A serving of blueberry *(V. corymbosum)* acutely improves peripheral arterial dysfunction in young smokers and non-smokers: a randomized controlled crossover pilot study

Cristian Del Bo'[1*], Valeria Deon[1], Jonica Campolo[2], Claudia Lanti[1], Marina Parolini[2], Marisa Porrini[1], Dorothy Klimis-Zacas[3] and Patrizia Riso[1]

[1]Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition, Milan, Italy; [2]CNR Institute of Clinical Physiology, CardioThoracic and Vascular Department, ASST Great Metropolitan Hospital Niguarda, Milan, Italy. [3]School of Food and Agriculture, University of Maine, ME, USA.

*Dr Cristian Del Bo', PhD, Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition, Via G. Celoria 2, 20133 Milan, Italy, Tel: +39 02503 16727; Fax: +39 02503 16721. Email: cristian.delbo@unimi.it

ABSTRACT

Several studies have documented the important role of polyphenol-rich foods in the modulation of vascular remodelling and function. This study aimed to evaluate the capacity of a single portion of blueberry (*V. corymbosum)* to acutely improve peripheral arterial dysfunction in a group of young volunteers.

Twenty-four healthy male (12 non-smokers and 12 smokers) were recruited for two different randomized, controlled, crossover pilot acute studies. In the first study, non-smokers were exposed to a control treatment (C; 300 mL of water with sugar) and a blueberry treatment (BB; 300 g of blueberry). In the second study, smokers underwent 3 different protocols: 1-smoking treatment (S); 2-control treatment (CS; 300 mL of water with sugar + smoking); 3-blueberry treatment (BS; 300 g of blueberry + smoking). Each treatment (1 day long) was separated by a one week washout period. Blood pressure, peripheral arterial function (reactive hyperemia index, RHI, a marker of endothelial function) and arterial stiffness (digital augmentation index, dAix and dAix normalized by considering a heart rate of 75 bpm, dAix@75) were measured before and after each treatment.

In the first study, the consumption of blueberry and control treatment acutely increased peripheral arterial function in the group of non-smokers. The improvement in RHI was higher and significantly different after blueberry treatment compared to control treatment (54.8±8.4% BB *vs.* 28.2±8.3% C; p=0.01). No effects were observed for markers of arterial stiffness, blood pressure and heart rate.

Acute cigarette smoke significantly increased blood pressure and heart rate, while no significant effect was registered in peripheral arterial function and stiffness. The intake of blueberry and control treatment before a cigarette did not counteract the increase in blood pressure and heart rate, while it significantly improved peripheral arterial function. In particular, a significant increase was observed following BS (35.2±7.5% RHI; p=0.02) and CS treatment (34.6±11.9% RHI; p=0.02) when compared to only smoking treatment. No difference between BS and CS was detected.

In conclusion, the intake of blueberry and control treatment acutely improved peripheral arterial dysfunction both in smoker and non-smoker subjects. Further studies should be performed to confirm the results obtained and reveal the potential mechanisms of blueberry in the improvement of endothelial function.

Keywords: Blueberry, arterial function, arterial stiffness, blood pressure, smoker and non-smoker subjects

Introduction

Endothelial dysfunction is characterized by an imbalance between vasodilator and vasoconstrictor mediators resulting in altered vascular tone, organ blood flow and peripheral vascular resistance.[1] The endothelium, in response to mechanical and hormonal stimuli, releases vasoconstrictor and vasodilator agents that regulate vasomotor function by inducing vasoconstriction or vasodilation.[2] Nitric oxide (NO) is one of the most important molecules involved in the process of vasodilation. When endothelial damage occurs, the bioavailability of NO decreases causing vasoconstriction of the endothelium.[2] Endothelial dysfunction represents the first step leading to the development of atherosclerosis.[1] Convincing evidence supports the pivotal role of smoking, as source of oxidative stress, in the development of ED.[3] Cigarette smoke causes a temporal increase in blood pressure, heart rate and acute endothelial damage, and vascular and systemic inflammation.[4]

Blueberries are a rich source of polyphenolic compounds such as phenolic acids and anthocyanins involved in the modulation of several functional and metabolic pathways related to endothelial function.[5-8] For example, anthocyanins (ACNs) have been reported to induce the expression and activity of numerous enzymes involved in NO metabolism.[7] In particular, ACNs have documented to stimulate endothelial nitric oxide synthase (eNOS) and to decrease endothelial NADPH oxidase activity and $O_2^{\cdot-}$ levels as a result of haem oxygenase-induction.[7] Furthermore, ACNs have been shown to reduce the expression of a plethora of pro-inflammatory agents involved in the adhesion of monocytes to endothelial cells by inhibiting redox-sensitive transcription factor NF-kB and by eliciting cell adaptive responses involving the transcription factor Nrf2.[7,9-11]

The role of blueberries in the modulation of vascular function has been evaluated in different acute and chronic intervention studies.[5,12-17] Stull et al.[12] reported that a 6-week intervention with blueberries (22.5g of freeze-dried blueberry powder, providing 290 mg of anthocyanins) smoothie (twice/day) improved endothelial function in subjects with metabolic syndrome. We previously documented that a 6-week consumption of 250 mL of a wild blueberry drink (25g of freeze-dried blueberry powder, providing 375 mg of anthocyanins) failed to show an improvement of peripheral

arterial function, a marker of endothelial function, in the whole group of subjects with cardiovascular risk factors.[17] The beneficial effects were documented only in smokers and in those with endothelial dysfunction.[17] Recently, we showed that a serving of 300 g of blueberry purée (providing about 300 mg of anthocyanins) was able to counteract the temporary impairment of endothelial function induced by acute cigarette smoke in a group of smoker volunteers with normal endothelial function.[14] Based on our previous research, we evaluated whether the consumption of the same portion of blueberries could also improve endothelial function also in subjects with a dysfunctional endothelium (single risk factor) and in those exposed to oxidative stress (smoking) and showing an impairment in peripheral arterial function (double risk factor). To this aim, two randomized, controlled, pilot studies (one in a group of smokers and the other one in non-smoker subjects) were performed in which the effect of a single blueberry portion on peripheral arterial function was evaluated.

Materials and Methods

Subject selection

Twenty-four healthy male (12 non-smokers and 12 smokers) subjects with peripheral arterial dysfunction (RHI < 1.67) were recruited from the student population of the University of Milan according to the following criteria: 20–30 years of age, reactive hyperemia index, as marker of peripheral arterial function (RHI < 1.67), moderate physical activity (up to 25-30 min per day of brisk walk or jog) and alcohol consumption (no more than 14 drinks of wine or beer per week). Smokers were homogeneous for smoking habits (about 15 cigarettes per day). Exclusion criteria were hypertension (systolic pressure >140 mm Hg and diastolic pressure >90 mm Hg), fasting hyperglycemia (>10 mmol/L), hypercolesterolemia (high total serum cholesterol, >5.17 mmol/L; high low density lipoprotein cholesterol, >3.36 mmol/L), hypertriglyceridemia (>1.69 mmol/L), overweight/obese (BMI >25 kg/m^2) based on American Heart Association guidelines.[18] Subjects with normal endothelial function (RHI >1.67) were automatically excluded from the study.

Moreover, diabetic patients, subjects with renal insufficiency, constipation, diarrhea or gastrointestinal problem or diseases, were not included. Subjects with traumas of the arms or hands, fingers, atopic dermatitis, thyroid disturbance, depression, anxiety, palpitations, asthma, and chronic backache were excluded. Other exclusion criteria were as follow: allergies, high (>5 portions/day) or low (<2 portions/day) intake of fruit and vegetables, specific diet (e.g. vegetarian, vegan or macrobiotic), specific aversion to blueberries or their products, use of drugs, supplements, medications during the last month. The study was performed in accordance to the ethical standards established in the 1964 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan. All participants signed the informed consent form. The study was registered at www.isrctn.org as ISRCTN59129089.

Food preparation and composition

Fresh blueberry (*Vaccinium corymbosum* L. "Brigitta"), from a single batch, was purchased and immediately stored at -20°C until use. A portion (300 g) of frozen blueberry was thawed at +4°C overnight and provided to the participants. It contained 27 g of total sugars, 856 mg of total phenolic acids, 309 mg of total anthocyanins, 30 mg of chlorogenic acid and 2.4 mg of ascorbic acid. The control treatment was prepared by suspending 16.4 g of fructose and 10.6 g of glucose (the same amount and type of sugars contained in the blueberry) in 300 ml of water. No bioactive compounds were added.

Experimental design

Prior to the intervention, a 10-day run-in period was performed. Subjects were deprived of polyphenol-rich foods such as chocolate, berries, 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 reduce a potential effect on vascular function. The day before the

experiment, breakfast, lunch and dinner were standardized to provide adequate energy/macronutrient intake, taking into account Italian dietary habits.[14] All participants refrained from physical activity the day before the experiment, while smokers were asked to maintain their smoking habits as reported in the questionnaire but refrain from cigarettes the morning of the experiment.

Study 1- Twelve nonsmokers with peripheral arterial dysfunction were randomly divided into 2 groups of 6 subjects each: group 1 was assigned to the sequence BB treatment/wash-out/C treatment, whereas group 2 followed the sequence C/wash-out/BB treatment. The study consisted of a repeated measure 2-arm randomized-controlled trial (**Fig. 1A**). Each protocol was separated by a 7-day wash-out period. RHI levels were assessed in the morning after overnight fasting and following the consumption of 300 g blueberries or control treatment (BB or C, respectively). The protocol was designed to measure vascular function (peripheral arterial function and arterial stiffness) 120 min after blueberry/control intake by considering our previous observations on the specific time-point effect on endothelial function observed following the intake of the same portion of blueberry.[14] The evaluation of peripheral arterial function and arterial stiffness was performed at baseline (T = 0) and after the intake of blueberry and control treatment (T = 120 min). Systolic (S) and diastolic (D) blood pressure (BP), and heart rate (HR) were measured in duplicate as follows: before BB and C intake (T = 0 min), after BB and C intake (T = 100 min), and following the measurements of endothelial function and arterial stiffness (T = 120 min).

Study 2-Twelve smokers with peripheral arterial dysfunction were randomly 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. The study consisted of a repeated measure 3-arm randomized-controlled trial (**Fig. 1B**). Each protocol was separated by a-7 day wash-out period. The day of the experiment, baseline RHI levels were assessed early in the morning after overnight fasting and without smoking. Successively, peripheral arterial function was assessed after smoking (S) or following the consumption of 300 g blueberries or control treatment and smoking (BS or CS

150 respectively). The protocol was designed to measure reactive peripheral arterial function and
151 stiffness 120 min after blueberry/control intake (corresponding to 20 minutes after cigarette
152 smoking) according to our previous publication.[14] Systolic (S) and diastolic (D) blood pressure
153 (BP), and heart rate (HR) were measured in duplicate at baseline (T = 0 min), before smoking (T =
154 100 min), 5 min after smoking one cigarette (T = 105 min), and following the measurements of
155 endothelial function and arterial stiffness (T = 120 min).
156
157 **Evaluation of blood pressure, heart rate, peripheral arterial function and arterial stiffness**
158 Peripheral arterial function was performed by a non-invasive plethysmographic method (Endo-PAT
159 2000, Itamar Medical Ltd, Caesarea, Israel) as previously reported in detail.[14] During the
160 assessment, participants were in a supine position in a comfortable, dimly lit and temperature-
161 controlled room. After application of the occlusion cuff to the dominant arm and finger tip probes to
162 the index fingers of each hand, the study began with a 5 minute baseline, 5 minutes of occlusion,
163 and last 5 minutes of post-occlusion measurements (hyperemic period). Occlusion of the brachial
164 artery was performed on the dominant upper arm (at least 60 mmHg above the systolic blood
165 pressure; minimally 200 mmHg and maximally 300 mmHg). The Endo-PAT system generates
166 automatically a value of reactive hyperemia index (RHI) as index of the endothelial-dependent
167 flow-mediated dilation (FMD; gold standard method for the evaluation of endothelial function).[19-20]
168 A RHI value less than 1.67 provides a sensitivity of 82% and a specificity of 77% for diagnosing
169 endothelial dysfunction.[21] The Endo-PAT device also provides dAix, strongly correlated to aortic
170 Aix, calculated from the shape of the pulse wave recorded by the probes during baseline.[22] Because
171 Aix is influenced in an inverse and linear manner by heart rate, the dAix was automatically
172 normalized by considering a heart rate of 75 bpm (dAix@75).
173
174 **Statistical analysis**

The sample size was calculated taking into account the expected variation of RHI as the primary endpoint considered. Based on our previous observation,[14] twelve subjects were calculated to be sufficient to evaluate a difference of RHI after a blueberry intake of 0.30 (standard deviation 0.35), with alpha = 0.05 and a statistical power of 80%. In addition, the repeated measures design reduces the variance of estimates of treatment-effects allowing to use fewer subjects. Finally, the number of subjects enrolled was comparable to that reported in other studies evaluating the role of cocoa, chocolate and mango in the modulation of endothelial function through PAT technology.[23-26] Results for each treatment are reported as the percentage change (i.e. [after treatment − before treatment]/before treatment × 100). Mean changes are described as a mean with 95% CI. Variables were analyzed by one way ANOVA with time or treatment as dependent factors. Differences were 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 reported as mean values and standard error of the mean (SEM). Statistical analysis was performed by means of the STATISTICA software (Statsoft Inc., Tulsa, OK, US).

Results

Study 1

Baseline characteristic of non-smoker subjects

The anthropometric and clinical characteristics at baseline of the 12 healthy non-smoker subjects enrolled are reported in **Table 1**. All volunteers presented values of RHI lower than 1.67 (cut-off to discriminate an endothelial dysfunction). Blood pressure, heart rate and BMI were in the normal range.

Effect of blueberry and control treatments on blood pressure, heart rate, arterial function and arterial stiffness in non-smokers

The mean percentage changes pre- to post-treatment following the intake of blueberry and control are presented in **Table 2**. Both treatments did not have a significant impact on SBP, DPB and HR. The effect of blueberry and control treatment on RHI (A), dAix (B) and dAix@75 (C) is reported in **Figure 2**. Both treatments had a favorable effect on RHI showing an improvement compared to baseline (*time effect*). In particular, 10 out of 12 subjects (83%) reversed their RHI impairment following blueberry treatment, while 7 out of 12 subjects (58%) did similarly following the control treatment. The mean percentage change pre- to post-treatment following the BB treatment for RHI was +54.8% (95% CI: +37.9%, +71.7%) and +28.2% (95% CI: +11.5%, +44.9%) following C treatment (**Fig. 2A**). This increase was higher and significantly different after BB when compared to C treatment (RHI, $p = 0.011$; *treatment effect*). On the contrary, no significant effect was observed for dAix and dAix@75 as markers of arterial stiffness (**Fig. 2B and 2C).**

Study 2

Baseline characteristics of smoker subjects

The anthropometric and clinical characteristics of the 12 smoker subjects enrolled are reported in **Table 3**. All volunteers were apparently healthy with blood pressure, heart rate and BMI in the normal range while the levels of peripheral arterial function were lower than 1.67, implying impaired peripheral arterial function.

Effect of smoking on blood pressure, heart rate, arterial function and arterial stiffness in smokers.

Smoking induced a significant temporary increase in the levels of SBP (from 117.1 ± 4.40 mmHg to 129.6 ± 2.99 mmHg; $p = 0.006$), DBP (from 74.2 ± 4.00 to 83.2 ± 3.32; p = 0.03) and HR (from 65.2 ± 3.58 beat min^{-1} to 75.3 ± 5.07 beat min^{-1}; $p = 0.04$). The rise was registered after 5 min from smoking and the values dropped to baseline after 20 min (**Fig. 3**). **Table 4** reports the levels of RHI,

dAix and dAix@75 before and after smoking. No significant effect on markers of arterial function and stiffness was observed after acute cigarette smoking.

Effect of blueberry and control treatments on blood pressure, heart rate, arterial function and stiffness in smokers

The effect of BS and C treatment on blood pressure and heart rate in smoker volunteers is reported in **Table 5**. No significant effect in the mean percentage change pre- to post-treatment was observed on SBP, DPB and HR following the three interventions (S *vs.* CS *vs.* BS). **Figure 4** shows the effect of S, BS and CS treatment on RHI (A), dAix (B) and dAix@75 (C). ANOVA revealed a significant effect of the treatment for the variable RHI (p = 0.03; **Fig. 4A**). In particular, the mean percentage change pre- to post-treatment was +8.38% (95% CI: −4.57%, +21.3%) following S treatment, +34.6% (95% CI: +10.6%, +58.7%) following CS treatment and +35.2% (95% CI: +20.2%, +50.3%) following BS treatment. Post-hoc analysis (*LSD* test) showed that consumption of a single serving of blueberry and control significantly reversed the impairment of RHI **(Fig. 4B)** when compared to S treatment (BS *vs.* S, p = 0.022 and CS *vs.* S, p = 0.023**).**

However, BS and CS treatments did not differ in their effect (p = 0.954). No significant variation was detected for dAix and dAix@75 following the three treatments **(Fig. 4C)**.

Discussion

Several studies have emphasized the role of polyphenol-rich foods in the modulation of vascular function. Encouraging results have been obtained following the consumption of a serving of coffee,[27-28] tea,[29-30] dark chocolate and cocoa powder,[25,31-33] and grape,[34] while few and conflicting are those reported after the intake of different types of berries.[15-16, 35-38] For example, Dohadwala and colleagues documented a significant increase in vascular function 4 h after the consumption of a cranberry juice (480 ml, providing 835 mg total polyphenols and 94 mg anthocyanins) in a group of subjects with coronary artery disease.[36] Rodriguez-Mateos et al.[15] showed that the consumption

of blueberry baked products (containing a total of 34 g of wild blueberry powder, equivalent to 240 g of fresh blueberry) improved endothelial function at 1, 2 and 6 h in healthy volunteers. In contrast, we previously failed to observe a beneficial effect on vascular function 1 h after the intake of a portion (300 g) of blueberry (providing 300 mg of anthocyanins) in young, healthy subjects with normal endothelial function.[37] Also, Jin et al.,[38] reported that vascular reactivity was not affected 2 h from the intake of 250 ml blackcurrant juice drink in a group of healthy volunteers. In the present pilot studies, we documented that the administration of 300 g of blueberry acutely improved peripheral arterial function both in smoker and non-smoker subjects after 2 h from the consumption. Discrepancies among studies may be dependent on the type and the dose of berry (e.g. cranberry *vs.* blackcurrant *vs.* blueberry), the experimental design, and/or the subject characteristics (e.g. healthy *vs.* subjects with cardiovascular risk factors/endothelial dysfunction), the length of time between berry intake and the evaluation of the endothelial function (e.g. 1 h *vs.* 2, 4 or 6 h), and the mode of measuring endothelial function (e.g. flow mediated dilation *vs.* peripheral arterial function). Another important variable to be considered may be the peak time of absorption of bioactives/metabolites and their blood concentration. Rodriguez-Matheos et al.[16] reported that the administration of different doses of blueberry polyphenols (766, 1278, and 1791 mg total blueberry polyphenols, equivalent to 240, 400, and 560 g fresh blueberries, respectively) increased endothelial function at 1-2 h and 6 h in a group of healthy subjects and these beneficial effects were closely linked to the increase of circulating levels of polyphenol metabolites in a time- and dose-dependent manner. In our study, we did not measure the absorption of blueberry bioactives and their metabolic products; however, we previously documented that the intake of the same blueberry portion, increased anthocyanin absorption at 1 h, reaching the maximum peak at 1.5-2 h from consumption.[39] These findings could explain the modulation of endothelial function observed at 2 h in our subjects.

It is widely recognized that cigarette smoking causes acute and chronic vascular damage. We reported that the smoke of one cigarette, temporarily induces vascular dysfunction in a group of

young smokers.[40] In the present study, acute cigarette smoke did not further induce detrimental effects on vascular function in smokers with established endothelial dysfunction. Positively, the intake of blueberries reversed this condition, in agreement with our previous publication.[14] Similar results were also reported by Schwarz and colleagues documenting that pre-consumption of red wine prevented most of the negative vascular effects of acute smoking in a group of young healthy non-smokers.[41] In our study, the beneficial effects observed following the consumption of blueberry were not significantly different compared to those observed after the intake of control drink. The lack of a significant difference in RHI between blueberry and control treatment, together with the apparent increase observed also in the non-smoker group following the intake of control drink, could be attributed to different factors. For example, it has been reported that the amount and type of sugar may affect endothelial function.[42-43] The consumption of both blueberry and control drink, providing the same amount of glucose and fructose, brought blood glucose to a comparable elevation within the first 15 min from intake and dropped to baseline after 1 h, as documented in a subgroup of subjects (data not shown). Since the evaluation of arterial function was performed 2 h from the intake of blueberry and control drink, we can exclude a direct contribution of sugars for the above observation. However, different studies reported a possible involvement of insulin and glucagon, two hormones secreted in response to blood sugar levels, in the modulation of vascular function especially at microvascular level.[44-46] The secretion of insulin may induce changes in microcirculatory tone, activate the eNOS pathway and consequently lead to vasodilation.[44-45] On the other hand, glucagon can trigger the formation of cAMP, which induces the formation of NO playing a pivotal role in vasorelaxation.[46] Since PAT technology measures endothelial function at the microvascular level, and subjects did not consume food apart from the blueberry and control drinks) during the entire duration of the experiment, the involvement of insulin or glucagon cannot ruled out.

Arterial stiffness represents a significant determinant of pulse pressure and elasticity of the blood vessels.[47] Numerous studies found that chronic smoking increases arterial stiffness.[48] The loss

303 of elasticity of the artery walls reduces its compensatory ability to absorb the pulsatile energy and
304 the wave propagation effects, that influence peripheral wave reflection. This inability for
305 compensatory response, results in the gradual increase in blood pressure with age, leading to the
306 development of isolated systolic hypertension and to an increase of cardiovascular risk. The Endo-
307 PAT system provides the value of augmentation index (AI) and the value of digital AI, standardized
308 for heart rate (dAI@75), as markers of arterial stiffness. Digital AI reflects changes in vessel
309 diameter, blood pressure and heart rate.[49] In our experimental conditions, we documented that the
310 subjects involved did not show an impairment in arterial stiffness, probably due to their young age.
311 Acute cigarette smoke, as well as the intake of a portion of blueberry, seem to be insufficient to
312 alter arterial stiffness in accordance with previous observations.[14] However, other studies reported
313 a significant effect following medium/long term intervention with blueberries.[50-51] Johnson et al.,[50]
314 showed that 8 weeks of blueberry consumption (22 g freeze-dried blueberry powder, providing
315 about 844 mg phenolics and 470 mg anthocyanins) reduced arterial stiffness in postmenopausal
316 women with pre- and stage 1-hypertension. McAnulty and colleagues reported that 6 weeks
317 consumption of 25 g of a whole blueberry powder (equivalent to 250 g fresh berries, not
318 characterized for phenolic compounds) improved arterial stiffness in sedentary men and women.[51]
319 Recent research emphasized the role blueberries in the control of blood pressure in subjects
320 with pre-hypertension, hypertension and/or metabolic syndrome.[50-53] We previously reported that
321 the intake of a portion of blueberry was unable to affect blood pressure in a group of healthy
322 subjects,[37] while we documented its capability to counteract the increase in systolic blood pressure
323 induced by acute cigarette smoke.[14] In the present study, we confirmed the effect of smoking on
324 blood pressure and heart rate in accordance with the literature, but we failed to demonstrate the
325 ability of blueberries to counterbalance this impairment, probably due to the small number of
326 subjects enrolled. Similar results have been reported by McAnulty and colleagues that documented
327 no significant effect on blood pressure following an acute and chronic intervention with blueberries
328 in smokers.[54]

Possible study limitations are the small number of subjects, the lack of information regarding the circulating levels of insulin, glucagon and anthocyanins as potential mechanisms underpinning the improvement of endothelial function. Finally, another factor maybe the absence of a real placebo as control treatment.

In conclusion, this study documented that one portion of blueberries (300 g) can acutely improve endothelial function in young smokers and non-smokers with endothelial dysfunction. Additional, acute and chronic intervention studies should be performed to confirm the results obtained and reveal the potential mechanisms of action through which blueberries can affect endothelial function. Moreover, since sugars have been shown to positively influence the endothelial response, the role of insulin and glucagon must be evaluated in the future. Even though this pilot study concludes that blueberries can overcome the endothelial dysfunction associated with cigarette smoking, the authors do encourage people to stop smoking in order to reduce all risks associated with this habit.

Author contribution

Cristian Del Bo' performed the study and drafted the manuscript; Valeria Deon and Claudia Lanti enrolled the subjects and contributed to performing the study; Marisa Porrini and Patrizia Riso designed the study and provided funding. Jonica Campolo, Marina Parolini and Dorothy Klimis Zacas contributed to the study concept and design. All the authors critically revised the manuscript and declare no conflict of interest.

Acknowledgements

This study was supported by intramural fundings and by a grant (Rif. Pratica 2010.2303) from Cariplo Foundation. We are grateful to all our volunteers for their time and commitment.

References

1. A. Sandoo, J. J. van Zanten, G. S. Metsios, D. Carroll, G. D. Kitas. The endothelium and its role in regulating vascular tone, *Open Cardiovasc. Med. J.,* 2010, **4**, 302-312.

2. D. Tousoulis, A. M. Kampoli, C. Tentolouris, N. Papageorgiou, C. Stefanadis, The role of nitric oxide in endothelial function, *Curr. Vasc. Pharmacol.,* 2012, **10**, 4-18.

3 B. Messner & D. Bernhard, Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis, *Arterioscler. Thromb. Vasc. Biol.,* 2014, **34**, 509-515.

4. S. Salahuddin, D. Prabhakaran, A. Roy, Pathophysiological mechanisms of tobacco-related CVD. *Glob. Heart* 2012, **7**, 113-120.

5. B. R. Cutler, C. Petersen, P. V. Anandh Babu. Mechanistic insights into the vascular effects of blueberries: Evidence from recent studies, *Mol. Nutr. Food Res.,* 2017, **61**, doi: 10.1002/mnfr.201600271.

6. A. Kerimi & G. Williamson. At the interface of antioxidant signalling and cellular function:key polyphenol effects, *Mol. Nutr. Food Res.,* 2016, **60**, 1770-1788.

7. A.Speciale, F. Cimino, A. Saija, R. Canali, F. Virgili, Bioavailability and molecular activities of anthocyanins as modulators of endothelial function, *Genes Nutr.,* 2014, **9**, 404.

8. M. Edwards, C. Czank, G. M. Woodward, A. Cassidy, C. D. Kay, Phenolic metabolites of anthocyanins modulate mechanisms of endothelial function. *J. Agric. Food Chem.,* 2015, **63**, 2423-2431.

9. E.F. Warner, M. J, Smith, Q, Zhang, K. S. Raheem, D. O'Hagan, M. A. O'Connell, C. D. Kay. Signatures of anthocyanin metabolites identified in humans inhibit biomarkers of vascular inflammation in human endothelial cells, *Mol. Nutr. Food Res.,* 2017, doi: 10.1002/mnfr.201700053.

10. S. Vendrame & D. Klimis-Zacas. Anti-inflammatory effect of anthocyanins via modulation of nuclear factor-κB and mitogen-activated protein kinase signaling cascades. *Nutr. Rev.,* 2015, **73**, 348-358.

11.A, Aboonabi & I. Singh, Chemopreventive role of anthocyanins in atherosclerosis via activation of Nrf2-ARE as an indicator and modulator of redox. Biomed. Pharmacother., 2015, 72, 30-36.

12. A. J. Stull, K. C. Cash, C. M. Champagne, A. K., Gupta, R. Boston, R. A. Beyl, W. D. Johnson, W.T. Cefalu, Blueberries improve endothelial function, but not blood pressure, in adults with metabolic syndrome: a randomized, double-blind, placebo-controlled clinical trial, Nutrients, 2015, 7, 4107-4123.

13. S. A. Johnson, A. Figueroa, N. Navaei, A. Wong, R. Kalfon, L. T. Ormsbee, R. G. Feresin, M. L. Elam, S. Hooshmand, M.E. Payton, B. H. Arjmandi. Daily blueberry consumption improves blood pressure and arterial stiffness in postmenopausal women with pre- and stage 1-hypertension: a randomized, double-blind, placebo-controlled clinical trial. *J. Acad. Nutr. Diet.*, 2015, **115**, 369-377.

14. C. Del Bo', M. Porrini, D. Fracassetti, J. Campolo, D. Klimis-Zacas, P. Riso. A single serving of blueberry (V. corymbosum) modulates peripheral arterial dysfunction induced by acute cigarette smoking in young volunteers: a randomized-controlled trial. *Food Funct.*, 2014, **5**, 3107-3116.

15. A. Rodriguez-Mateos, R. Del Pino-García, T. W. George, A. Vidal-Diez, C. Heiss, J. P. Spencer. Impact of processing on the bioavailability and vascular effects of blueberry (poly)phenols, *Mol. Nutr. Food Res.*, 2014, **58**, 1952-1961.

16. A. Rodriguez-Mateos, C. Rendeiro, T. Bergillos-Meca, S. Tabatabaee, T. W. George, C. Heiss, J. P. Spencer, Intake and time dependence of blueberry flavonoid-induced improvements in vascular function: a randomized, controlled, double-blind, crossover intervention study with mechanistic insights into biological activity, *Am. J. Clin. Nutr.*, 2013, **98**, 1179-1191.

17. P. Riso, D. Klimis-Zacas, C. Del Bo', D. Martini, J. Campolo, S. Vendrame, P. Møller P, S. Loft, R. De Maria, 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.

18. American Heart Association. http://www.heart.org/HEARTORG/ . Accessed 25 July 2017.

406 19. A. L. Axtell, F. A. Gomari, J. P. Cooke. Assessing endothelial vasodilator function with the
407 Endo-PAT 2000. *J. Vis. Exp.*, 2010,**44**, pii: 2167.
408 20. D. Martini, S. Rossi, B. Biasini, I. Zavaroni, G. Bedogni, M. Musci, C. Pruneti, G. Passeri, M.
409 Ventura, S. Di Nuzzo, D. Galli, P. Mirandola, M. Vitale, A. Dei Cas, R. C .Bonadonna, D. Del Rio.
410 Claimed effects, outcome variables and methods of measurement for health claims proposed under
411 European Community Regulation 1924/2006 in the framework of protection against oxidative
412 damage and cardiovascular health. *Nutr. Metab. Cardiovasc. Dis.*, 2017, **27**, 473-503.
413 21. P. O. Bonetti, Attenuation of digital reactive hyperaemia in patients with early and advanced
414 coronary artery disease, *J. A. A. C.*, 2005, **45**, 407A.
415 22. S. Munir, A. Guilcher, T. Kamalesh, B. Clapp, S. Redwood, M. Marber and P. Chowienczyk,
416 Peripheral augmentation index defines the relationship between central and peripheral pulse
417 pressure, *Hypertension*, 2008, **51**, 112–118.
418 23. S. Buchwald-Werner, C. Schön, S. Frank, C. Reule. Effects of Mangifera indica (Careless) on
419 microcirculation and glucose metabolism in healthy volunteers, *Planta Med.*, 2017. doi: 10.1055/s-
420 0043-103017.
421 24. A. Gerstgrasser, S. Röchter, D. Dressler, C. Schön, C. Reule, S. Buchwald-Werner. In Vitro
422 activation of eNOS by Mangifera indica (Careless™) and determination of an effective dosage in a
423 randomized, double-blind, human pilot study on microcirculation. *Planta Med.*, 2016, **82**, 298-304.
424 25. D. D. Mellor, L. A. Madden, K. A. Smith, E. S. Kilpatrick, S. L., High-polyphenol chocolate
425 reduces endothelial dysfunction and oxidative stress during acute transient hyperglycaemia in Type
426 2 diabetes: a pilot randomized controlled trial, *Diabet. Med.*, 2013, **30**, 478-483.
427 26. H. Schroeter, C. Heiss, J. Balzer, P. Kleinbongard, C. L. Keen, N. K. Hollenberg, H. Sies, C.
428 Kwik-Uribe, H. H. Schmitz, M. Kelm, (-)-Epicatechin mediates beneficial effects of flavanol-rich
429 cocoa on vascular function in humans. *Proc. Natl. Acad. Sci. U S A.* 2006, **103**, 1024-1029.

27. N. C. Ward, J. M. Hodgson, R. J. Woodman, D. Zimmermann, L. Poquet, A. Leveques, L. Actis-Goretta, I. B. Puddey, K. D. Croft, Acute effects of chlorogenic acids on endothelial function and blood pressure in healthy men and women, *Food Funct.*, 2016, **7**, 2197-2203.

28. R. Ochiai, Y. Sugiura, K. Otsuka, Y. Katsuragi, T. Hashiguchi T., Coffee bean polyphenols ameliorate postprandial endothelial dysfunction in healthy male adults, *Int. J. Food Sci. Nutr.*, 2015, **66**, 350-354.

29. M. Lorenz, F. Rauhut, C. Hofer, S. Gwosc, E. Müller, D. Praeger, B. F. Zimmermann, K. D. Wernecke, G. Baumann, K. Stangl, V. Stangl, Tea-induced improvement of endothelial function in humans: No role for epigallocatechin gallate (EGCG), *Sci. Rep.*, 2017, **7**, 2279.

30. L. P. Nogueira, J. F. Nogueira Neto, M. R. Klein, A. F. Sanjuliani, Short-term effects of green tea on blood pressure, endothelial function, and metabolic profile in obese prehypertensive women: a crossover randomized clinical trial. *J. Am. Coll. Nutr.*, 2017, **36**, 108-115.

31. A. M. N. D. C. Teixeira, L. A. Luzia, S. J. de Souza, A. de Almeida Petrilli, P. M. Pontilho, J. M. P. de Souza, A. A. C. Segurado, P. Efraim, C. M. Picone, The impact of dark chocolate intake on arterial elasticity in individuals with HIV/AIDS undergoing ART: a randomized, double-blind, crossover trial, *Food Funct.*, 2017, doi: 10.1039/c6fo01681b.

32. S. G. West, M. D. McIntyre, M. J. Piotrowski, N. Poupin, D. L. Miller, A. G. Preston, P. Wagner, L. F. Groves, A. C. Skulas-Ray, Effects of dark chocolate and cocoa consumption on endothelial function and arterial stiffness in overweight adults. *Br. J. Nutr.*, 2014, **111**, 653-661.

33. G. Rull, Z. N. Mohd-Zain, J. Shiel, M. H. Lundberg, D. J. Collier, A. Johnston, T. D. Warner, R. Corder, Effects of high flavanol dark chocolate on cardiovascular function and platelet aggregation, *Vascul. Pharmacol.*, 2015, **71**, 70-78.

34. S. H. Li, H. B. Tian, H. J. Zhao, L. H. Chen, L. Q. Cui, The acute effects of grape polyphenols supplementation on endothelial function in adults: meta-analyses of controlled trials. *PLoS One*, 2013, **8**, e69818.

455 35. A Rodriguez-Mateos, C. Heiss, G. Borges, A. Crozier A, Berry (poly)phenols and
456 cardiovascular health. *J. Agric. Food Chem.*, 2014, **62**, 3842-3851.
457 36. M. M. Dohadwala, M. Holbrook, N. M. Hamburg, S. M. Shenouda, W. B. Chung, M. Titas, M.
458 A. Kluge, N. Wang, J. Palmisano, P. E. Milbury, J. B. Blumberg, J. A. Vita. Effects of cranberry
459 juice consumption on vascular function in patients with coronary artery disease. *Am. J. Clin. Nutr.*,
460 2011, **93**, 934-940.
461 37. C. Del Bo', P. Riso, J. Campolo, P. Møller, S. Loft, D. Klimis-Zacas, A. Brambilla, A. Rizzolo,
462 M. Porrini, A single portion of blueberry (Vaccinium corymbosum L) improves protection against
463 DNA damage but not vascular function in healthy male volunteers, *Nutr. Res.*, 2013, **33**, 220-227.
464 38. Y. Jin, D. Alimbetov, T. George, M. H. Gordon, J. A. Lovegrove. A randomised trial to
465 investigate the effects of acute consumption of a blackcurrant juice drink on markers of vascular
466 reactivity and bioavailability of anthocyanins in human subjects. *Eur. J. Clin. Nutr.*, 2011, **65**, 849-
467 856.
468 39. C. Del Bo', P. Riso, A. Brambilla, C. Gardana, A. Rizzolo, P. Simonetti, G. Bertolo, D. Klimis-
469 Zacas, M. Porrini. Blanching improves anthocyanin absorption from highbush blueberry (
470 Vaccinium corymbosum L.) purée in healthy human volunteers: a pilot study. *J. Agric. Food*
471 *Chem.*, 2012, **60**, 9298-9304.
472 40. C. Del Bo', J. Campolo, M. Porrini, D. Fracassetti, M. Parolini, D. Klimis-Zacas and P. Riso,
473 Acute cigarette smoking impairs microvascular function in young moderate smokers: a model for
474 studying vasoactive properties of food bioactives, *PharmaNutrition*, 2014, **2**, 1–7.
475 41. V. Schwarz, K. Bachelier, S. H. Schirmer, C. Werner, U. Laufs, M. Böhm, Red wine prevents
476 the acute negative vascular effects of smoking, *Am. J. Med.*, 2017, **130**, 95-100.
477 42. A. Major-Pedersen, N. Ihlemann, T. S. Hermann, B. Christiansen, H. Dominguez, B. Kveiborg,
478 D. B. Nielsen, O. L. Svendsen, L. Køber, C. Torp-Pedersen, Effects of oral glucose load on
479 endothelial function and on insulin and glucose fluctuations in healthy individuals, *Exp. Diabetes*
480 *Res.*, 2008, **2008**, 672021.

481 43. E. Jovanovski, A. Zurbau, V. Vuksan, Carbohydrates and endothelial function: is a low-
482 carbohydrate diet or a low-glycemic index diet favourable for vascular health? *Clin. Nutr. Res.*,
483 2015, **4**, 69-75.
484 44. J. Woerdeman, R. I. Meijer, E. C. Eringa, T. Hoekstra, Y. M. Smulders, E. H. Serné, Insulin
485 sensitivity determines effects of insulin and meal ingestion on systemic vascular resistance in
486 healthy subjects, *Microcirculation*, 2016, **23**, 62-68.
487 45. M. P de Boer, R. I. Meijer, I. Newman, C. D. Stehouwer, E. C. Eringa, Y. M. Smulders, E. H.
488 Serné, Insulin-induced changes in microvascular vasomotion and capillary recruitment are
489 associated in humans, *Microcirculation*, 2014, **21**, 380-387.
490 46. W. F. Graier, K. Groschner, K. Schmidt, W. R. Kukovetz, Increases in endothelial cyclic AMP
491 levels amplify agonist-induced formation of endothelium-derived relaxing factor (EDRF), *Biochem.*
492 *J.*, 1992, 288(Pt 2), 345-349.
493 47. A. Avolio, Arterial stiffness, *Pulse*, 2013, **1**, 14–28.
494 48. R. J. Doonan, A. Hausvater, C. Scallan, D. P. Mikhailidis, L. Pilote, S. S. Daskalopoulou, The
495 effect of smoking on arterial stiffness, *Hypertens. Res.*, 2010, **33**, 398-410.
496 49. I. B. Wilkinson, H. MacCallum, L. Flint, J. R. Cockcroft, D. E. Newby, D. J. Webb, The
497 influence of heart rate on augmentation index and central arterial pressure in humans, *J. Physiol.*,
498 2000, **525**, 263–270.
499 50. S. A. Johnson, A. Figueroa, N. Navaei, A. Wong, R. Kalfon, L. T. Ormsbee, R. G. Feresin, M.
500 L. Elam, S. Hooshmand, M. E. Payton, B. H. Arjmandi, Daily blueberry consumption improves
501 blood pressure and arterial stiffness in postmenopausal women with pre- and stage 1-hypertension:
502 a randomized, double-blind, placebo-controlled clinical trial, *J. Acad. Nutr. Diet.*, 2015, **115**, 369-
503 377
504 51. L. S. McAnulty, S. R. Collier, M. J. Landram, D. S. Whittaker, S. E. Isaacs, J. M. Klemka, S. L.
505 Cheek, J. C. Arms, S. R. McAnulty, Six weeks daily ingestion of whole blueberry powder increases

506 natural killer cell counts and reduces arterial stiffness in sedentary males and females, *Nutr. Res.*,
507 2014, **34**, 577-584.
508 52. A. Basu, M. Du, M. J. Leyva, K. Sanchez, N. M. Betts, M. Wu, C. E. Aston, T. J. Lyons,
509 Blueberries decrease cardiovascular risk factors in obese men and women with metabolic
510 syndrome, *J. Nutr.*, 2010, **140**, 1582-1587.
511 53.Y. Zhu, J. Sun, W. Lu, X. Wang, X. Wang, Z. Han, C. Qiu, Effects of blueberry supplementation
512 on blood pressure: a systematic review and meta-analysis of randomized clinical trials, *J. Hum.*
513 *Hypertens.*, 2017, **31**, 165-171.
514 54. S. R McAnulty, L.S. McAnulty, J. D. Morrow, D. Khardouni, L. Shooter, J. Monk, S. Gross, V.
515 Brown, Effect of daily fruit ingestion on angiotensin converting enzyme activity, blood pressure,
516 and oxidative stress in chronic smokers, *Free Radic. Res.*, 2005, **39**, 1241-1248.

Table 1- Characteristics of non-smoker subjects at baseline

Variables	Mean ± SEM[1]
Age (years)	24.2 ± 1.2
Height (cm)	175.8 ± 1.4
Weight (kg)	70.5 ± 2.1
BMI (kg/m^2)	22.5 ± 1.2
SBP (mm Hg)	116.9 ± 3.2
DBP (mm Hg)	75.3 ± 2.9
HR (beat/min)	61.8 ± 5.3
RHI	1.41 ± 0.07
dAix(%)	-14.6 ± 2.7
dAix@75 (%)	-20.0 ± 5.8

Legend

[1]N=12. Data are reported as mean ± SEM; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; RHI, reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm; SEM, standard error of the mean

Table 2 – Mean percentage variation (Δ) of systolic blood pressure (SBP), diastolic blood pressure (DPB) and heart rate (HR) in non-smokers following the intake of blueberry (BB) and control (C) treatment.[1]

	Δ% BB	Δ% C	p value[2]
SBP	-0.89 ± 0.91	-2.93 ± 2.03	0.236
DBP	-2.76 ± 2.33	-1.38 ± 1.04	0.431
HR	-1.08 ± 1.84	0.56 ± 4.05	0.869

Legend

[1] N =12. Data are expressed as means ± SEM. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; BB, blueberry treatment; C, control treatment; SEM, standard error of the mean

[2]Overall *p* value for t-TEST with STATISTICA software (Statsoft Inc., Tulsa, OK, US).

539

Table 3- Characteristics of smoker subjects at baseline

Variables	Mean ± SEM[1]
Age (years)	24.5 ± 1.9
Height (cm)	180.1 ± 1.3
Weight (kg)	70.7 ± 1.2
BMI (kg/m^2)	22.9 ± 1.1
Smoke (cigarettes/day)	15 ± 0.4
SBP (mm Hg)	118.2 ± 2.9
DBP (mm Hg)	75.7 ± 2.7
HR (beat/min)	64.9 ± 5.1
RHI	1.47 ± 0.05
dAix(%)	-12.7 ± 2.5
dAix@75 (%)	-18.2 ± 5.0

Legend

[1]N=12. Data are reported as mean ± SEM; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; RHI, reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm; SEM, standard error of the mean

Table 4 - Arterial function and arterial stiffness before and 20 min after smoking a cigarette in smokers

	Before smoking	20 min After smoking	p value[1]
RHI	1.47 ± 0.05	1.58 ± 0.07	0.324
dAix (%)	-12.7 ± 2.5	-15.6 ± 3.5	0.455
dAix@75 (%)	-18.2 ± 5.0	-18.8 ± 4.9	0.895

Legend

[1] N =12. Data are expressed as means ± SEM. RHI, reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm.

[1]Overall *p* value for t-TEST with STATISTICA software (Statsoft Inc., Tulsa, OK, US).

563 **Table 5** – Mean percentage variation (Δ) of systolic blood pressure (SBP), diastolic blood pressure

564 (DPB) and heart rate (HR) in smokers measured during each treatment.[1]

	Δ% S	Δ% BS	Δ% CS	p value[2]
SBP	11.3 ± 3.5	4.13 ± 1.27	5.38 ± 2.52	0.174
DBP	13.6 ± 5.5	4.09 ± 3.05	2.35 ± 2.34	0.130
HR	15.5 ± 3.7	9.80 ± 4.50	10.8 ± 5.1	0.961

Legend

[1] N =12. Data are expressed as mean ± SEM. S, smoking treatment; CS, control-drink + smoking treatment; BS, blueberry intake + smoking treatment. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate;

565 [2]Overall *p* value for ANOVA with STATISTICA software (Statsoft Inc., Tulsa, OK, US).

566

Figure 1-Experimental designs

Figure legend

A) non-smokers study: two arms, randomized controlled crossover design; B) smokers study: three arms, randomized controlled crossover design.

Figure 2- Mean percent variation of RHI (A), dAix (B), dAix@75(B) measured during blueberry (BB) and control (C) treatment.[1]

Figure legend

[1]Data are expressed as mean ± SEM. C, control treatment; BB, blueberry treatment; RHI, 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 ($p \leq 0.01$).

Figure 3-Variation of systolic and diastolic blood pressure (3A), and heart rate (3B) during acute cigarette smoking over time.[1]

Figure legend

[1]Data are expressed as mean ± SEM. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

[2]Overall *p* value for ANOVA with STATISTICA software (Statsoft Inc., Tulsa, OK, US).

*Data are significantly different.

590 **Figure 4-** Mean percent variation of RHI (A), dAix (B), dAix@75 (C) measured during each
591 treatment.
592
593 **Figure legend**
594 [1]Data are expressed as mean ± SEM. S, smoking treatment; CS, control-drink + smoking treatment;
595 BS, blueberry intake + smoking treatment; RHI, reactive hyperemia index; dAix, digital
596 augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm.
597 [a,b] Graphs with different letters are significantly different from other treatments ($p \leq 0.01$).
598