

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

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16 **ABSTRACT**

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

21 Twenty-four healthy male (12 non-smokers and 12 smokers) were recruited for two different
22 randomized, controlled, crossover pilot acute studies. In the first study, non-smokers were exposed
23 to a control treatment (C; 300 mL of water with sugar) and a blueberry treatment (BB; 300 g of
24 blueberry). In the second study, smokers underwent 3 different protocols: 1-smoking treatment (S);
25 2-control treatment (CS; 300 mL of water with sugar + smoking); 3-blueberry treatment (BS; 300 g
26 of blueberry + smoking). Each treatment (1 day long) was separated by a one week washout period.

27 Blood pressure, peripheral arterial function (reactive hyperemia index, RHI, a marker of endothelial
28 function) and arterial stiffness (digital augmentation index, dAix and dAix normalized by
29 considering a heart rate of 75 bpm, dAix@75) were measured before and after each treatment.

30 In the first study, the consumption of blueberry and control treatment acutely increased peripheral
31 arterial function in the group of non-smokers. The improvement in RHI was higher and
32 significantly different after blueberry treatment compared to control treatment (54.8±8.4% BB vs.
33 28.2±8.3% C; p=0.01). No effects were observed for markers of arterial stiffness, blood pressure
34 and heart rate.

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

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

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

47 **Introduction**

48 Endothelial dysfunction is characterized by an imbalance between vasodilator and vasoconstrictor
49 mediators resulting in altered vascular tone, organ blood flow and peripheral vascular resistance.¹

50 The endothelium, in response to mechanical and hormonal stimuli, releases vasoconstrictor and
51 vasodilator agents that regulate vasomotor function by inducing vasoconstriction or vasodilation.²

52 Nitric oxide (NO) is one of the most important molecules involved in the process of vasodilation.

53 When endothelial damage occurs, the bioavailability of NO decreases causing vasoconstriction of
54 the endothelium.² Endothelial dysfunction represents the first step leading to the development of

55 atherosclerosis.¹ Convincing evidence supports the pivotal role of smoking, as source of oxidative
56 stress, in the development of ED.³ Cigarette smoke causes a temporal increase in blood pressure,
57 heart rate and acute endothelial damage, and vascular and systemic inflammation.⁴

58 Blueberries are a rich source of polyphenolic compounds such as phenolic acids and anthocyanins
59 involved in the modulation of several functional and metabolic pathways related to endothelial
60 function.⁵⁻⁸ For example, anthocyanins (ACNs) have been reported to induce the expression and

61 activity of numerous enzymes involved in NO metabolism.⁷ In particular, ACNs have documented
62 to stimulate endothelial nitric oxide synthase (eNOS) and to decrease endothelial NADPH oxidase
63 activity and O₂⁻ levels as a result of haem oxygenase-induction.⁷ Furthermore, ACNs have been

64 shown to reduce the expression of a plethora of pro-inflammatory agents involved in the adhesion
65 of monocytes to endothelial cells by inhibiting redox-sensitive transcription factor NF-κB and by
66 eliciting cell adaptive responses involving the transcription factor Nrf2.^{7,9-11}

67 The role of blueberries in the modulation of vascular function has been evaluated in different acute
68 and chronic intervention studies.^{5,12-17} Stull et al.¹² reported that a 6-week intervention with
69 blueberries (22.5g of freeze-dried blueberry powder, providing 290 mg of anthocyanins) smoothie

70 (twice/day) improved endothelial function in subjects with metabolic syndrome. We previously
71 documented that a 6-week consumption of 250 mL of a wild blueberry drink (25g of freeze-dried
72 blueberry powder, providing 375 mg of anthocyanins) failed to show an improvement of peripheral

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

85

86 **Materials and Methods**

87 **Subject selection**

88 Twenty-four healthy male (12 non-smokers and 12 smokers) subjects with peripheral arterial
89 dysfunction (RHI < 1.67) were recruited from the student population of the University of Milan
90 according to the following criteria: 20–30 years of age, reactive hyperemia index, as marker of
91 peripheral arterial function (RHI < 1.67), moderate physical activity (up to 25-30 min per day of
92 brisk walk or jog) and alcohol consumption (no more than 14 drinks of wine or beer per week).
93 Smokers were homogeneous for smoking habits (about 15 cigarettes per day). Exclusion criteria
94 were hypertension (systolic pressure >140 mm Hg and diastolic pressure >90 mm Hg), fasting
95 hyperglycemia (>10 mmol/L), hypercholesterolemia (high total serum cholesterol, >5.17 mmol/L;
96 high low density lipoprotein cholesterol, >3.36 mmol/L), hypertriglyceridemia (>1.69 mmol/L),
97 overweight/obese (BMI >25 kg/m²) based on American Heart Association guidelines.¹⁸ Subjects
98 with normal endothelial function (RHI >1.67) were automatically excluded from the study.

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

109

110 **Food preparation and composition**

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

118

119 **Experimental design**

120 Prior to the intervention, a 10-day run-in period was performed. Subjects were deprived of
121 polyphenol-rich foods such as chocolate, berries, red wine and red to blue fruits, and green tea.
122 Volunteers were asked to limit their intake of coffee to three cups per day, as well as caffeine-rich
123 beverages (e.g. energy drinks), to reduce a potential effect on vascular function. The day before the

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

129 **Study 1-** Twelve nonsmokers with peripheral arterial dysfunction were randomly divided into 2
130 groups of 6 subjects each: group 1 was assigned to the sequence BB treatment/wash-out/C
131 treatment, whereas group 2 followed the sequence C/wash-out/BB treatment. The study consisted of
132 a repeated measure 2-arm randomized-controlled trial (**Fig. 1A**). Each protocol was separated by a
133 7-day wash-out period. RHI levels were assessed in the morning after overnight fasting and
134 following the consumption of 300 g blueberries or control treatment (BB or C, respectively). The
135 protocol was designed to measure vascular function (peripheral arterial function and arterial
136 stiffness) 120 min after blueberry/control intake by considering our previous observations on the
137 specific time-point effect on endothelial function observed following the intake of the same portion
138 of blueberry.¹⁴ The evaluation of peripheral arterial function and arterial stiffness was performed at
139 baseline (T = 0) and after the intake of blueberry and control treatment (T = 120 min). Systolic (S)
140 and diastolic (D) blood pressure (BP), and heart rate (HR) were measured in duplicate as follows:
141 before BB and C intake (T = 0 min), after BB and C intake (T = 100 min), and following the
142 measurements of endothelial function and arterial stiffness (T = 120 min).

143 **Study 2-** Twelve smokers with peripheral arterial dysfunction were randomly assigned to 3 different
144 groups: S- smoking treatment; BS- blueberry treatment (300 g of blueberry) + smoking; CS- control
145 treatment (300 mL of water with sugar) + smoking. The study consisted of a repeated measure 3-
146 arm randomized-controlled trial (**Fig. 1B**). Each protocol was separated by a 7 day wash-out period.
147 The day of the experiment, baseline RHI levels were assessed early in the morning after overnight
148 fasting and without smoking. Successively, peripheral arterial function was assessed after smoking
149 (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**

175 The sample size was calculated taking into account the expected variation of RHI as the primary
176 endpoint considered. Based on our previous observation,¹⁴ twelve subjects were calculated to be
177 sufficient to evaluate a difference of RHI after a blueberry intake of 0.30 (standard deviation 0.35),
178 with $\alpha = 0.05$ and a statistical power of 80%. In addition, the repeated measures design reduces
179 the variance of estimates of treatment-effects allowing to use fewer subjects. Finally, the number of
180 subjects enrolled was comparable to that reported in other studies evaluating the role of cocoa,
181 chocolate and mango in the modulation of endothelial function through PAT technology.²³⁻²⁶
182 Results for each treatment are reported as the percentage change (i.e. [after treatment – before
183 treatment]/before treatment \times 100). Mean changes are described as a mean with 95% CI. Variables
184 were analyzed by one way ANOVA with time or treatment as dependent factors. Differences were
185 considered significant at $p \leq 0.05$; post-hoc analysis of differences between treatments was assessed
186 by the *Least Significant Difference (LSD)* test with $p \leq 0.05$ as the level of statistical significance.
187 Data are reported as mean values and standard error of the mean (SEM). Statistical analysis was
188 performed by means of the STATISTICA software (Statsoft Inc., Tulsa, OK, US).

189

190 **Results**

191 **Study 1**

192 **Baseline characteristic of non-smoker subjects**

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

197

198 **Effect of blueberry and control treatments on blood pressure, heart rate, arterial function and** 199 **arterial stiffness in non-smokers**

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

211

212 **Study 2**

213 **Baseline characteristics of smoker subjects**

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

218

219 **Effect of smoking on blood pressure, heart rate, arterial function and arterial stiffness in** 220 **smokers.**

221 Smoking induced a significant temporary increase in the levels of SBP (from 117.1 ± 4.40 mmHg to
222 129.6 ± 2.99 mmHg; $p = 0.006$), DBP (from 74.2 ± 4.00 to 83.2 ± 3.32 ; $p = 0.03$) and HR (from
223 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
224 smoking and the values dropped to baseline after 20 min (**Fig. 3**). **Table 4** reports the levels of RHI,

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

227

228 **Effect of blueberry and control treatments on blood pressure, heart rate, arterial function and** 229 **stiffness in smokers**

230 The effect of BS and C treatment on blood pressure and heart rate in smoker volunteers is reported
231 in **Table 5**. No significant effect in the mean percentage change pre- to post-treatment was observed
232 on SBP, DPB and HR following the three interventions (S vs. CS vs. BS). **Figure 4** shows the effect
233 of S, BS and CS treatment on RHI (A), dAix (B) and dAix@75 (C). ANOVA revealed a significant
234 effect of the treatment for the variable RHI ($p = 0.03$; **Fig. 4A**). In particular, the mean percentage
235 change pre- to post-treatment was +8.38% (95% CI: -4.57%, +21.3%) following S treatment,
236 +34.6% (95% CI: +10.6%, +58.7%) following CS treatment and +35.2% (95% CI: +20.2%,
237 +50.3%) following BS treatment. Post-hoc analysis (*LSD* test) showed that consumption of a single
238 serving of blueberry and control significantly reversed the impairment of RHI (**Fig. 4B**) when
239 compared to S treatment (BS vs. S, $p = 0.022$ and CS vs. S, $p = 0.023$).

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

242

243 **Discussion**

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

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

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

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

301 Arterial stiffness represents a significant determinant of pulse pressure and elasticity of the
302 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

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

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

342

343 **Author contribution**

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

349

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353

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517 **Table 1-** Characteristics of non-smoker subjects at baseline

518

519	Variables	Mean \pm SEM ¹
520	Age (years)	24.2 \pm 1.2
521	Height (cm)	175.8 \pm 1.4
522	Weight (kg)	70.5 \pm 2.1
523	BMI (kg/m ²)	22.5 \pm 1.2
524	SBP (mm Hg)	116.9 \pm 3.2
525	DBP (mm Hg)	75.3 \pm 2.9
526	HR (beat/min)	61.8 \pm 5.3
527	RHI	1.41 \pm 0.07
528	dAix(%)	-14.6 \pm 2.7
529	dAix@75 (%)	-20.0 \pm 5.8
530		

531

532

533 **Legend**

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

538

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

	$\Delta\%$ BB	$\Delta\%$ C	p value ²
SBP	-0.89 \pm 0.91	-2.93 \pm 2.03	0.236
DBP	-2.76 \pm 2.33	-1.38 \pm 1.04	0.431
HR	-1.08 \pm 1.84	0.56 \pm 4.05	0.869

Legend

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

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

540 **Table 3-** Characteristics of smoker subjects at baseline

541

542	Variables	Mean \pm SEM ¹
543	Age (years)	24.5 \pm 1.9
544	Height (cm)	180.1 \pm 1.3
545	Weight (kg)	70.7 \pm 1.2
546	BMI (kg/m ²)	22.9 \pm 1.1
547	Smoke (cigarettes/day)	15 \pm 0.4
548	SBP (mm Hg)	118.2 \pm 2.9
549	DBP (mm Hg)	75.7 \pm 2.7
550	HR (beat/min)	64.9 \pm 5.1
551	RHI	1.47 \pm 0.05
552	dAix(%)	-12.7 \pm 2.5
553	dAix@75 (%)	-18.2 \pm 5.0
554		

555

556 **Legend**

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

561

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.

562 ¹Overall *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

	$\Delta\%$ S	$\Delta\%$ BS	$\Delta\%$ CS	p value ²
SBP	11.3 \pm 3.5	4.13 \pm 1.27	5.38 \pm 2.52	0.174
DBP	13.6 \pm 5.5	4.09 \pm 3.05	2.35 \pm 2.34	0.130
HR	15.5 \pm 3.7	9.80 \pm 4.50	10.8 \pm 5.1	0.961

Legend

¹ N =12. Data are expressed as mean \pm 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 ²Overall *p* value for ANOVA with STATISTICA software (Statsoft Inc., Tulsa, OK, US).

566

567 **Figure 1-Experimental designs**

568

569 **Figure legend**

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

572

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

575

576 **Figure legend**

577 ¹Data are expressed as mean \pm SEM. C, control treatment; BB, blueberry treatment; RHI, reactive
578 hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index
579 standardized for heart rate of 75 bpm.

580 ^{a,b} Graphs with different letters are significantly different from other treatments ($p \leq 0.01$).

581

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

584

585 **Figure legend**

586 ¹Data are expressed as mean \pm SEM. SBP, systolic blood pressure; DBP, diastolic blood pressure;
587 HR, heart rate.

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

589 *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 ¹Data are expressed as mean \pm 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