



Cite this: DOI: 10.1039/c4fo00570h

A single serving of blueberry (*V. corymbosum*) modulates peripheral arterial dysfunction induced by acute cigarette smoking in young volunteers: a randomized-controlled trial

Cristian Del Bo,^a Marisa Porrini,^a Daniela Fracassetti,^a Jonica Campolo,^b Dorothy Klimis-Zacas^c and Patrizia Riso^{*a}

Cigarette smoking causes oxidative stress, hypertension and endothelial dysfunction. Polyphenol-rich foods may prevent these conditions. We investigated the effect of a single serving of fresh-frozen blueberry intake on peripheral arterial function and arterial stiffness in young smokers. Sixteen male smokers were recruited for a 3-armed randomized-controlled study with the following experimental conditions: smoking treatment (one cigarette); blueberry treatment (300 g of blueberry) + smoking; control treatment (300 mL of water with sugar) + smoking. Each treatment was separated by one week of wash-out period. The blood pressure, heart rate, peripheral arterial function (reactive hyperemia and Framingham reactive hyperemia), and arterial stiffness (digital augmentation index, digital augmentation index normalized for a heart rate of 75 bpm) were measured before and 20 min after smoking with Endo-PAT2000. Smoking impaired the blood pressure, heart rate and peripheral arterial function, but did not affect the arterial stiffness. Blueberry consumption counteracted the impairment of the reactive hyperemia index induced by smoking ($-4.4 \pm 0.8\%$ blueberry treatment vs. $-22.0 \pm 1.1\%$ smoking treatment, $p < 0.01$) and Framingham reactive hyperemia ($+28.3 \pm 19.2\%$ blueberry treatment vs. $-42.8 \pm 20.0\%$ smoking treatment, $p < 0.0001$), and the increase of systolic blood pressure ($+8.4 \pm 0.02\%$ blueberry treatment vs. $+13.1 \pm 0.02\%$ smoking treatment, mmHg, $p < 0.05$) after cigarette smoking. No effect was observed for arterial stiffness and other vital signs. In conclusion, data obtained suggest a protective role of blueberry on reactive hyperemia, Framingham reactive hyperemia, and systolic blood pressure in subjects exposed to smoke of one cigarette. Future studies are necessary to elucidate the mechanisms involved.

Received 28th June 2014,
Accepted 3rd September 2014

DOI: 10.1039/c4fo00570h

www.rsc.org/foodfunction

Introduction

Several studies have documented that both active and passive cigarette smoke exposure induces endothelial dysfunction, an early phenomenon involved in the atherosclerotic process.^{1–3} The mechanism of endothelial dysfunction could be mediated by several substances that constitute the particulate (tar) and gaseous phase of the cigarette⁴ and that are involved in the production of radical oxygen species (ROS). In this regard, ROS induces oxidative stress and inflammation with detrimental consequences on the bioavailability of nitric oxide (NO), the

most important vasodilator produced by endothelial cells.⁴ The reduction of NO causes an increase in blood pressure³ and arterial wall stiffness,⁵ one of the underlying pathophysiological mechanisms of the cardiovascular process.⁵ Arterial stiffness is considered a predictor of cardiovascular events in the general population,⁶ and its measurement provides information about the functional and structural vascular changes not only at the level of the aorta, but also at the microvascular level.⁶ In fact, the augmentation index (Aix) is widely used as a surrogate measure of arterial stiffness and as a composite index of arterial dysfunction.⁷

Polyphenols, such as anthocyanins (ACNs), present in high amounts in berries, are recognized as potential bioactive compounds able to counteract ROS production by reducing oxidative stress and inflammation.^{8,9} Moreover, ACNs have been proposed as mediators of NO production, thus playing a crucial role in the modulation of arterial stiffness, endothelial function and blood pressure.^{10,11} Most of the evidence on health and vascular benefits of polyphenols derives from

^aUniversità degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition, Milano, Italy.

E-mail: patrizia.riso@unimi.it; Fax: +39-02-50316721; Tel: +39-02-50316726

^bCNR Institute of Clinical Physiology, CardioThoracic and Vascular Department, Niguarda Ca' Granda Hospital, Milan, Italy

^cDepartment of Food Science and Human Nutrition, University of Maine, Orono, Maine, USA

in vitro and *ex vivo* studies,^{12,13} while in humans the results are still inconclusive.^{14–23} On the whole, an improvement of endothelial function has been observed in several studies after a single administration of polyphenol rich-foods and/or bioactive compounds compared to chronic dietary intervention studies.^{15,21–23} It is clear that several factors related to the type of population enrolled (*e.g.* age, sex, dietary habits, physical activity, risk factors and exposure to oxidative stress) could contribute to different results obtained both in short and long term studies. In addition, the specific experimental protocol used, or the different methodologies applied to determine endothelial function [*e.g.* peripheral arterial tone (PAT) vs. brachial artery ultrasound (BAUS)] can be important variables.

We recently developed an *in vivo* experimental model to study peripheral arterial function following a stressor/insult. The experimental protocol involves the evaluation of the Reactive Hyperemia Index (RHI) and blood pressure response in smokers exposed to smoke from one cigarette. Through PAT technology measurements, we demonstrated an impairment of peripheral arterial function 20 min after smoking.²⁴ The same model may be exploited to investigate the vasoactive properties of bioactive components when introduced before the stress, causing dysfunction (*i.e.* smoking one cigarette). Thus, the aim of the present study is to explore the effect of a single serving of fresh-frozen blueberry (300 g) on markers of peripheral arterial function and blood pressure in young and healthy smokers.

Methods

Preparation of blueberry and control treatment

Fresh blueberries (*Vaccinium corymbosum* L. “Brigitta”) from a single batch were purchased, sorted and immediately frozen by the individually quick freezing technique (Thermolab, Codogno, Italy) and stored at $-20\text{ }^{\circ}\text{C}$ until use. For the study, 300 g of frozen blueberry was thawed at $+4\text{ }^{\circ}\text{C}$ overnight and provided to the participants. Since blueberry contained 16 g fructose and 11 g glucose, the control treatment sample was prepared by suspending the same amount of sugars in 300 mL of water. No bioactive compounds were added to the control.

Sugars, anthocyanins, total phenolics and vitamin C determination in blueberry

The sugar (glucose and fructose) content was quantified by ultra-high pressure liquid chromatography–mass spectrometry as previously described.²⁵ Individual ACNs and chlorogenic acid were analyzed by high performance liquid chromatography (HPLC),²⁵ while total phenolic compounds were analyzed by the Folin–Ciocalteu assay and expressed as gallic acid equivalents (mg per 100 g).²⁶ Vitamin C (ascorbic acid) was extracted and determined by HPLC analysis as previously described.²⁷

Subject recruitment

Sixteen healthy male smokers, with an average age of 23.6 ± 2.9 and BMI of $23.0 \pm 1.9\text{ kg m}^{-2}$, were recruited from the

student population of the University of Milan according to the following criteria: 20–30 years of age, homogeneous for smoking habit (about 15 cigarettes per day, 270 packs containing 20 cigarettes per year), physical activity (25–30 min per day of brisk walk or jog) and alcohol consumption (up to 10–14 drinks of wine or beer per week). Subjects were recruited on the basis of an interview by a dietitian to evaluate their dietary habits. This was obtained by means of a food frequency questionnaire previously published²⁸ and revised focusing on polyphenol-rich foods (*e.g.* chocolate, green tea) with particular attention paid to berry consumption. Exclusion criteria were hypertension (systolic blood pressure $> 140\text{ mmHg}$ and/or diastolic blood pressure $> 90\text{ mmHg}$), fasting hyperglycaemia ($> 5.5\text{ mmol L}^{-1}$), hypertriglyceridemia ($\text{TG} \geq 1.69\text{ mmol L}^{-1}$) and hypercholesterolemia (total serum cholesterol (TSC) $\geq 5.17\text{ mmol L}^{-1}$, low HDL cholesterol (HDL-C) $< 1.03\text{ mmol L}^{-1}$, high LDL cholesterol (LDL-C) $\geq 3.36\text{ mmol L}^{-1}$), endothelial dysfunction (RHI < 1.67) and overweight (BMI $\geq 25\text{ kg m}^{-2}$). Other exclusion criteria were history of cardiovascular, coronary, diabetes, hepatic, renal, or gastrointestinal diseases, traumas of the arms or hands, fingers, atopic dermatitis, thyroid disturbance, depression, anxiety, palpitations and chronic backache. Subjects were excluded if they were taking supplements, drugs or medications for at least one month before the beginning of the study. The study was performed in accordance with the ethical standards established in the 1964 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan. Moreover, this study was registered at <http://www.isrctn.org> as ISRCTN59129089. All participants signed the informed consent form.

Experimental design

Volunteers were selected for a repeated measure 3-armed randomized-controlled study and assigned to 3 different groups: S- smoking treatment; BS- blueberry treatment (300 g of blueberry) + smoking; CS- control treatment (300 mL of water with sugar) + smoking. Each protocol was separated by 7 days of wash-out period (Fig. 1). All subjects ($n = 16$) completed the three treatments. The control treatment was chosen since it was reported that sugar intake may affect endothelial function.²⁹ Both blueberry and control products presented similar glycaemic response within the first 15 min following their consumption and dropped to the baseline after 1 h (data not shown). Subjects were deprived of polyphenol-rich foods 10 days before experimentation. Specific attention was devoted to foods such as chocolate, berry fruits (*i.e.* blueberries, cranberries, raspberries, blackcurrants, and elderberries), red wine and red to blue fruits, and green tea. Volunteers were asked to limit their intake of coffee to three cups per day, as well as caffeine-rich beverages (*e.g.* energy drinks), to standardize their intake and reduce a potential effect on vascular function. The day before the experiment and during the trial, breakfast, lunch and dinner were standardized. Breakfast consisted of milk and biscuits (*i.e.* shortbread) while lunch was composed of two sandwiches (one with cooked ham and cheese and one with raw ham). During dinner, subjects could eat pasta or rice

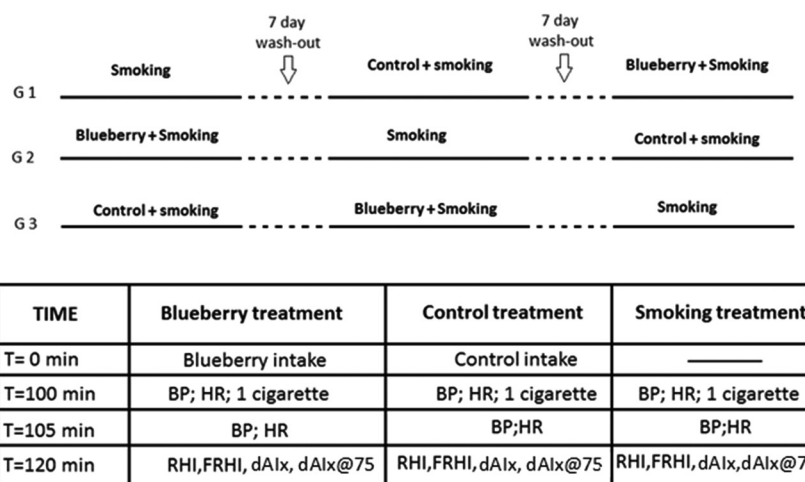


Fig. 1 Randomized experimental design ($n = 16$). Sixteen subjects for each group; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm; G, groups; F-RHI, Framingham reactive hyperemia index; HR, heart rate; BP, blood pressure; RHI, reactive hyperemia index.

with butter and cheese, and a steak with potatoes and two slices of white bread. The dinner was consumed by 9.00 pm. Only one cup of coffee was allowed at the end of dinner. No alcoholic drinks or soft drinks were permitted. Overall the meals were standardized in order to provide adequate energy/macronutrient intake, limiting polyphenols and taking into account Italian dietary habits. Moreover, all participants were asked to refrain from physical activity from the day before the experiment and to continue smoking the number of cigarettes per day as declared in the questionnaire.

For the present study, peripheral arterial function was measured in two consecutive days. This protocol was chosen to avoid multiple measurements (involving 5 min arterial occlusion through cuff inflation) in a short time-period, because it could promote vasodilation through NO production between test and re-test evaluation.³⁰ In addition, we excluded an inter-day variability demonstrating a within-subject repeatability of the measurement of vascular function²⁰ as also reported by other authors.^{31,32} Therefore, baseline levels were assessed on the first day early in the morning in volunteers who had fasted overnight. On the second day, vascular function was assessed after subjects smoked one cigarette (S) or consumed 300 g blueberry or the control treatment, followed by one cigarette smoking (BS or CS respectively). The cigarette, containing approximately 6 mg of tar by volume, 0.5 mg of nicotine and 0.9 mg of carbon monoxide, was smoked for 100 min after blueberry or control consumption. The protocol is described in Fig. 1 and was designed to measure peripheral arterial function 120 min after blueberry intake (*i.e.* 20 min after smoking); the protocol was chosen by considering previous observations on the beneficial effect on endothelial function observed at this specific time-point following the intake of a polyphenol-rich food.^{15,21} The reactive hyperemia index (RHI) and digital augmentation index (dAix) were tested 20 min after smoking ($T = 120$ min). The systolic (S) and diastolic (D) blood pressure (BP), and heart rate (HR) were measured before smoking

($T = 100$ min) and 5 min after smoking one cigarette ($T = 105$ min) and at the end of the endothelial function measurement ($T = 120$ min).

Determination of peripheral arterial function and arterial stiffness

Endothelial-dependent vasodilation in the small finger arteries was assessed by a non-invasive plethysmographic method (Endo-PAT2000, Itamar Medical Ltd, Caesarea, Israel) based on the registration of the pulsatile blood volume in the fingertips of both hands.³³

Briefly, subjects were in the supine position with both hands in the same level in a comfortable, thermoneutral environment. The arterial systolic and diastolic blood pressure and heart rate frequency were measured before starting the test. A blood pressure cuff was placed on one upper arm (study arm), while the contralateral arm served as a control (control arm). After a 10 min equilibration period, the blood pressure cuff on the study arm was inflated to 60 mmHg above the systolic pressure for 5 min. The cuff was then deflated to induce RH while the signals from both PAT channels (Probe 1 and Probe 2) were recorded by a computer. The RHI, an index of the endothelial-dependent flow-mediated dilation, was derived automatically in an operator independent manner, as the ratio of the average pulse wave amplitude during hyperaemia (60 to 120 s of the post-occlusion period) to the average pulse wave amplitude during the baseline in the occluded hand was divided by the same values in the control hand and then multiplied by a baseline correction factor. A RHI value of 1.67 provides a sensitivity of 82% and a specificity of 77% for diagnosing endothelial dysfunction.³³ In addition to the RHI we have also reported in our paper the Framingham RHI (F-RHI), which was automatically calculated using, a different post-occlusion hyperaemia period (90 to 120 s) without the baseline correction factor. The F-RHI, that has been shown to correlate with other CVD risk markers,^{34,35} was expressed as a natural

log of the resulting ratio. The EndoPAT device also generates dAix, strongly correlated to aortic Aix, calculated from the shape of the pulse wave recorded by the probes during baseline.³⁶ Because Aix is influenced in an inverse and linear manner by heart rate, the dAix was automatically normalized by considering a heart rate of 75 bpm (dAix@75).

Biochemical measurements

Blood samples were drawn and immediately centrifuged at 1000g for 15 min for serum separation and stored at -80°C until analysis. A general laboratory clinical assessment was performed in serum, including evaluation of the lipid profile (TAG, TSC, LDL-C and HDL-C), and glucose. All these parameters were determined using standard laboratory methods as previously described.¹⁴

Statistical analysis

The sample size has been calculated taking into account the expected variation of RHI as the primary endpoint considered. Based on our previous observations,^{14,24} sixteen subjects were calculated to be sufficient to evaluate a difference of RHI after a blueberry intake of 0.30 (standard deviation 0.40), with $\alpha = 0.05$ and a statistical power of 80%. Moreover, the “repeated measures” experimental design in which each subject acts as its own control allows reduction of the error variance.

Statistical analysis was performed by means of the STATISTICA software (Statsoft Inc., Tulsa, OK, US). The Shapiro–Wilk test was applied to verify the normal distribution of the variables. Data of the variables under study were analyzed by one way ANOVA with time (before and after smoking) or treatment (smoking *vs.* consuming a portion of blueberry + smoking *vs.* consuming a control drink + smoking) as dependent factors. The variables of the treatment were reported as the percentage change (*i.e.* [after treatment – before treatment]/before treatment $\times 100$). The mean changes are described as a mean with 95% CI. Differences are considered significant at $p \leq 0.05$; *post-hoc* analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as the level of statistical significance. Data are presented as mean values of the standard error of the mean (SEM).

Results

Baseline characteristics of the subjects

The anthropometric and clinical characteristics of the sixteen subjects enrolled in the study are reported in Table 1. The lipid profile (TAG, TSC, LDL-C and HDL-C), glucose, BP, RHI (>1.67) and BMI were in the normal range.

Composition and characteristics of blueberry and control treatments

The fresh-frozen blueberries provided 27 g of total sugars (16.4 g of fructose and 10.6 g of glucose), 309 mg of ACNs (malvidin-galactoside, delphinidin-galactoside, cyanidin-galactoside, petunidin-galactoside and malvidin-arabinoside) were the

Table 1 Anthropometric and clinical characteristics of the subjects at the baseline ($n = 16$)

Variables	Mean \pm SEM
Age (years)	23.6 \pm 0.7
Height (cm)	178.1 \pm 1.7
Weight (kg)	73.1 \pm 2.3
BMI (kg m^{-2})	23.0 \pm 0.5
Smoke (cigarettes per day)	15 \pm 1
SBP (mmHg)	116.0 \pm 1.7
DBP (mmHg)	76.1 \pm 2.1
HR (beat min^{-1})	63.3 \pm 2.9
RHI	2.23 \pm 0.07
F-RHI	0.65 \pm 0.07
dAix (%)	-8.6 \pm 2.0
dAix@75 (%)	-18.4 \pm 2.2
TSC (mmol L^{-1})	4.13 \pm 0.08
HDL-C (mmol L^{-1})	1.43 \pm 0.10
LDL-C (mmol L^{-1})	2.20 \pm 0.10
TAG (mmol L^{-1})	1.01 \pm 0.08
Glucose (mmol L^{-1})	4.34 \pm 0.17

SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for the heart rate of 75 bpm; TSC, total serum cholesterol.

dominant compounds), 856 mg of total phenolic acids, 30 mg of chlorogenic acid and 2.4 mg of ascorbic acid. The control provided the same amount and type of sugars but no bioactive compounds (Table 2).

Effect of smoking on the reactive hyperemia index and arterial stiffness

The values of RHI, F-RHI, dAix and dAix@75 before and after smoking are reported in Table 3. Peripheral arterial function, measured through the digital hyperemic response by the RHI, was impaired after smoking. Smoking induced a significant reduction of endothelial function and in 9 out of 16 subjects the RHI indicated endothelial dysfunction ($\text{RHI} < 1.67$). A significant impairment was also observed for F-RHI. The F-RHI reduction occurred in 13 out of 16 subjects, while a small increase with respect to the baseline value was observed in 3 subjects. Regarding dAix, a significant ($p = 0.003$) reduction was also observed (Table 3), while no significant ($p = 0.819$) effect was detected after normalization for the heart rate (dAix@75).

Effect of smoking on the blood pressure and heart rate

Smoking a single cigarette significantly increased the levels of SBP (from 116.0 ± 1.7 mmHg to 131.7 ± 1.6 mmHg; $p = 0.0001$), DBP (from 76.1 ± 2.1 to 83.5 ± 1.9 ; $p = 0.005$), and HR (from 63.3 ± 2.9 beat min^{-1} to 70.7 ± 2.9 beat min^{-1} ; $p = 0.047$). This effect was transitional and the values dropped to the baseline at the last measurement.

Effect of blueberry and control treatments on the reactive hyperemia index and arterial stiffness

The mean percentage variation values of RHI (A), F-RHI (B), dAix (C), and dAix@75 (D) for each treatment are reported in

Table 2 Nutritional composition of blueberry and control treatments

	Blueberry	Control
Sugars (g per 100 g)		
Fructose	5.46 ± 0.10	5.46
Glucose	3.57 ± 0.18	3.57
Total phenolic compounds (mg per 100 g)	242.4 ± 23.9	—
Chlorogenic acid (mg per 100 g)	30.1 ± 1.2	—
Total anthocyanins (mg per 100 g)	116.1 ± 6.9	—
Mv-3-gal	31.19 ± 1.55	
Mv-3-glc	2.72 ± 0.08	
Mv-3-ara	16.71 ± 0.80	
Dp-3-gal	19.0 ± 2.04	
Dp-3-glc	0.58 ± 0.11	
Cy-3-gal	15.50 ± 1.27	
Cy-3-glc	0.51 ± 0.02	
Cy-3-ara	1.77 ± 0.06	
Pt-3-gal	12.31 ± 1.44	
Pt-3-glc	2.36 ± 0.10	
Peo-3-gal	8.07 ± 0.30	
Peo-3-glc	1.26 ± 0.04	
Vitamin C (mg per 100 g)	0.8 ± 0.1	—

Data are expressed as means ± SD. Mv-3-gal, malvidin-3-galactoside; Mv-3-glc, malvidin-3-glucoside; Mv-3-ara, malvidin-3-arabinoside; Dp-3-gal, delphinidin-3-galactoside; Dp-3-glc, delphinidin-3-glucoside; Cy-3-gal, cyanidin-3-galactoside; Cy-3-glc, cyanidin-3-glucoside; Cy-3-ara, cyanidin-3-arabinoside; Pt-3-gal, petunidin-3-galactoside; Pt-3-glc, petunidin-3-glucoside; Peo-3-gal, peonidin-3-galactoside; Peo-3-glc, peonidin-3-glucoside.

Table 3 Arterial function and arterial stiffness measured before and 20 min after smoking a cigarette ($n = 16$)^a

	Before smoking	20 min after smoking	p value ^b
RHI	2.23 ± 0.08	1.64 ± 0.07	0.0001
F-RHI	0.65 ± 0.08	0.31 ± 0.07	0.002
dAix (%)	-7.8 ± 2.1	-14.1 ± 1.8	0.003
dAix@75 (%)	-18.8 ± 2.2	-19.1 ± 2.2	0.819

^a Data are expressed as mean ± SEM. RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for a heart rate of 75 bpm. ^b Overall p value for one-way ANOVA with STATISTICA (Statsoft Inc., Tulsa, OK, US).

Fig. 2(A–D). Repeated measures ANOVA revealed a significant effect of treatment for the variable RHI ($p = 0.0006$), and F-RHI ($p = 0.003$) while no effect was observed for dAix and dAix@75 ($p = 0.20$ and $p = 0.79$, respectively). The mean percentage change pre- to post-treatment for RHI was -25.2% (95% CI: -34%, -16.2%) following S treatment, -17.5% (95% CI: -26%, -8.9%) following CS treatment and -6.6% (95% CI: -13%, -0.5%) following BS treatment (Fig. 2A). As reported for smoking (see the previous paragraph), a similar reduction of RHI was also observed in 14 out of 16 subjects following CS treatment, while a small increase compared to the baseline was documented in 2 subjects. Reduced impairment of endothelial dysfunction was observed in 11 out of 16 subjects following BS treatment compared to the baseline, while in 5 subjects RHI increased.

The mean percentage change pre- to post-treatment for F-RHI was -42.7% (95% CI: -85.4%, -0.15%) for S treatment,

-8.1% (95% CI: -36.5%, +20.3%) for CS treatment and +28.3% (95% CI: -12.6%, +69.2%) for BS treatment (Fig. 2B). *Post-hoc* analysis (LSD test) revealed that consumption of a single serving of blueberry significantly counteracted the reduction of RHI and F-RHI after S treatment (BS vs. S, $p = 0.0001$ and $p = 0.0008$, respectively). However, the reduction was significantly different with respect to CS treatment (BS vs. CS, $p = 0.01$) for RHI, but not for F-RHI (BS vs. CS, $p = 0.06$). No effect was observed between S vs. CS treatment for both the variables (RHI, $p = 0.09$ and F-RHI, $p = 0.08$).

Effect of blueberry and control treatments on the systolic and diastolic blood pressure, and heart rate

The mean percentage variation for SBP, DBP and HR for each treatment 5 min after smoking is reported in Fig. 3(A–C). Statistical analysis revealed a significant effect of treatment for SBP ($p = 0.01$). The mean percentage change between the pre- and post-treatment was +13.1% (95% CI: 10.5%, 15.7%) after S treatment, +12.7% (95% CI: 10.2%, 15.2%) after CS treatment, and +8.4% (95% CI: 5.4%, 11.4%) after BS treatment (Fig. 3A). *Post-hoc* analysis (LSD test) showed that the consumption of a single blueberry portion counteracted significantly the increment of SBP after S treatment (BS vs. S, $p = 0.008$). This effect was also significantly different with respect to CS treatment (BS vs. CS, $p = 0.01$) while no significant difference was observed between S and CS ($p = 0.90$). No effect was observed after blueberry intake for the variables DBP and HR among the three treatments ($p = 0.71$ and $p = 0.50$, respectively).

Discussion

In the present study we have documented that acute smoking can significantly reduce peripheral arterial function and increase blood pressure and heart rate in healthy male smoker volunteers. The deleterious effects observed are in accordance with those found in several studies^{1–3} and with our previous observations.²⁴ Endothelial dysfunction could be related to multiple compounds following combustion of tobacco smoke that elevate the levels of vasoconstrictors such as vascular endothelial growth factors and endothelin-1, reduce NO levels, and increase oxidative stress.⁴

We demonstrated that a single 300 g serving of fresh-frozen blueberry could counteract the endothelial dysfunction induced by smoking, when measured 2 h after blueberry consumption. These results are in accordance with those obtained by Karatzi *et al.*³⁷ who documented the capacity of red wine and dealcoholized red wine to counterbalance the endothelial dysfunction, induced after 30 and 60 min from smoking, in young healthy smokers. In addition, our results are also in accordance with the previous observations in which polyphenol-rich foods, such as chocolate and cranberries, demonstrated to affect vascular function 2 hours after consumption.^{15,21} These beneficial effects could be dependent on the absorption of bioactive compounds. In a previous study, we demonstrated that one serving (300 g) of blueberries

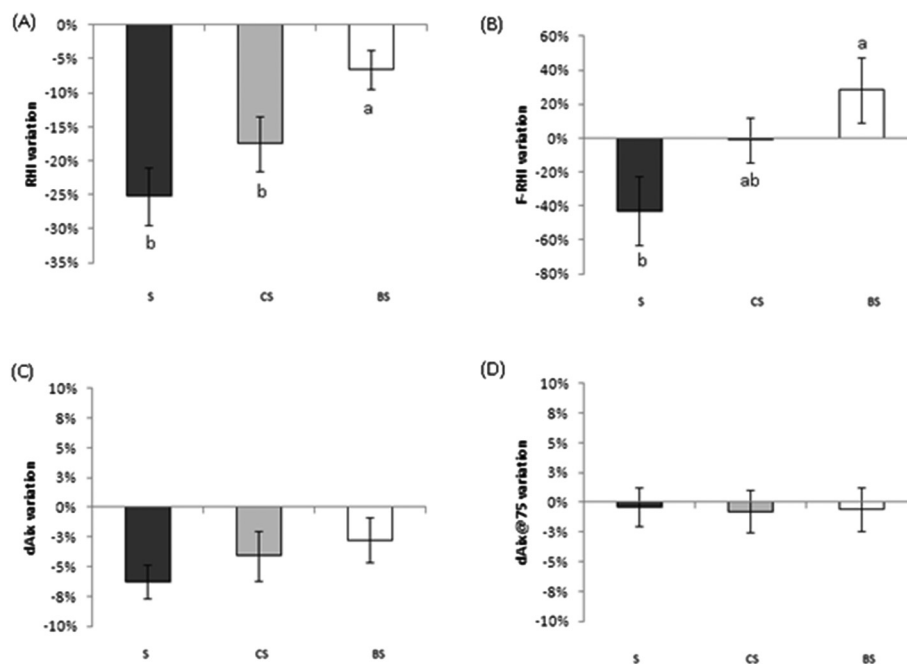


Fig. 2 Mean percent variation of RHI (A), F-RHI (B), dAix (C), and dAix@75 (D) measured during each treatment ($n = 16$). Data are expressed as mean \pm SEM. S, smoking treatment; CS, control + smoking treatment; BS, blueberry + smoking treatment; RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm. ^{a,b}Graphs with different letters are significantly different from other treatments ($p \leq 0.01$).

could increase ACN plasma levels up to 2 h from the time of intake.³⁸ Thus, the beneficial effects on endothelial function could be related to the kinetics of absorption of polyphenol compounds. In this regard, many studies demonstrated that ACNs are rapidly absorbed in blood (generally within 2–3 hours) reaching nanomolar concentrations that tend to disappear within the first 4–6 hours from food intake. In the meantime, ACN metabolite concentrations increase in plasma as an effect of endogenous metabolic pathways already after 2 h from their consumption.³⁹ Thus, an important parameter to be considered, while performing short-term studies, is the length of time between the intake of food/supplement and the measurement of peripheral arterial function. In this regard, in a previous study, we failed to demonstrate modulation of endothelial function 1 h after 300 g blueberry consumption in non-smoking male subjects.²⁰ In the present study circulating levels of ACNs or phenolic compounds were not measured, thus we cannot postulate a casual effect of the above compounds in the modulation of RHI.

As far as long term intervention studies are concerned, results are still inconclusive. We recently reported that 6 weeks of wild blueberry drink consumption failed to significantly alter vascular function in subjects with cardiovascular risk factors,¹⁴ even though half of the population experienced an improvement. Similar results have been observed by other authors after intervention with cranberries¹⁵ and apples.¹⁶ One possible explanation could be related to different protocols used [different times of exposure to bioactive compounds, markers related to vascular function (flow mediated dilation

vs. peripheral arterial function), methodologies (PAT vs. BAUS), and different study populations] as mentioned previously. However, we cannot exclude that the conflicting results on modulation of endothelial function can be due to differences in food sources and the amount and type of polyphenol considered. In this context positive effects on endothelial function after dark chocolate and/or flavonol intake seem to derive from medium-long intervention studies.^{37,38,40–42} Results available suggest that the vasodilatory and vasoprotective mechanisms of polyphenols include improved bioavailability of vasodilators (*i.e.* NO, endothelium-derived hyperpolarizing factor and prostacyclin), inhibition of the synthesis of vasoconstrictor endothelin-1 in endothelial cells and the inhibition of expression of pro-angiogenic factors such as vascular endothelial growth factor and matrix metalloproteinase-2 in smooth muscle cells.^{43,44}

In the present study, we documented that even though smoking reduced dAix, no effect was observed after normalization for heart beats. Our findings are in agreement with several studies where acute smoking did not affect arterial stiffness in young smokers;⁴⁵ in contrast studies performed in older smokers showed an increase in arterial stiffness.⁴⁵ Thus, the age of volunteers can be a critical factor in the outcome, since young people have more elastic walls able to counteract the vasoconstriction induced by smoking.^{45,46}

It has been suggested that consumption of polyphenol-rich foods may reduce and improve arterial stiffness;^{47,48} in the present study the intake of blueberry did not affect this parameter. Our results are in accordance with those obtained

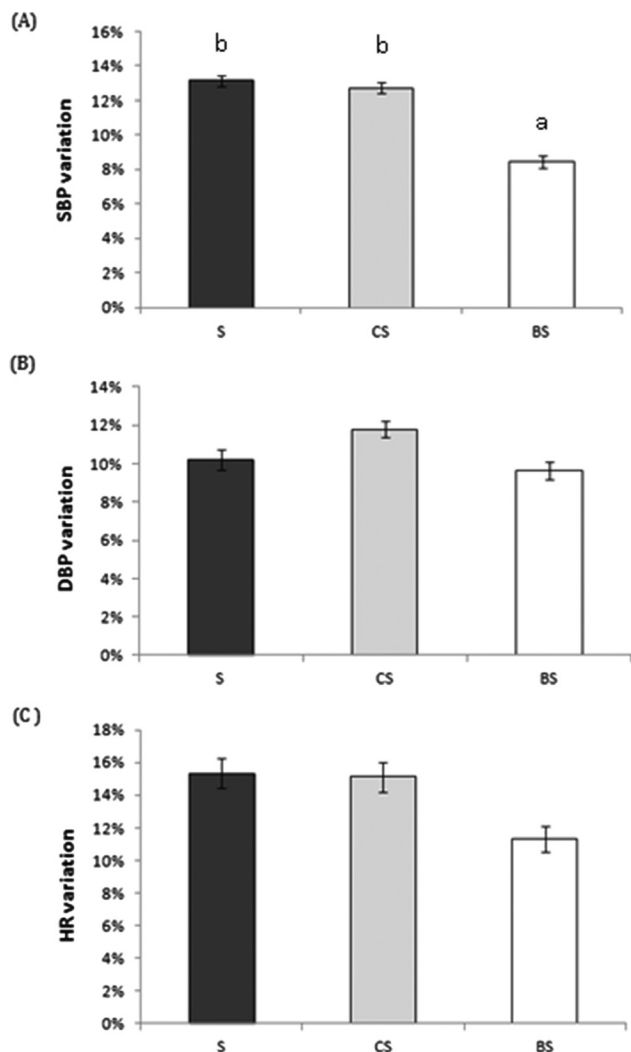


Fig. 3 Mean percent variation of SBP (A), DBP (B) and HR (C) measured during each treatment ($n = 16$). Data are expressed as mean \pm SEM. S, smoking treatment; CS, control + smoking treatment; BS, blueberry + smoking treatment; SBP, systolic blood pressure; DPB, diastolic blood pressure; HR, heart rate. ^{a,b}Graphs with different letters are significantly different from other treatments ($p \leq 0.05$).

by Mathew *et al.*⁴⁹ in which no effect on arterial stiffness was observed following consumption of a high-fat meal and pomegranate juice extract, in contrast to the results obtained by Karatzi *et al.*⁴⁸ who documented modulation of arterial stiffness following an acute consumption of polyphenol-rich beer.

Short-term smoking can increase the blood pressure and heart rate. In the present study, we demonstrated that acute cigarette smoking impaired the blood pressure and heart rate. These changes were observed 5 min after smoking and were not apparent 30 min later. This is in accordance with the results obtained by Lekakis *et al.*² and Stefanadis *et al.*⁵⁰ who documented a prompt increment in heart rate and blood pressure during the first 5 min after smoking attributed to an increase in circulating levels of catecholamines that reach a

maximum concentration 5–10 min after smoking, and return to baseline levels after 30 min.⁵⁰

In this context, we have demonstrated that the consumption of blueberry before smoking can counteract the increase of SBP compared to the control, supporting the potential beneficial effect of polyphenol compounds on the modulation of blood pressure.

Several studies indicate that diets rich in antioxidant compounds can improve the blood pressure. A recent meta-analysis has reported for the first time that the intake of polyphenol and ACN-rich foods is associated with low levels of blood pressure.¹¹ Similar results were also observed by Mathew *et al.*⁴⁹ who documented that the consumption of an active drink (containing a pomegranate extract) resulted in suppression of the postprandial increase in systolic blood pressure following a high-fat meal. In contrast, two recent dietary intervention studies reported that 4-week consumption of an ACN-extract did not reduce the levels of blood pressure in healthy and pre-hypertensive men.^{51,52}

Conclusion

In conclusion, we documented that blueberries may prevent peripheral arterial dysfunction induced by acute cigarette smoking in young volunteers. These results confirm previous observations on the protective role of blueberry in the modulation of vascular function, emphasizing the contribution of berry fruit consumption especially in people exposed to oxidative stress such as smokers. However, we should point out that blueberry consumption cannot be considered a means of preventing health consequences due to smoking; this can only be realized by smoking cessation and/or prevention. Prospective short-term studies in larger samples are needed to confirm blueberry's beneficial effects and to underline the mechanisms involved in the modulation of vascular function. Moreover, long term interventions are required to clarify the effect of regular berry fruit consumption justifying possible dietary recommendations.

Author contributions

The authors' contributions are as follows: Cristian Del Bo' and Daniela Fracassetti performed the study, analyzed the data and drafted the manuscript; Marisa Porrini and Patrizia Riso obtained funding, contributed to the study concept and design, supervised the study, and critically revised the manuscript; Jonica Campolo and Dorothy Klimis-Zacas contributed to the study concept and design and critically revised the manuscript. None of the authors had any conflict of interest.

Abbreviations

ACNs	Anthocyanins
dAix	Digital augmentation index

dAix@75	Digital augmentation index normalized for the heart rate
DBP	Diastolic blood pressure
F-RHI	Framingham reactive hyperemia index
HPLC	High performance liquid chromatography
HR	Heart rate
NO	Nitric oxide
RHI	Reactive hyperemia index
SEM	Standard error of the mean
SBP	Systolic blood pressure
TSC	Total serum cholesterol

Acknowledgements

This study was supported by a grant (Rif. Pratica 2010.2303) from Cariplo Foundation. We thank Ms Alice Zhao and Ms Anisia Canavesio for their technical support and contributions to the study. We are grateful to all our volunteers for their time and commitment.

References

- R. L. Bard, J. T. Dvorchak, N. Kaciroti, S. A. Lustig and R. D. Brook, Is acute high-dose secondhand smoke exposure always harmful to microvascular function in healthy adults?, *Prev. Cardiol.*, 2010, **13**, 175–179.
- J. Lekakis, C. Papamichael, C. Vemmos, J. Nanas, D. Kontoyannis, S. Stamatelopoulos and S. Mouloupoulos, Effect of acute cigarette smoking on endothelium-dependent brachial artery dilatation in healthy individuals, *Am. J. Cardiol.*, 1997, **79**, 529–531.
- I. Gül, H. Karapinar, M. Yarlioglu, I. Ozdogru, M. G. Kaya, A. Yilmaz, O. O. Turgut, I. Tandogan and N. K. Eryol, Acute effects of passive smoking on endothelial function, *Angiology*, 2011, **62**, 245–247.
- A. Csiszar, A. Podlutzky, M. S. Wolin, G. Losonczy, P. Pacher and Z. Ungvari, Oxidative stress and accelerated vascular aging: implications for cigarette smoking, *Front. Biosci.*, 2009, **14**, 3128–3144.
- R. M. Fitch, R. Vergona, M. E. Sullivan and Y. X. Wang, Nitric oxide synthase inhibition increases aortic stiffness measured by pulse wave velocity in rats, *Cardiovasc. Res.*, 2001, **51**, 351–358.
- J. Nürnberger, A. Keflioglu-Scheiber, A. M. Opazo Saez, R. R. Wenzel, T. Philipp and R. F. Schäfers, Augmentation index is associated with cardiovascular risk, *J. Hypertens.*, 2002, **20**, 2407–2414.
- S. Laurent and P. Boutouyrie, Arterial stiffness: a new surrogate end point for cardiovascular disease?, *J. Nephrol.*, 2007, **20**(Suppl. 12), S45–S50.
- T. Tsuda, Dietary anthocyanin-rich plants: Biochemical basis and recent progress in healthy benefits studies, *Mol. Nutr. Food Res.*, 2012, **56**, 159–170.
- D. Grassi, G. Desideri and C. Ferri, Flavonoids: antioxidants against atherosclerosis, *Nutrients*, 2010, **2**, 889–902.
- C. D. Kay, L. Hooper, P. A. Kroon, E. B. Rimm and A. Cassidy, Relative impact of flavonoid composition, dose and structure on vascular function: a systematic review of randomised controlled trials of flavonoid-rich food products, *Mol. Nutr. Food Res.*, 2012, **56**, 1605–1616.
- A. Jennings, A. A. Welch, S. J. Fairweather-Tait, C. Kay, A. M. Minihane, P. Chowienczyk, B. Jiang, M. Cecelja, T. Spector, A. Macgregor and A. Cassidy, Higher anthocyanin intake is associated with lower arterial stiffness and central blood pressure in women, *Am. J. Clin. Nutr.*, 2012, **96**, 781–788.
- M. C. Lazzè, R. Pizzala, P. Perucca, O. Cazzalini, M. Savio, L. Forti, V. Vannini and L. Bianchi, Anthocyanidins decrease endothelin-1 production and increase endothelial nitric oxide synthase in human endothelial cells, *Mol. Nutr. Food Res.*, 2006, **50**, 44–51.
- S. de Pascual-Teresa, D. A. Moreno and C. García-Viguera, Flavonols and anthocyanins in cardiovascular health: A review of current evidence, *Int. J. Mol. Sci.*, 2010, **11**, 1679–1703.
- P. Riso, D. Klimis-Zacas, C. Del Bo', D. Martini, J. Campolo, S. Vendrame, P. Møller, S. Loft, R. De Maria and M. Porrini, Effect of a wild blueberry (*Vaccinium angustifolium*) drink intervention on markers of oxidative stress, inflammation and endothelial function in humans with cardiovascular risk factors, *Eur. J. Nutr.*, 2013, **52**, 949–961.
- M. M. Dohadwala, M. Holbrook, N. M. Hamburg, S. M. Shenouda, W. B. Chung, M. Titas, M. A. Kluge, N. Wang, J. Palmisano, P. E. Millbury, J. B. Blumberg and J. A. Vita, Effects of cranberry juice consumption on vascular function in patients with coronary artery disease, *Am. J. Clin. Nutr.*, 2011, **93**, 934–940.
- S. Auclair, G. Chironi, D. Milenkovic, P. C. Hollman, C. M. Renard, J. L. Mégnien, J. Gariépy, J. L. Paul, A. Simon and A. Scalbert, The regular consumption of a polyphenol-rich apple does not influence endothelial function: a randomized double-blind trial in hypercholesterolemic adults, *Eur. J. Clin. Nutr.*, 2010, **64**, 1158–1165.
- J. Barona, J. C. Aristizabal, C. N. Blesso, J. S. Volek and M. L. Fernandez, Grape polyphenols reduce blood pressure and increase flow-mediated vasodilation in men with metabolic syndrome, *J. Nutr.*, 2012, **142**, 1626–1632.
- L. A. van Mierlo, P. L. Zock, H. C. van der Knaap and R. Draijer, Grape polyphenols do not affect vascular function in healthy men, *J. Nutr.*, 2010, **140**, 1769–1773.
- R. Moreno-Luna, R. Muñoz-Hernandez, M. L. Miranda, A. F. Costa, L. Jimenez-Jimenez, A. J. Vallejo-Vaz, F. J. Muriana, J. Villar and P. Stiefel, Olive oil polyphenols decrease blood pressure and improve endothelial function in young women with mild hypertension, *Am. J. Hypertens.*, 2012, **25**, 1299–1304.
- C. Del Bo', P. Riso, J. Campolo, P. Møller, S. Loft, D. Klimis-Zacas, A. Brambilla, A. Rizzolo and M. Porrini, A single portion of blueberry (*Vaccinium corymbosum* L) improves protection against DNA damage but not vascular function in healthy male volunteers, *Nutr. Res.*, 2013, **33**, 220–227.

- 21 L. Loffredo, R. Carnevale, L. Perri, E. Catasca, T. Augelletti, R. Cangemi, F. Albanese, C. Piccheri, C. Nocella, P. Pignatelli and F. Violi, NOX2-mediated arterial dysfunction in smokers: acute effect of dark chocolate, *Heart*, 2011, **97**, 1776–1781.
- 22 J. Oyama, T. Maeda, K. Kouzuma, K. R. Ochiai, I. Tokimitsu, Y. Higuchi, M. Sugano and N. Makino, Green tea catechins improve human forearm endothelial dysfunction and have antiatherosclerotic effects in smokers, *Circ. J.*, 2010, **74**, 578–588.
- 23 C. E. Berryman, J. A. Grieger, S. G. West, C. Y. Chen, J. B. Blumberg, G. H. Rothblat, S. Sankaranarayanan and P. M. Kris-Etherton, Acute consumption of walnuts and walnut components differentially affect postprandial lipemia, endothelial function, oxidative stress, and cholesterol efflux in humans with mild hypercholesterolemia, *J. Nutr.*, 2013, **143**, 788–794.
- 24 C. Del Bo', J. Campolo, M. Porrini, D. Fracassetti, M. Parolini, D. Klimis-Zacas and P. Riso, Acute cigarette smoking impairs microvascular function in young moderate smokers: a model for studying vasoactive properties of food bioactives, *PharmaNutrition*, 2014, **2**, 1–7.
- 25 C. Del Bo', S. Ciappellano, D. Klimis-Zacas, D. Martini, C. Gardana, P. Riso and M. Porrini, Anthocyanin absorption, metabolism and distribution from a wild blueberry-enriched diet (*Vaccinium angustifolium*) is affected by diet duration in the Sprague-Dawley rat, *J. Agric. Food Chem.*, 2010, **58**, 2491–2497.
- 26 N. Pellegrini, F. Visioli, S. Buratti and F. Brighenti, Direct analysis of total antioxidant activity of olive oil and studies on the influence of heating, *J. Agric. Food Chem.*, 2001, **49**, 2532–2538.
- 27 P. Riso, F. Visioli, C. Gardana, S. Grande, A. Brusamolino, F. Galvano, G. Galvano and M. Porrini, Effects of blood orange juice intake on antioxidant bioavailability and on different markers related to oxidative stress, *J. Agric. Food Chem.*, 2005, **53**, 941–947.
- 28 M. Porrini, M. G. Gentile and F. Fidanza, Biochemical validation of a self-administered semi-quantitative food-frequency questionnaire, *Br. J. Nutr.*, 1995, **74**, 323–333.
- 29 A. Ceriello, K. Esposito, L. Piconi, M. Ihnat, J. Thorpe, R. Testa, A. G. Bonfigli and D. Giugliano, Glucose “peak” and glucose “spike”: Impact on endothelial function and oxidative stress, *Diabetes Res. Clin. Pract.*, 2008, **82**, 262–267.
- 30 M. C. Corretti, T. J. Anderson, E. J. Benjamin, D. Celermajer, F. Charbonneau, M. A. Creager, J. Deanfield, H. Drexler, M. Gerhard-Herman, D. Herrington, P. Vallance, J. Vita, R. Vogel and International Brachial Artery Reactivity Task Force, Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force, *J. Am. Coll. Cardiol.*, 2002, **39**, 257–265.
- 31 S. Onkelinx, V. Cornelissen, K. Goetschalckx, T. Thomaes, P. Verhamme and L. Vanhees, Reproducibility of different methods to measure the endothelial function, *Vasc. Med.*, 2012, **17**, 79–84.
- 32 Y. Reisner, R. Lusky, Y. Shay-El, R. Schnall and S. Herscovici, Reproducibility of endothelial function and arterial stiffness assessed using finger peripheral arterial tonometry, *Eur. Heart J.*, 2008, **29**(Suppl. 1), 491.
- 33 P. O. Bonetti, Attenuation of digital reactive hyperaemia in patients with early and advanced coronary artery disease, *J. A. A. C.*, 2005, **45**, 407A.
- 34 N. M. Hamburg, M. J. Keyes, M. G. Larson, R. S. Vasan, R. Schnabel, M. M. Pryde, G. F. Mitchell, J. Sheffy, J. A. Vita and E. J. Benjamin, Cross-sectional relations of digital vascular function to cardiovascular risk factors in The Framingham Heart Study, *Circulation*, 2008, **117**, 2467–2474.
- 35 N. M. Hamburg and E. J. Benjamin, Assessment of endothelial function using digital pulse amplitude tonometry, *Trends Cardiovasc. Med.*, 2009, **19**, 6–11.
- 36 S. Munir, A. Guilcher, T. Kamalesh, B. Clapp, S. Redwood, M. Marber and P. Chowienczyk, Peripheral augmentation index defines the relationship between central and peripheral pulse pressure, *Hypertension*, 2008, **51**, 112–118.
- 37 K. Karatzi, C. Papamichael, E. Karatzis, T. G. Papaioannou, P. T. Voidonikola, J. Lekakis and A. Zampelas, Acute smoking induces endothelial dysfunction in healthy smokers. Is this reversible by red wine's antioxidant constituents?, *J. Am. Coll. Nutr.*, 2007, **26**, 10–15.
- 38 C. Del Bo', P. Riso, A. Brambilla, C. Gardana, A. Rizzolo, P. Simonetti, G. Bertolo, D. Klimis-Zacas and M. Porrini, Blanching improves anthocyanin absorption from high-bush blueberry (*Vaccinium corymbosum* L.) purée in healthy human volunteers: a pilot study, *J. Agric. Food Chem.*, 2012, **60**, 9298–9304.
- 39 C. D. Kay, G. Mazza, B. J. Holub and J. Wang, Anthocyanin metabolites in human urine and serum, *Br. J. Nutr.*, 2004, **91**, 933–942.
- 40 J. Balzer, T. Rassaf, C. Heiss, P. Kleinbongard, T. Lauer, M. Merx, N. Heussen, H. B. Gross, C. L. Keen, H. Schroeter and M. Kelm, Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients a double-masked, randomized, controlled trial, *J. Am. Coll. Cardiol.*, 2008, **51**, 2141–2149.
- 41 K. Davison, A. M. Coates, J. D. Buckley and P. R. Howe, Effect of cocoa flavanols and exercise on cardiometabolic risk factors in overweight and obese subjects, *Int. J. Obes.*, 2008, **32**, 1289–1296.
- 42 C. Heiss, S. Jahn, M. Taylor, W. M. Real, F. S. Angeli, M. L. Wong, N. Amabile, M. Prasad, T. Rassaf, J. I. Ottaviani, S. Mihardja, C. L. Keen, M. L. Springer, A. Boyle, W. Grossman, S. A. Glantz, H. Schroeter and Y. Yeghiazarians, Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease, *J. Am. Coll. Cardiol.*, 2010, **56**, 218–224.
- 43 V. B. Schini-Kerth, C. Auger, J. H. Kim, N. Etienne-Selloum and T. Chataigneau, Nutritional improvement of the endo-

- thelial control of vascular tone by polyphenols: role of NO and EDHF, *Pflugers Arch.*, 2010, **459**, 853–862.
- 44 J. C. Stoclet, T. Chataigneau, M. Ndiaye, M. H. Oak, J. El Bedoui, M. Chataigneau and V. B. Schini-Kerth, Vascular protection by dietary polyphenols, *Eur. J. Pharmacol.*, 2004, **500**, 299–313.
- 45 R. C. Seet, W. M. Loke, C. M. Khoo, S. E. Chew, W. L. Chong, A. M. Quek, E. C. Lim and B. Halliwell, Acute effects of cigarette smoking on insulin resistance and arterial stiffness in young adults, *Atherosclerosis*, 2012, **224**, 195–200.
- 46 N. Rehill, C. R. Beck, K. R. Yeo and W. W. Yeo, The effect of chronic tobacco smoking on arterial stiffness, *Br. J. Clin. Pharmacol.*, 2006, **61**, 767–773.
- 47 G. Ruel, A. Lapointe, S. Pomerleau, P. Couture, S. Lemieux, B. Lamarche and C. Couillard, Evidence that cranberry juice may improve augmentation index in overweight men, *Nutr. Res.*, 2013, **33**, 41–49.
- 48 K. Karatzi, V. G. Rontoyanni, A. D. Protogerou, A. Georgoulia, K. Xenos, J. Chrysou, P. P. Sfikakis and L. S. Sidossis, Acute effects of beer on endothelial function and hemodynamics: A single-blind, crossover study in healthy volunteers, *Nutrition*, 2013, **29**, 1122–1126.
- 49 A. S. Mathew, G. M. Capel-Williams, S. E. Berry and W. L. Hall, Acute effects of pomegranate extract on postprandial lipaemia, vascular function and blood pressure, *Plant Foods Hum. Nutr.*, 2012, **67**, 351–357.
- 50 R. Stefanadis, E. Tsiamis, C. Vlachopoulos, C. Stratos, K. Toutouzas, C. Pitsavos, S. Marakas, H. Boudoulas and P. Toutouzas, Unfavorable effect of smoking on the elastic properties of the human aorta, *Circulation*, 1997, **95**, 31–38.
- 51 S. S. Hassellund, A. Flaa, L. Sandvik, S. E. Kjeldsen and M. Rostrup, Effects of anthocyanins on blood pressure and stress reactivity: a double-blind randomized placebo-controlled crossover study, *J. Hum. Hypertens.*, 2012, **26**, 396–404.
- 52 S. S. Hassellund, A. Flaa, S. E. Kjeldsen, I. Seljeflot, A. Karlsen, I. Erlund and M. Rostrup, Effects of anthocyanins on cardiovascular risk factors and inflammation in pre-hypertensive men: a double-blind randomized placebo-controlled crossover study, *J. Hum. Hypertens.*, 2013, **27**, 100–106.