimulation, a standardized lower ADP concentration (3 $\mu\text{mol/L}$) was defined as suboptimal and used for all measurements. For ex vivo studies, effects on platelet aggregation observed after treatment or control interventions are expressed as the percentage change in area under the aggregation curve after consumption of extract or placebo, as compared with baseline values.

Ex vivo coagulometry

Clotting time measurements were made at each timepoint. Two clotting factors, prothrombin and thrombin, were used to provide measurements reflecting the status of the coagulation cascade, independent of platelet function. Prothrombin time (PT) and thrombin clotting time (TCT) estimations were performed on a coagulometer (CoaData 4001; Helena Biosciences).

Supplementary measurements

Plasma fibrinopeptide A concentration was measured by using a competitive enzyme-linked immunosorbent assay (Zymutest FPA assay; HYPHEN BioMed, Neuville-sur-Oise, France) after removal of fibrinogen by bentonite treatment. CRP concentration was measured by using a semiquantitative latex agglutination assay (Dade Behring, Milton Keynes, United Kingdom), which allowed classification of sample CRP status as either normal or elevated (>6 mg CRP/L).

Statistical analysis

Data are presented as means \pm SEMs. Differences in the effects of test substances on platelet function in vitro were evaluated by using analysis of variance (ANOVA) after adjustment for multiple comparisons by using the Tukey method. For ex vivo measurements, all data from blood samples in which fibrinopeptide A or CRP was above defined values (6 $\mu\text{g/L}$ and 6 mg/L, respectively) were eliminated (30% data point loss). Preliminary assessment of the data distribution was carried out by inspecting histograms and producing normal plots. Baseline platelet aggregation and coagulometry data were analyzed for differences by ANOVA with the use of treatment and sex as factors. Data from postintervention timepoints were analyzed as changes from baseline; this unbalanced data set was analyzed by using the method of residual maximum likelihood (REML; equivalent to a 2-factor ANOVA for balanced data) with the use of time and treatment volume as factors. The presence of different ADP concentrations was not treated as a factor in the analysis—data collected under different conditions were analyzed separately. $P < 0.05$ was considered significant. We used GENSTAT for WINDOWS statistical software (version 8.1; VSN International Ltd, Hemel Hempstead, United Kingdom) for statistical analyses.

RESULTS

Profile of in vitro activity of tomato extract components

Effects on platelet aggregation

Isolation of tAF from the inactive matrix of the fresh tomato juice significantly concentrated the observed bioactivity, with >50 -fold increases in half-maximal inhibitory concentration values for ADP and collagen-mediated aggregation and >10 -fold increases for arachidonic acid and thrombin-mediated aggregation (Table 1). Partition of tAF into subfractions AF1, AF2, and AF3 allowed the range of components with antiplatelet activity to be observed for the first time (Figure 1) and confirmed that these components had significantly different modes of action in vitro (Table 1). The fractions AF1, AF2, and AF3 contributed 32%, 13%, and 55% to the total dry matter of tAF, respectively. The different proportional contributions of each subfraction to the total activity shown by tAF are represented in Figure 2.

Characterization of many of the individual compounds showing antiplatelet activity has been carried out by using chromatographic and spectroscopic methods. AF1 is highly active against ADP- and collagen-mediated platelet aggregation, and it contains a range of nucleosides and derivatives, including adenosine, cytidine, inosine, guanosine, AMP, and guanosine monophosphate (GMP). Several other components that inhibit platelet aggregation have been isolated but not yet identified. Subfraction AF2 shows significantly less inhibition of ADP- and collagen-induced aggregation but significantly greater inhibition of thrombin-induced aggregation than do the other subfractions (Table 1). AF2 contains a range of low-molecular-weight compounds, many of which are sensory components of the tomato and are present at very low concentrations. The main components of the AF3 subfraction are phenolic compounds. Simple phenolic acids (eg, ferulic and caffeic acids) and some glycosidic derivatives (eg, chlorogenic acid) have been identified. In addition, several flavonoids have been isolated, including quercetin,



TABLE 1

Inhibition of platelet aggregation by tomato aqueous extract, the total active fraction (tAF) isolated from the aqueous extract, and its subfractions AF1–AF3¹

Extract or subfraction	Inhibition of platelet aggregation: IC50			
	ADP (10 μ mol/L)	Collagen (4 mg/L)	Arachidonic acid (500 mg/L)	Thrombin (2 U/L)
Tomato aqueous extract ²	2.0 \pm 0.1 ^a	2.7 \pm 0.1 ^a	6.8 \pm 0.1 ^a	8.0 \pm 0.1 ^a
tAF ³	0.05 \pm 0.01 ^b	0.05 \pm 0.01 ^b	0.5 \pm 0.1 ^b	0.7 \pm 0.1 ^b
AF1 ⁴	0.06 \pm 0.01 ^b	0.04 \pm 0.01 ^b	0.9 \pm 0.1 ^c	1.0 \pm 0.1 ^c
AF2 ⁴	0.40 \pm 0.01 ^c	0.59 \pm 0.01 ^c	0.5 \pm 0.1 ^b	0.2 \pm 0.1 ^d
AF3 ⁴	0.17 \pm 0.01 ^d	0.30 \pm 0.01 ^d	0.1 \pm 0.0 ^d	0.8 \pm 0.1 ^b

¹ All values are $\bar{x} \pm$ SEM. Half-maximal inhibitory concentration (IC50) values represent the final concentration (g/L) of test substance required in platelet-rich plasma or washed platelet suspension for inhibition of platelet aggregation by 50% under standardized conditions. Experiments were carried out in platelet-rich plasma (ADP, collagen, and arachidonic acid) or washed platelet suspensions (thrombin), adjusted to give a platelet count of $(300 \pm 20) \times 10^9$ platelets/L. Means in a column with different superscript letters are significantly different, $P < 0.001$ (ANOVA followed by Tukey's post hoc test).

² $n = 5$, all agonists.

³ $n = 10$ (ADP and collagen) and 5 (arachidonic acid and thrombin).

⁴ $n = 10$ (ADP and collagen) and 3 (arachidonic acid and thrombin).

kaempferol, and luteolin. Quercetin is present largely as its glycosides, of which rutin is the most abundant. This subfraction inhibits arachidonate-induced aggregation to a significantly greater extent than do the other subfractions (Table 1); it also contains products of Maillard degradation reactions, which can vary according to the starting composition of the tomato juice and the exact conditions used during fractionation.

To evaluate the intersubject heterogeneity of platelet responses to tAF and derivatives before ex vivo studies, we examined the range of in vitro inhibition of ADP and collagen-mediated platelet aggregation in PRP from 10 healthy subjects after incubation with tAF and AF1–AF3 (Table 2). The wide range of the in vitro responses to each test fraction reflects the different baseline platelet activities of a group of normal healthy

subjects. Despite the range of responses, fractions tAF and AF1–AF3 showed significantly different inhibitory potentials toward ADP-mediated aggregation, which reflected their half-maximal inhibitory concentration values. Similar patterns and in vitro levels of inhibition were observed for collagen-mediated aggregation (Table 2).

Effects on platelet activation

Within the subject group, baseline (ie, control sample) fibrinogen-binding capacity, which reflects the accessibility of

TABLE 2

Inhibition of ADP- and collagen-induced platelet aggregation in vitro after incubation of platelet-rich plasma (PRP) from healthy subjects with the tomato extract's total active fraction (tAF) and its subfractions AF1–AF3 as compared with control values¹

Component	Inhibition of platelet aggregation	
	7.5 μ mol ADP/L	3 mg Collagen/L
	%	
tAF		
Difference from control	-64.9 \pm 3.6 ^{2,a}	-67.1 \pm 9.3 ^a
Interquartile range	-73.7 to -55.5	-89.9 to -58.4
AF1		
Difference from control	-67.2 \pm 3.6 ^a	-74.6 \pm 9.8 ^a
Interquartile range	-75.8 to -62.5	-92.5 to -67.2
AF2		
Difference from control	-19.6 \pm 7.5 ^b	-14.3 \pm 6.1 ^b
Interquartile range	-33.0 to -5.8	-17.6 to -2.8
AF3		
Difference from control	-30.0 \pm 6.0 ^b	-25.1 \pm 9.0 ^b
Interquartile range	-44.6 to -16.7	-31.6 to -7.7

¹ All test fractions were used at a final incubation concentration of 0.1 g PRP/L. $n = 10$ for all measurements. Means in a column with different superscript letters are significantly different, $P < 0.001$ (ANOVA followed by Tukey's post hoc test). No significant differences were detected for the test fraction \times agonist interaction ($P > 0.05$) when the inhibition of ADP-induced aggregation and that of collagen-induced aggregation were compared for each fraction.

² $\bar{x} \pm$ SEM (all such values).

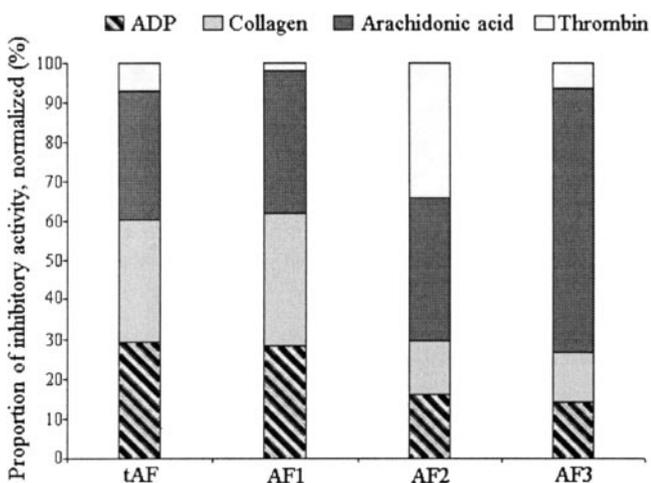


FIGURE 2. Proportional inhibitory activity shown by the total active fraction (tAF) and its subfractions (AF1, AF2, and AF3) toward ADP- (10 μ mol/L), collagen- (4 mg/L), arachidonic acid- (500 mg/L), and thrombin- (2U/L) mediated platelet aggregation. A comparison ($n = 5$) was made between the antiplatelet activity of the tAF at a final concentration of 1.98 g/L and the antiplatelet activities of each subfraction at final concentrations of 0.58, 0.24, and 1.00 g/L, respectively. These concentrations reflected the proportional contribution of each subfraction to the total active fraction. Resulting data were normalized.

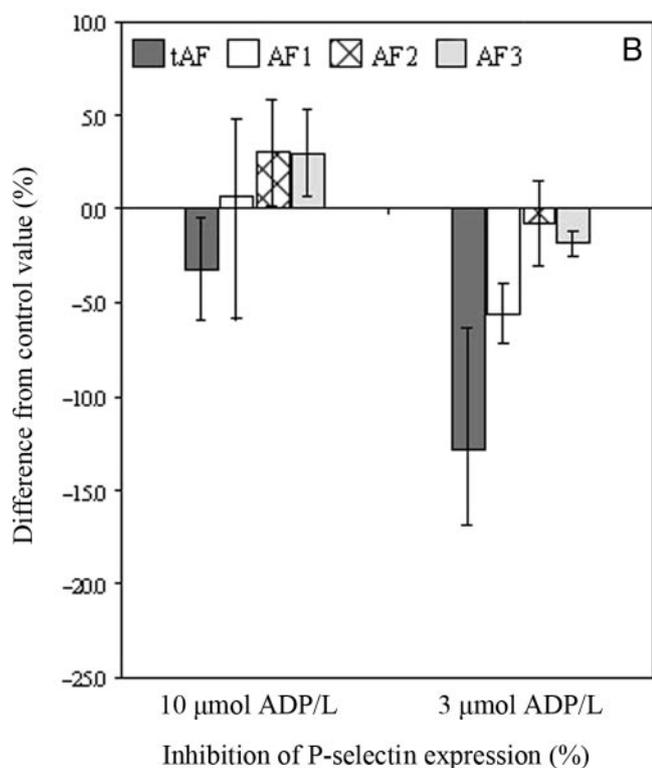
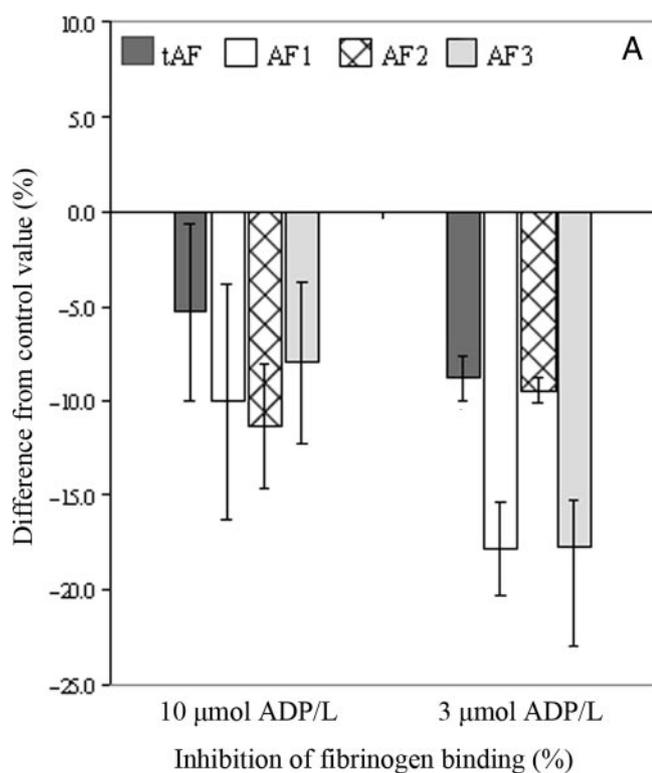


FIGURE 3. Inhibition of ADP-induced expression of the platelet activation markers glycoprotein IIb/IIIa (GPIIb/IIIa) (A) and P-selectin (B) after incubation of whole blood with the tomato extract's total active fraction (tAF) and its subfractions (AF1, AF2, and AF3). All inhibitors were used at a final concentration of 0.05 g/L; for each measurement, $n = 5$. For both GPIIb/IIIa and P-selectin measurements, no significant differences were detected between the 2 agonist concentrations, and the fraction \times agonist concentration interaction also was not significant ($P > 0.05$, ANOVA). Across agonist

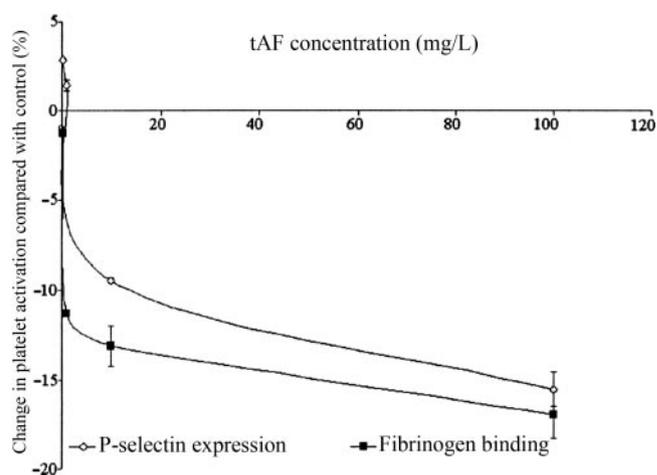


FIGURE 4. Observed dose response of the inhibition of platelet activation induced by 3 μ mol ADP/L after incubation of platelets with the tomato extract's total active fraction (tAF) at concentrations ranging from 0.0 to 0.1 g/L. Data are shown for inhibition of activation as measured by reduction in fibrinogen-binding capacity and P-selectin expression. $n = 3$.

the platelet surface glycoprotein IIb/IIIa (GPIIb/IIIa) complex, was in the range of 46.3% to 76.1% (median: 68.3%) when 10 μ mol ADP/L was used as agonist and in the range of 48.7% to 72.9% (median: 60.1%) when 3 μ mol ADP/L was used. Baseline P-selectin expression after stimulation with 10 μ mol ADP/L was found to be in the range of 49.6% to 72.5% P-selectin-positive platelets (median: 58.6%). When 3 μ mol ADP/L was used as agonist, 41.2–68.2% P-selectin-positive platelets were recorded (median: 51.1%). The experimental variance was $<5\%$ for measurement of both activation markers. Incubation of diluted whole blood with tAF and AF1–AF3 resulted in lower expression of the active conformation of GPIIb/IIIa on the platelet surface, as shown by a significantly lower fibrinogen-binding capacity induced by ADP agonist, than was seen in control samples ($P < 0.001$; **Figure 3A**). For all test fractions, similar effects were recorded for 3 and 10 μ mol ADP/L, and no significant fraction \times ADP concentration interaction was seen. Comparison of the individual effects of tAF and its subfractions showed that no one test fraction gave effects significantly different from the others. Significantly less activation-induced P-selectin expression than in control samples was also observed after incubation with tAF and AF1–AF3 ($P < 0.001$; **Figure 3B**). In this case, the effect of tAF on P-selectin expression (across agonist concentrations) differed significantly from the effects of each of the subfractions AF1–AF3, although no differences were detected between the individual subfractions (**Figure 3B**). A dose response was observed in the inhibition of both platelet activation markers by tAF (final concentration range: 0–100 mg/L) when 3 μ mol ADP/L as agonist was used to stimulate platelet activation (**Figure 4**).

concentrations, test fractions significantly inhibited both platelet activation markers, with results significantly different from control ($P < 0.001$, ANOVA). No difference in the extent of GPIIb/IIIa inhibition was observed between the 4 test fractions (A; $P > 0.05$, ANOVA followed by Tukey's post hoc test). With regard to P-selectin inhibition, tAF was significantly different from each of AF1, AF2, and AF3 (B; $P < 0.001$, ANOVA followed by Tukey's post hoc test); however, the 3 subfractions did not differ significantly.

TABLE 3Baseline platelet aggregation and coagulometry characteristics of the subjects¹

	Subject group (10 M, 13 F)
Optimal ADP ²	
AUC (AU)	49 339 ± 812 ³
Interquartile range	48 484–51 860
Suboptimal ADP ⁴	
AUC (AU)	41 684 ± 2056
Interquartile range	36 043–49 307
PT	
Clotting time (s)	14.4 ± 0.2
Interquartile range	13.8–15.1
TCT	
Clotting time (s)	18.1 ± 0.2
Interquartile range	17.7–18.9

¹ PT, prothrombin time; TCT, thrombin clotting time; AUC, area under the curve; AU, arbitrary units. No significant differences between male and female subjects were observed at baseline ($P > 0.05$ for all variables, ANOVA).

² Values given represent the area under the aggregation curve induced by 7.5–8.5 μmol ADP agonist/L (the optimal concentration).

³ $\bar{x} \pm \text{SEM}$ (all such values).

⁴ Values given represent the area under the aggregation curve induced by 3 μmol ADP agonist/L (the suboptimal concentration).

Ex vivo study: time course of the observed acute antiplatelet effect

Baseline characteristics of subjects selected by prestudy screening

The average baseline platelet aggregation and clotting time data for the subject group are shown in **Table 3**. For simplicity, the terms “optimal” and “suboptimal” are applied to the different ADP agonist concentrations used in ex vivo measurements (*see* Subjects and Methods). Screening of subjects for high platelet activity, in an attempt to reduce the expected heterogeneity of the data set (as seen in Table 2), resulted in a subject group that was highly responsive to stimulation with suboptimal concentrations of ADP. No significant differences in either platelet function or clotting times were observed between male and female subjects at baseline. Clotting times were within the normal reference range.

Stability of cannulation: effects on activation of the hemostatic system

Maintenance of cannula patency for a period of 7 h without an anticoagulant rinse was difficult. For 9 interventions, the full timecourse was not completed and the study came to a premature end at 3 h. Even when blood samples were successfully drawn from the cannula, the saline rinse was often insufficient to prevent a small amount of clotting. Blood samples at affected timepoints were subsequently shown to be activated during or after phlebotomy, and data from these samples was discarded. In all, 38 (30%) of 128 data points were discarded because of activation during sampling.

Diurnal changes in platelet responsiveness to agonist over time

The measured changes in platelet aggregation (optimal or suboptimal ADP concentration) 1.5, 3, and 6 h after supplementation

TABLE 4The effect of supplementation with control or tomato extract treatment on ex vivo platelet aggregation induced by ADP agonist over a 7-h period¹

	1.5 h	3 h	6 h
% change			
Optimal ADP			
Overall			
Tomato extract ²	0.3 ± 0.8	-2.9 ± 1.4	-2.4 ± 1.6
Control ³	1.2 ± 0.8	5.0 ± 3.2	-1.9 ± 2.3
50 mL			
Tomato extract ⁴	-1.0 ± 1.3	-4.7 ± 3.1	-4.5 ± 2.5
Control ⁵	1.1 ± 1.2	7.9 ± 7.9	-2.7 ± 2.8
200 mL			
Tomato extract ⁶	1.4 ± 0.8	-1.5 ± 0.6	-0.4 ± 1.9
Control ⁷	1.3 ± 1.1	3.0 ± 2.8	1.3
Suboptimal ADP			
Overall			
Tomato extract ²	-7.4 ± 2.4	-20.0 ± 4.9 ⁸	-21.3 ± 6.2 ⁸
Control ³	0.3 ± 1.2	4.4 ± 2.2	0.9 ± 2.1
50 mL			
Tomato extract ⁴	-9.3 ± 4.6	-23.1 ± 9.8 ⁸	-27.2 ± 12.1 ⁸
Control ⁵	-0.5 ± 1.5	6.8 ± 1.5	1.2 ± 2.7
200 mL			
Tomato extract ⁶	-5.7 ± 2.0	-17.8 ± 5.1 ⁸	-15.4 ± 3.0 ⁸
Control ⁷	1.6 ± 2.3	2.9 ± 3.4	-0.4

¹ All values are $\bar{x} \pm \text{SEM}$ or \bar{x} . Values represent the change from baseline aggregation. Analysis was carried out by using a residual maximum likelihood model with interaction terms for time and volume. The treatment \times time interaction was significant at suboptimal ADP ($P = 0.014$) but not at optimal ADP ($P > 0.05$), and the treatment \times time \times volume interaction was not significant at either agonist concentration.

² 1.5 h, $n = 17$; 3 h, $n = 14$; 6 h, $n = 10$.

³ 1.5 h, $n = 8$; 3 h, $n = 5$; 6 h, $n = 5$.

⁴ 1.5 h, $n = 8$; 3 h, $n = 6$; 6 h, $n = 5$.

⁵ 1.5 h, $n = 5$; 3 h, $n = 2$; 6 h, $n = 4$.

⁶ 1.5 h, $n = 9$; 3 h, $n = 8$; 6 h, $n = 5$.

⁷ 1.5 h, $n = 3$; 3 h, $n = 3$; 6 h, $n = 1$.

⁸ Significantly different from the control supplement, $P < 0.001$ (residual maximum likelihood).

with control or tomato extract drinks, expressed as the percentage change ($\Delta\%$) from baseline values, are summarized in **Table 4**. No significant differences from baseline status were observed in the control group over time, which indicated that diurnal changes in platelet response to ADP agonist were not significant in this subject group.

Effects of treatment with tomato extract supplements on platelet aggregation

ADP-induced platelet aggregation was significantly lower than baseline values after supplementation with tomato extract but not after supplementation with the control drink. The overall differences between the tomato extract and control drinks were significant at both optimal (overall mean $\Delta\%$ in tomato extract group: $-1.58 \pm 0.71\%$; overall mean $\Delta\%$ in control group: $2.10 \pm 1.15\%$; $P = 0.03$) and suboptimal (overall mean $\Delta\%$ in tomato extract group: $-15.23 \pm 2.19\%$; overall mean $\Delta\%$ in control group: $1.86 \pm 3.56\%$; $P < 0.001$) ADP concentrations. Table 4 shows the changes from baseline aggregation observed at each timepoint for the tomato extract group. When platelets were stimulated with suboptimal concentrations of ADP, significant differences from the control group were observed for the

TABLE 5

The effect of supplementation with control or tomato extract treatment on ex vivo prothrombin time (PT) and thrombin clotting time (TCT) over a 7-h period¹

	1.5 h	3 h	6 h
	% change		
PT			
Tomato extract ²	0.8 ± 1.8	2.9 ± 2.0	2.7 ± 2.3
Control ³	0.3 ± 2.7	1.7 ± 3.4	2.1 ± 4.1
TCT			
Tomato extract ²	-2.9 ± 1.2	1.3 ± 1.3	-0.7 ± 1.6
Control ³	1.9 ± 1.8	-1.4 ± 2.3	-1.1 ± 2.7

¹ All values are $\bar{x} \pm \text{SEM}$. No significant differences between tomato extract and control groups were observed ($P > 0.05$, residual maximum likelihood), and the treatment \times time and treatment \times time \times volume interactions were not significant for this data set.

² 1.5 h, $n = 17$; 3 h, $n = 14$; 6 h, $n = 10$.

³ 1.5 h, $n = 8$; 3 h, $n = 5$; 6 h, $n = 5$.

tomato extract group at 3 and 6 h. When optimal ADP concentrations were used, the time \times treatment interaction was not significant at any timepoint. No significant differences arising from the different carrier volumes were observed in either the control or tomato extract group at any timepoint (Table 4).

The persistence of the observed effect was greater than anticipated, and therefore it is not possible to pinpoint the peak effect from this data set. A small bridging study ($n = 15$) was conducted to measure the extent of the antiplatelet effect 12, 18, and 24 h after consumption of the tomato extract supplement drink (data not shown). At 18 h after supplementation, platelet function had returned to baseline in all subjects.

Coagulometry

No significant differences from baseline values in clotting time were observed in the control group over the course of the 7-h cannulation period (Table 5). No significant difference was detected between the effects of the tomato extract and the control drinks on coagulation variables.

DISCUSSION

Earlier work by Dutta-Roy et al (23), Yamamoto et al (30), and Lazarus and Garg (31) found that aqueous, delipidated tomato extracts were potent antiplatelet agents in vitro and that a proportion of the observed activity could be linked to the nucleoside adenosine. To account for the full range of antiplatelet activities shown, it was concluded that additional bioactive compounds must be present (23, 30). We have separated the active components of tomato juice (tAF) from generic matrix compounds and identified groups of components that are responsible for different antiplatelet effects in vitro. Results show that specific subfractions of tAF are of great interest for future work. Whereas fraction AF1 shows high activity against both ADP- and collagen-mediated platelet aggregation, nucleoside derivatives may have a short half-life in the gut or bloodstream, which limits their physiologic effectiveness. However, fractions AF2 and AF3 have a broader spectrum of activity against platelet agonists, and they contain a wide range of components, some of which are known to be bioavailable. Some of the flavonoid derivatives present in AF3 have established antiplatelet activity in vitro,

although they are present at concentrations lower than those that other studies have shown to be effective (32–34). Most of the isolated components with antiplatelet activity are not flavonoid derivatives, and their characterization and individual antiplatelet activity profiles remain to be reported.

Universal occurrence of tAF components has been established in tomatoes of various cultivars, places of origin, and times of harvest. Yamamoto et al (30) reported cultivar-dependent variation in the antiplatelet activity of tomato extracts. We observed that stage of ripeness has a more significant effect on active component concentration than does either cultivar or place of origin. Extracts produced from green tomatoes of all cultivars examined were less active and contained lower concentrations of AF2 and AF3 components than did extracts produced from corresponding ripe tomatoes (data not shown).

A possible mechanism by which tomato extract components inhibit different pathways of platelet aggregation has been suggested from our observation that tAF and its subfractions prevent activation of integrin $\alpha_{\text{IIb}}\beta_3$ (ie, GPIIb/IIIa). Expression of this integrin is specific to platelets and megakaryocytes. Its activation is essential for platelet aggregation, which it mediates by binding fibrinogen or von Willebrand factor (vWF) (35, 36). In resting platelets, GPIIb/IIIa is maintained in a low-affinity state for ligand binding, which allows platelets to circulate freely (37). On activation, receptor-specific inside-out signals are generated, which activates GPIIb/IIIa and increases its affinity for adhesive ligands (38, 39). Inhibition of the GPIIb/IIIa activation step—which is common to multiple aggregation pathways—could underlie the wide-ranging effects of tAF. Recently, Lazarus and Garg (31) showed that basal platelet cyclic AMP concentrations are unaltered by tomato extract active components in vitro, which suggests that phospholipase C enzyme family-mediated cascade reactions (which can affect GPIIb/IIIa activation) may be at the root of the observed antiaggregatory activity. The effects of AF1–AF3 on platelet receptor-binding capacity as well as on phosphoinositide metabolism and shape change must also be examined before mechanisms can be confidently suggested.

This work has shown that, in addition to preventing platelet activation and aggregation, tAF reduces the expression of P-selectin (ie, CD62P) on the platelet surface in response to ADP-induced platelet activation in whole blood. In resting platelets, P-selectin is localized in the membranes of platelet α -granules (40). On platelet activation, it is redistributed to the platelet surface, where it initiates adhesion to leukocytes (41). Under conditions of blood flow and shear stress, this glycoprotein promotes platelet cohesion and stabilizes newly formed aggregates (42). Thus, tAF components can potentially affect the size and longevity of platelet aggregates. The observed effects on P-selectin also imply that tAF components inhibit the release of α -granule components in activated platelets, which affects many proaggregatory molecules. Some protein kinase C isoforms may be inhibited (43).

The objectives of the reported time-course study were, first, to ascertain whether a significant acute effect of tAF components could be detected ex vivo in healthy volunteers; second, to ascertain the time course of the effect and establish whether diurnal changes in platelet function would affect its detection; and third, to examine whether supplement volume would affect the observed platelet responses. Thus, to summarize the main outcomes of the study, a significant change in platelet function over time

was observed in response to supplementation with tomato extract. This change was significant 3 h after extract consumption, and control subjects did not show an equivalent effect. The decrease in platelet response to ADP agonist after supplementation was most sensitively detected at suboptimal concentrations of ADP. No changes in clotting time variables were detectable in any of the subject groups between treatments or over time, which indicates that the adequacy of the extrinsic system was not affected and implies that supplementation with tomato extract should not result in a prolonged bleeding time under normal conditions.

Many studies have suggested that circadian rhythms in plasma catecholamines, blood pressure, and blood viscosity can lead to a diurnal variation in the platelet aggregation response to an agonist—in particular, epinephrine (44, 45). A similar effect for ADP agonist would be expected to mask any observed response to tomato components *ex vivo*. However, in the current study, neither baseline platelet response to ADP nor baseline clotting times were significantly altered by diurnal influences over a 7-h period, and *ex vivo* effects of administered tomato extract were seen without adjustments to platelet measurements. Altering the carrier beverage volume did not cause a significant difference in *ex vivo* platelet response to tomato extract between study groups. Lack of statistical significance may have been due to losses of data at later timepoints.

Our work supports suggestions that observed cardioprotective effects of tomato consumption could be linked to modulation of platelet function. Localization of previously reported *in vitro* activities to specific groups of water-soluble tomato components has further elucidated possible antiplatelet mechanisms, and *ex vivo* studies have shown that some of the relevant components are bioavailable and retain activity *in vivo*. The timecourse of the acute effects observed in healthy volunteers suggests that, when removed from the fresh fruit matrix and consumed in a small volume, which would facilitate absorption from the gut, the tAF-containing tomato extract causes a reduction in *ex vivo* platelet response to agonist within 3 h. This suppression of platelet function could be beneficial in preventing thrombotic and proinflammatory events associated with activated platelets. 

We thank the volunteers for their participation and interest in this study and the staffs of the Rowett Institute (Aberdeen, United Kingdom) and, in particular, of the Rowett Institute's Human Nutrition Unit for their support in volunteer management and phlebotomy. We also thank the following persons for their help and expertise: Brent Williams for advice and discussions during the early stages of the study; Graham Horgan for advice on statistical analysis; and Stan Heptinstall and Isobel Ford for their help during *in vitro* method development.

NO and LC were responsible for the study design, and JIB, DJW, and AKD provided critical input; JIB was the ethics committee representative and was responsible for critical review of the study design and the manuscript; NO and LC were responsible for laboratory data collection; LC, NO, and MvL conducted the hematologic measurements; MvL was responsible for developing the laboratory assays, subject recruitment and coordination, and blood collection; NO was responsible for data analysis and interpretation and for drafting the manuscript; and MvL, DJW, and AKD provided critical review of the manuscript. NO and LC were fully funded employees of Proxavis plc; MvL was contracted to Proxavis plc for the study period; and JIB, DJW, and AKD are members of the Proxavis Ltd Scientific Advisory Board and serve as scientific and clinical advisors to the board.

REFERENCES

- Heart disease and stroke statistics—2003 update. Dallas, TX: American Heart Association, 2003.
- Gartside P, Glueck C. The important role of modifiable dietary and behavioral characteristics in the causation and prevention of coronary heart disease hospitalization and mortality: the prospective NHANES I follow-up study. *J Am Coll Nutr* 1995;14:71–9.
- Hu F, Rimm E, Stampfer M, Ascherio A, Spiegelman D, Willet W. Prospective study of major dietary patterns and risk of coronary heart disease in men. *Am J Clin Nutr* 2000;72:912–21.
- Joshiyura K, Hu F, Manson J, et al. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann Intern Med* 2001;134:1106–14.
- Kerver J, Yang E, Bianchi L, Song W. Dietary patterns associated with risk factors for cardiovascular disease in healthy US adults. *Am J Clin Nutr* 2003;78:1103–10.
- Kris-Etherton P, Harris W, Appel I. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Scientific statement of the American Heart Association Nutrition Committee. *Circulation* 2002;106:2747–57.
- Hertog M, Feskens E, Hollman P, Katan M, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* 1993;342:1007–11.
- Rahman K. Historical perspective on garlic and cardiovascular disease. *J Nutr* 2001;131:977S–9S.
- Bucher H, Hengstler P, Schindler C, Meier G. n–3 Polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. *Am J Med* 2002;112:298–304.
- Osganian S, Stampfer M, Rimm E, Spiegelman D, Manson J, Willet W. Dietary carotenoids and risk of coronary artery disease in women. *Am J Clin Nutr* 2003;77:1390–9.
- Dutta-Roy AK. Dietary components and human platelet activity. *Platelets* 2002;13:67–75.
- Wilcox J, Catignani G, Lazarus S. Tomatoes and cardiovascular health. *Crit Rev Food Sci Nutr* 2003;43:1–18.
- Weisburger J. Evaluation of evidence on the role of tomato products in disease prevention. *Proc Soc Exp Biol Med* 1998;218:140–3.
- Agarwal S, Rao A. Tomato lycopene and low density lipoprotein oxidation: a human dietary intervention study. *Lipids* 1998;33:981–4.
- Arab L, Steck S. Lycopene and cardiovascular disease. *Am J Clin Nutr* 2000;71(suppl):1691S–5S.
- Rissanen T, Voutilainen S, Nyyssonen K, Salonen J. Lycopene, atherosclerosis and coronary heart disease. *Exp Biol Med* 2002;227:900–7.
- Agarwal S, Rao A. Tomato lycopene and its role in human health and chronic diseases. *Can Med Assoc J* 2000;163:739–44.
- Sesso H, Liu S, Gaziano J, Buring J. Dietary lycopene, tomato-based food products and cardiovascular disease in women. *J Nutr* 2003;133:2336–41.
- De Lorgeril M, Salen P, Martin J, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. *Circulation* 1999;99:779–85.
- Kohlmeier L, Kark J, Gomez-Garcia E, et al. Lycopene and myocardial infarction risk in the EURAMIC study. *Am J Epidemiol* 1997;146:618–26.
- Rissanen T, Voutilainen S, Nyyssonen K, Salonen R, Kaplan G, Salonen J. Serum lycopene concentrations and carotid atherosclerosis: the Kuopio Ischaemic Heart Disease Risk Factor Study. *Am J Clin Nutr* 2003;77:133–8.
- Hak A, Stampfer M, Campos H, et al. Plasma carotenoids and tocopherols and risk of myocardial infarction in a low-risk population of US male physicians. *Circulation* 2003;108:802–7.
- Dutta-Roy A, Crosbie L, Gordon M. Effects of tomato extract on human platelet aggregation *in vitro*. *Platelets* 2001;12:218–27.
- Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation* 2001;104:365–72.
- Zaman A, Helft G, Worthley S, Badimon J. The role of plaque rupture and thrombosis in coronary artery disease. *Atherosclerosis* 2000;149:251–66.
- Ruggeri Z. Mechanisms initiating platelet thrombus formation. *Thromb Haemost* 1997;78:611–6.
- Osterud B. A global view on the role of monocytes and platelets in atherogenesis. *Thromb Res* 1997;85:1–22.
- Dutta-Roy A, Gordon M, Kelly C, et al. Inhibitory effect of *Ginkgo*



- biloba* extract on human platelet aggregation. *Platelets* 1999;10:298–305.
29. Storey R, May J, Wilcox R, Heptinstall S. A whole blood assay of inhibition of platelet aggregation by glycoprotein IIb/IIIa antagonists: comparison with other aggregation methodologies. *Thromb Haemost* 1999;82:1307–11.
 30. Yamamoto J, Taka T, Yamada K, et al. Tomatoes have natural anti-thrombotic effects. *Br J Nutr* 2003;90:1031–8.
 31. Lazarus S, Garg M. Tomato extract inhibits human platelet aggregation in vitro without increasing basal cAMP levels. *Int J Food Sci Nutr* 2004;249–56.
 32. Rein D, Paglieroni T, Pearson D, et al. Cocoa and wine polyphenols modulate platelet activation and function. *J Nutr* 2000;130:2120S–6S.
 33. Murphy K, Chronopoulos A, Singh I, et al. Dietary flavanols and procyanidin oligomers from cocoa (*Theobroma cacao*) inhibit platelet function. *Am J Clin Nutr* 2003;77:1466–73.
 34. Janssen K, Mensink R, Cox F, et al. Effects of the flavonoids quercetin and apigenin on hemostasis in healthy volunteers: results from an in vitro and a dietary supplement study. *Am J Clin Nutr* 1998;67:255–62.
 35. Shattil S, Kashiwagi H, Pampori N. Integrin signalling: the platelet paradigm. *Blood* 1998;91:2645–57.
 36. Phillips DR, Agin PP. Platelet membrane defects in Glanzmann's thrombasthenia. Evidence for decreased amounts of two major glycoproteins. *J Clin Invest* 1977;60:535–45.
 37. Tozer E, Hughes P, Loftus J. Ligand binding and affinity modulation of integrins. *Biochem Cell Biol* 1996;74:785–98.
 38. Leisner T, Wencel-Drake J, Wang W, Lam S. Bidirectional transmembrane modulation of integrin $\alpha_{IIb}\beta_3$ conformations. *J Biol Chem* 1999;274:12945–9.
 39. Cho M, Liu J, Pestina T, et al. The roles of $\alpha_{IIb}\beta_3$ -mediated outside-in signal transduction, thromboxane A2, and adenosine diphosphate in collagen-induced platelet aggregation. *Blood* 2003;101:2646–51.
 40. Stenberg P, McEver R, Shuman M, Jacques Y, Bainton D. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol* 1985;101:880–6.
 41. Hamburger S, McEver R. GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 1990;75:550–4.
 42. McEver R, Moore K, Cummings R. Leukocyte trafficking mediated by selectin-carbohydrate interactions. *J Biol Chem* 1995;270:11025–8.
 43. Furie B, Furie B, Flaumenhaft R. A journey with platelet P-selectin: the molecular basis of granule secretion, signalling and cell adhesion. *Thromb Haemost* 2001;86:214–21.
 44. Undar L, Akkoc N, Alakavuklar M, Cehreli C, Undar L. Flow cytometric analysis of circadian changes in platelet activation using anti-GMP-140 monoclonal antibody. *Chronobiol Int* 1999;16:335–42.
 45. Mores N, Martire M, Pistrutto G, et al. Platelet alpha 2-adrenoceptors and diurnal changes of platelet aggregability in hypertensive patients. *J Hypertens* 1994;12:939–45.

