1 **A single blueberry (V. *corymbosum*) portion does not affect markers of antioxidant defense and oxidative stress**
2 **in healthy volunteers following cigarette smoking**

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**Abstract**

We reported that a portion of blueberries reversed endothelial dysfunction induced by acute cigarette smoking. Since endothelial dysfunction is generally associated to a condition of oxidative stress, in the present study, we tried to elucidate the effect of the same portion of blueberries on markers of oxidative stress and antioxidant defense that we expected to be negatively modulated by smoking and involved in the endothelial dysfunction previously observed.

Fourteen out of 16 male healthy smokers previously enrolled, participated in a 3-armed randomized-controlled study with the following experimental conditions: smoking treatment (one cigarette); blueberry treatment (300 g of blueberries) + smoking; control treatment (300 mL of water with sugar) + smoking. Each treatment was separated by one week of wash-out period. Plasma vitamin (vitamin C, $B_{12}$ and folate) and aminothiol concentrations, endogenous (formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites) and oxidatively induced DNA damage (resistance to $H_2O_2$-induced DNA damage) in peripheral blood mononuclear cells (PBMCs) were measured at baseline and 20, 60, 90, 120 min and 24 h after smoking.

On the whole, analysis of variance did not show a significant effect of treatment on the modulation of markers of oxidative stress and antioxidant defense, but revealed an effect of time for plasma concentrations of vitamin C ($P = 0.003$), $B_{12}$ ($P < 0.001$), folate ($P < 0.001$), total cysteine ($P = 0.007$) and cysteine-glycine ($P = 0.010$) that increased following the three treatments. No significant effect of treatment was observed for the levels of FPG-sensitive sites ($P > 0.05$) and $H_2O_2$-induced DNA damage ($P > 0.05$) in PBMCs.

In conclusion, the consumption of a single blueberry portion failed to modulate markers of oxidative stress and antioxidant defense investigated in our experimental conditions. Further studies are necessary to confirm this finding and help clarifying the mechanisms of protection of blueberry against endothelial dysfunction.

**Keywords:** blueberry, antioxidant defense, oxidative stress, comet assay, smoker subjects

41 **Introduction**

42 Cigarette smoking is a well-known source of free radicals and chemical compounds such as reactive oxygen and
43 nitrogen species (ROS/RNS), hydrogen peroxide, superoxide, hydroxyl radicals, crotonaldehyde and acrolein (1). These
44 reactive and toxic substances, that usually constitute the particulate (tar) and gaseous phases of cigarettes, promote
45 oxidative stress with has detrimental consequences on structural and functional alterations of DNA, lipids, proteins and
46 carbohydrates (2). Oxidative stress is a common mediator in the pathogenicity of cardiovascular diseases including
47 hypertension, peripheral artery diseases, endothelial dysfunction and atherosclerosis (3-5). Smokers have high levels of
48 oxidized low-density lipoproteins and high circulating plasma levels of homocysteine, peroxinitrate and peroxinitrite
49 and inflammatory markers (1, 6, 7). Smoking and oxidative stress are known to affect the immune system by increasing
50 the levels of pro-inflammatory cytokines (1). Cigarette smoking has shown to augment the production of interleukin 6
51 and 8, C-reactive protein, tumor necrosis factor alpha, and to decrease the levels of anti-inflammatory markers as
52 interleukin 10 and adiponectin (1). In addition, smoking increases markers of endothelial dysfunction such as soluble
53 vascular cell adhesion molecule, soluble intercellular adhesion molecule, E-selectin and vascular endothelial growth
54 factor (8). Furthermore, the evidence consistently shows that smoking decreases circulating plasma levels of
55 micronutrients and antioxidants such as folate, vitamin C, E, $B_6$ and $B_{12}$, uric acid, glutathione, and carotenoids (9-11).

56 On the contrary, the intake of numerous bioactive molecules naturally present in fruits and vegetables seem to improve
57 nutritional status and antioxidant protection (12). In particular, the intake of dietary antioxidants such as vitamin C,
58 vitamin E and carotenoids has been documented to decrease several biomarkers of oxidative stress in smokers (12).
59 Blueberries are a rich source of polyphenol-bioactive compounds such as anthocyanins, proanthocyanidins and phenolic
60 acids (13). A plethora of *in vitro* and *in vivo* studies documented the capacity of these bioactives to reduce oxidative
61 stress by reducing DNA-oxidative damage, lipid peroxidation, and by influencing the expression and activities of
62 numerous antioxidant enzymes (13, 14). Moreover, epidemiological studies documented an inverse correlation between
63 consumption of polyphenol-rich foods and mortality from cardiovascular disease (15, 16). Consumption of polyphenol-
64 rich foods has been shown to improve different cardiovascular parameters such as blood pressure, lipid profile, vascular
65 function and inflammation (16-18).

66 To our knowledge, few studies examined the effect of foods/diet in the modulation of biomarkers of oxidative stress,
67 inflammation and endothelial function in subjects exposed to acute cigarette smoking. We recently reported that the
68 consumption of a single portion of blueberry counteracted the impairment of endothelial function and the increase in
69 blood pressure, induced by acute cigarette smoking, in young smoking volunteers (19). In the present study, the same
70 group of subjects participated in a further trial to investigate the effect of the intake of the same blueberry portion on
71 markers of antioxidant defense and oxidative stress. In particular, we selected those biomarkers that we expected to be

72 modulated after smoking, and that could help understanding the protective effect of blueberries against oxidative stress
73 and endothelial dysfunction.

74 **Material and Methods**

75 **Experimental design**

76 Sixteen healthy male smokers (23.6 ± 2.9 average of age and BMI of 23.0 ± 1.9 $kg/m^2$) were enrolled, from the student
77 population of the University of Milan, to participate in a trial evaluating the effect of fresh-frozen blueberries on
78 peripheral arterial function and arterial stiffness (19). Volunteers were selected according to smoking habits (about 15
79 cigarette/day), physical activity (at least 25-30 min per day of brisk walk or jog), alcohol consumption (up to 10-14
80 drinks of wine or beer per week) and dietary habits (homogeneous consumption of fruits and vegetables). Specifics
81 about subject recruitment, inclusion and exclusion criteria used are reported in details in Del Bo' *et al.* (19).

82 Fourteen out of 16 subjects previously enrolled gave their consent to participate in the second step of the trial and to
83 collect blood in order to perform further analysis.

84 The study was performed in accordance with the ethical standards established in the 2013 Declaration of Helsinki and
85 approved by the Ethics Committee of the University of Milan. Moreover, this study was registered at www.isrctn.org as
86 ISRCTN59129089. All participants signed an informed consent form.

87 The complete experimental design has been previously described (19). Briefly, volunteers followed a polyphenol-free
88 diet 10 days before experimentation. Subjects were deprived of foods with potential vasoactive properties such as
89 chocolate, berry fruits (i.e. blueberries, cranberries, raspberries, blackcurrants, and elderberries), red wine, red to blue
90 fruits, and green tea. The day before the experiment and during the trial, breakfast, lunch and dinner were standardized
91 [details are reported in Del Bo' *et al* (19)]. The dinner was consumed by 9.00 pm. Only one coffee was allowed at the
92 end of the dinner. No alcoholic drinks or soft drinks were permitted. Meals were standardized to provide adequate
93 energy/macronutrients intake, limiting polyphenols and taking into account Italian dietary habits. All the participants
94 refrained from physical activity from the day before the experiment and did not change their smoking habits (15
95 cigarette/day; smoking last cigarette at 11.00 pm).

96 The day of the experiment, fasted overnight subjects came to the facilities of the Division of Human Nutrition and
97 consumed a light breakfast (providing about 200 kcal) consisting of milk and biscuits (i.e. shortbread). Subjects were
98 allocated into three groups for a repeated measures 3-armed randomized-controlled study: S- Smoking treatment; BS-
99 Blueberry treatment (300 g of blueberry) + Smoking; CS- Control treatment (300 mL of water with sugar) + Smoking.
100 Blueberries and control drink were consumed 90 min after breakfast to avoid possible interference between milk
101 proteins and absorption of polyphenols. Each subject received all the three treatments separated by 7 days of wash-out
102 period [scheme of experimental design reported in Del Bo' *et al.* (19)]. The cigarette, containing approximately 6 mg of

103 Tar by volume, 0.5 mg of nicotine and 0.9 mg of carbon monoxide, was smoked 100 min after blueberry or control
104 consumption. This protocol was selected by considering previous observations on the detrimental effect of smoking on
105 peripheral arterial function (20 min after smoking) and the beneficial effect observed on endothelial function at 120 min
106 from the intake of blueberries (19). We hypothesized that the beneficial effects on endothelial function could be related
107 to the kinetics of absorption of polyphenol compounds that occurred up to 120 min from the blueberries consumption
108 (19). Blood was collected at baseline (before blueberries intake) and 20 min after smoking (120 min after
109 blueberry/control treatment). Additional blood samples were collected at 60, 90, 120 min after smoking (respectively
110 180, 210, 240 min after blueberry/control treatment) and after 24 h from the intervention.
111
112 **Preparation of blueberry and control treatment**
113 A single batch of fresh blueberries (*Vaccinium corymbosum L.* "Brigitta") were purchased, processed by Individually
114 Quick Freezing technique (Thermolab, Codogno, Italy), and stored at −20°C until use. On the study day, 300 g of
115 frozen blueberries (previously thawed at + 4°C overnight) was consumed by the volunteers. The blueberry portion
116 provided 27 g of total sugars (16.4 g of fructose and 10.6 g of glucose), 309 mg of ACNs, about 856 mg of total
117 phenolic acids, 30 mg of chlorogenic acid and 2.4 mg of ascorbic acid (19). The control treatment was prepared by
118 suspending the same amount of sugars provided by blueberry in 300 mL of water. No bioactive compounds were added
119 to the control.
120
121 **Variables under study**
122 The improvement of reactive hyperemia index (RHI), measured by a non-invasive plethysmographic method, was
123 considered as the primary endpoint [19]. The other variables under study were: markers of antioxidant defense
124 [aminothiol redox state such as glutathione (GSH), cysteine, cysteinylglycine (Cys-Gly), homocysteine (Hcy) in their
125 reduced and oxidized forms], nutritional markers (vitamin C, folate, vitamin $B_{12}$), marker of oxidative stress
126 (endogenous and oxidatively-induced DNA damage), urea, uric acid, aspartate aminotransferase (AST), alanine
127 aminotransferase (ALT) and gamma-glutamyltransferase (GGT). Moreover, screening for triglycerides, total
128 cholesterol, LDL and HDL-cholesterol, glucose and C-reactive protein was performed at the recruitment stage.
129
130 **Separation of plasma, serum and peripheral blood mononuclear cells**
131 Blood samples were collected into vacutainers containing heparin or K-EDTA as anticoagulant for plasma, or silicon
132 for serum. Plasma was separated within 30 min after collection while serum within 1 h by centrifugation at 1000 × g for
133 15 min at 4°C. Samples were aliquoted and stored at -80 °C until analysis. Peripheral blood mononuclear cells

134 (PMBCs) were obtained from 100 µL of whole blood gently mixed in micro tubes with 900 uL cold RPMI-1640
135 medium. Then, 100 µL Histopaque-1077 was carefully added to the bottom of the tube and centrifuged at 200 *x g* for 4
136 min at room temperature. Cells were collected and washed in 1 mL PBS solution. The samples were then centrifuged
137 for 10 sec at 5000 *x g* at room temperature to pellet the cells. Pellets were resuspended in PBS and immediately used for
138 the analysis.
139

140 **Evaluation of the biochemical parameters**

141 A general laboratory biochemical assessment was performed in serum including evaluation of hepatic function (AST,
142 ALT and GGT), lipid profile [triglycerides (TAG), total serum cholesterol (TSC), and HDL-cholesterol] and glucose
143 [20]. All these parameters were determined using standard laboratory methods. LDL cholesterol was calculated using
144 the Friedewald's method (LDL = total cholesterol – (HDL + 1/5 TG)).
145

146 **Evaluation of aminothiols, urea and urate in plasma and serum**

147 Plasma reduced and total aminothiols (Cys, Cys-Gly, Hcy, GSH and GSSG) were determined as described below.
148 Plasma reduced aminothiols were determined by prompt acidification with 10% trichloroacetic acid (1 : 1, v/v), protein
149 precipitation, and sample derivatization with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), a
150 specific derivatization reagent for –SH groups. Plasma total aminothiols were instead measured after a reducing step
151 with tri-n-butylphosphine, followed by sample derivatization with the same agent described above. Thiol concentrations
152 were determined by isocratic high-performance liquid chromatography (HPLC; Varian, Surrey, UK) on a Discovery
153 C18 column (250 × 4.6 mm I.D, Supelco, Sigma-Aldrich) and eluted with a solution of 0.1 mol/L potassium
154 dihydrogenphosphate-acetonitrile (92 : 8, v/v), pH 2.1, at a flow rate of 1 mL/min, as previously described (21, 22).
155 Fluorescence intensities were measured with excitation λ at 385 nm and emission λ at 515 nm, using a JASCO
156 fluorescence spectrophotometer.
157 Urea is evaluated by an UV enzymatic method based on its hydrolization to ammonia and $CO_2$ in presence of urease,
158 followed by the conversion of ammonia, 2-oxoglutarate and NADH in glutamate and NAD. The decrease in NADH
159 absorbance at 340 nm during the time is proportional to urea concentration. Uric acid levels were measured by an
160 enzymatic colorimetric method (Cobas Integra Uric Acid Cassette; Roche Diagnostics, Indianapolis, IN) on an
161 autoanalyzer (Cobas Integra 400; Roche Diagnostics).
162
163
164

165 **Evaluation of nutritional markers in plasma and serum**

166 The analysis of vitamin C in plasma was performed on 100 µL plasma (fresh sample in duplicate) to which 100 µL 167 MPA (10 %) solution was added. Samples were vortexed, centrifuged at 2200 × g for 2 min and the supernatant (50 µL) 168 was immediately analyzed by HPLC analysis. The chromatographic system consisted of a model 510 system pump 169 (Waters Corp., Milford, MA, USA), a 5mm Atlantis C18 column (250 x 4·6mm internal diameter; Waters, Dublin, 170 Republic of Ireland) and detection was achieved at 245 nm (UV-Vis detector Varian 9050; Varian Inc., Palo Alto, CA, 171 USA). Samples were eluted (1.4 mL $min^{-1}$) with a mobile phase of 0.1% formic acid. Chromatographic data were 172 acquired by a Millennium 4·0 Workstation (Waters Corp) (23).

173 Folate concentrations were determined by electrochemiluminescent immunoassay (Folate III) using Cobas 174 immunoassay analyzers (Roche). Briefly, serum samples were incubated with the folate pretreatment reagents and with 175 the ruthenium labeled folate binding protein to form a folate complex which is dependent upon the analyte 176 concentration. Streptavidin-coated microparticles and folate labeled with biotin were added to form a ruthenium labeled 177 folate binding protein-folate biotin complex. The entire complex was bound to the solid phase via interaction of biotin 178 and streptavidin. The reaction mixture was then aspirated into the measuring cell where the microparticles -were 179 magnetically captured onto the surface of the electrode. Unbound substances were washed away and application of a 180 voltage to the electrode induced-chemiluminescent emission which was measured by a photomultiplier. Results were 181 determined via a calibration curve.

182 Vitamins $B_{12}$ levels were measured by a competitive test principle using intrinsic factor specific for this 183 vitamin. As folate assessment, also Vitamin $B_{12}$ evaluation was performed with electrochemiluminescence 184 immunoassay (ECLIA) using Cobas immunoassay analyzers (Roche).

185

186 **Evaluation of FPG-sensitive sites and $H_2O_2$-induced DNA damage in PBMCs**

187 The levels of endogenous oxidized DNA bases in PBMCs were determined as FPG-sensitive sites; the protein detects 8- 188 oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases. Cells were 189 suspended with low melting point agarose (1.5% w/v) in Tris-acetate EDTA buffer, pH 7.4, at 37°C and immediately 190 pipetted ($10^4$ cells per gel) into frosted glass microscope slide (Richardson Supply Co., London, UK) precoated with a 191 layer of 1% (w/v) normal melting point agarose similarly prepared in Tris-acetate EDTA buffer. Slides were placed in 192 lysis buffer (2.5 M NaCl, 0.1M $Na_2EDTA$, 10 mM Trizma, 1% TRITON x-100, 1% dimethyl sulfoxide, 1% N- 193 Lauroylsarcosine sodium salt, pH 10) for 1 h at 4°C in the dark. One slide was treated with FPG enzyme (100 ng $mL^{-1}$, 194 for 45 min at 37°C) while the other slide acted as control (24, 25). For the determination of cell resistance against 195 oxidatively-induced DNA damage cells were treated with $H_2O_2$ (500 µmol $L^{-1}$ in PBS) or control PBS solution for 5

196 min, and placed in lysis buffer for 1 h at 4°C in the dark (25). Slides from both the treatments (FPG-sensitive sites and
197 $H_2O_2$-induced DNA damage) were placed and left for 40 min in the electrophoresis buffer (0.3 M NaOH, 1 mM
198 $Na_2EDTA$, 40 min at 4°C in the dark). Electrophoresis was performed at 1.1 V/$cm^2$ for 20 min. Slides were successively
199 neutralized (0.4 M tris-HCl, pH 7.5) for 15 min at 4°C in the dark, stained with ethidium bromide (2 μg $mL^{-1}$), washed
200 in PBS, drained, and covered with cover slips (25). One hundred comets from the two gels of each slide were
201 electronically captured using an epifluorescence microscope attached to a high sensitivity CCD video camera and to a
202 computer provided with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). The
203 levels of DNA damage were calculated as percentage of DNA in tail. For each sample, the percentage DNA in tail of
204 control cells (i.e. cells not treated with $H_2O_2$ or with FPG) was subtracted from the percentage DNA in tail of $H_2O_2$-
205 treated or FPG incubated cells, respectively (25).
206
207 **Evaluation of peripheral arterial function and arterial stiffness**
208 Peripheral arterial function (RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index) and arterial
209 stiffness (dAIx, digital augmentation index; dAIx@75, digital augmentation index normalized by considering a heart
210 rate of 75 bpm)-in the small finger arteries were assessed by a non-invasive plethysmographic method (Endo-PAT2000,
211 Itamar Medical Ltd., Caesarea, Israel) (19). Data previously obtained were correlated with markers of nutritional status,
212 antioxidant defense and oxidative stress.
213
214 **Statistical analysis**
215 Results are expressed as mean ± standard error of the mean (SEM) for continuous variables and frequency for
216 categorical variables. Data were tested for normality of distribution by the Kolmogorov–Smirnov test. Variables that
217 were not normally distributed were logarithmically transformed.
218 A repeated measures analysis of variance (ANOVA) was used to evaluate the effect of treatment (as between-subjects
219 factor) and time (as within-subject factor) on the levels of biochemical and functional parameters under study.
220 Moreover the interaction between treatment and time was considered to unravel the effect of S, BS and CS treatments
221 over time. To correct for multiple comparison, we used the Benjamini and Yekutieli false discovery rate method:
222 statistical significance was calculated using the formula $p = a/\Sigma(1/i)$, where $p= 0.05$, i ranges from 1 to N and N
223 represents the number of comparisons (26).
224 All analyses were performed using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL, USA). A two-tailed P value $<0.05$
225 was considered statistically significant.
226

227 **Results**

228 **Baseline characteristics of the study population**

229 Baseline characteristics of the fourteen subjects enrolled in the study are reported in **Table 1**. Lipid profile (TAG, TSC,
230 LDL-C and HDL-C), glucose, blood pressure, endothelial function (normal RHI value >1.67), body mass index (BMI)
231 and all other biochemical parameters were in the normal range. Six subjects showed a moderate hyperhomocysteinemia
232 (range 17.6-33.8 µL/L) with plasma total homocysteine (Hcy-pt) values ≥ 15 µmol/L (27). Moreover, three subjects had
233 folate levels (range 7.48-9.3 nmol/L) below 10 nmol/L suggesting folate deficiency, while two subjects were borderline
234 (28).

235

236 **Effect of treatment on plasma levels of aminothiols**

237 The levels of aminothiols in plasma for each treatment measured at baseline (time 0 min) and after 20, 60, 90, 120 and
238 1440 min from smoking are reported in **Figure 1**. On the whole, repeated measures ANOVA did not show significant
239 effects of *treatment* and of interaction *time x treatment*, but revealed a significant effect of time (after correction for
240 multiple testing) for plasma circulating levels of total (pt) Cys (P for time = 0.007, P for interaction = 0.889, P for
241 treatment = 0.673; **Figure 1A**) and Cys-Gly_pt (P = 0.010; P for interaction = 0.540, P for treatment = 0.408; **Figure
242 1B**) that increased following smoking treatment. No significant effect was documented for Cys_pr (P for time = 0.078,
243 P for interaction = 0.847, P for treatment = 0.990), Cys-Gly_pr (P for time = 0.694, P for interaction = 0.993, P for
244 treatment = 0.469), GSH_pr (P for time = 0.606, P for interaction = 0.735, P for treatment = 0.443), Hcy_pt (P for time
245 = 0.121, P for interaction = 0.804, P for treatment = 0.987), and Hcy_pr (P for time = 0.060, P for interaction = 0.791, P
246 for treatment = 0.908) after each treatment.

247

248 **Effect of treatment on serum and plasma levels of nutritional markers**

249 **Figure 2A-C** reports the levels of dietary markers (vitamin C, folate and vitamin $B_{12}$) measured in plasma and serum,
250 for each treatment, at baseline (time 0 min) and after 20, 60, 90, 120 min and 24 h from smoking. On the whole,
251 repeated measure ANOVA did not show significant effect of *treatment* and of interaction *time x treatment*, but revealed
252 a significant effect of *time* (after correction for multiple testing) for blood circulating levels of vitamin C (P for time =
253 0.003, P for interaction = 0.502, P for treatment = 0.829; **Figure 2A**), folate (P for time <0.001, P for interaction =
254 0.642, P for treatment = 0.642; **Figure 2B**) and vitamin $B_{12}$ (P for time <0.001, P for interaction = 0.051; P for
255 treatment = 0.879; **Figure 2C**) that increased following all the three treatments.

256

257 **Effect of treatments on the levels of background strand breaks, FPG-sensitive sites and $H_2O_2$-induced DNA**
258 **damage**
259 Results of the levels of strand breaks, FPG-sensitive sites and $H_2O_2$-induced DNA damage evaluated along time for
260 each treatment are reported in **Table 2**. Overall, repeated measure ANOVA did not show significant effect of *treatment*,
261 of *time*, and of interaction *time x treatm*ent for the levels of DNA damage also after correction for multiple testing.
262
263 **Effect of treatments on serum levels of urea, uric acid, ALT, AST and GGT activity**
264 The concentrations of urea, uric acid, AST, ALT and GGT in serum, for each treatment, evaluated at baseline (time 0
265 min) and after 20, 60, 90, 120 and 24 h from cigarette smoking are presented in **Table 3**. Overall, repeated measure
266 ANOVA did not show significant effects of *treatment* and of interaction *time x treatm*ent, but underlined a significant
267 effect of *time* (after correction for multiple testing) for urea concentration (P for time = 0.001), ALT activity (P for time
268 = 0.001) and GGT activity (P for time = 0.011), while no significant modulation was observed for uric acid (P for time
269 = 0.179) and AST activity (P for time = 0.054).
270
271 **Discussion**
272 We have previously documented that a portion of blueberries reversed endothelial dysfunction induced by acute
273 cigarette smoking (19). In the present study, we tried to elucidate the effect of the same portion of blueberries on
274 markers of oxidative stress and antioxidant defense that we expected to be modulated after smoking and that could
275 explain the protective effect against endothelial dysfunction following blueberries consumption. For these reasons, we
276 enrolled the same subjects to test the hypothesis that blueberries could affect several markers of oxidative stress and
277 antioxidant defense. However, the treatment with blueberries failed to demonstrate a modulation of the biomarkers
278 under study. Moreover, our results do not support a significant involvement of the levels of DNA damage, aminothiols,
279 vitamins (vitamin C, folate), uric acid (as contributors to antioxidant protection) in the improvement of endothelial
280 function. In fact, the modulation of the concentrations observed could not be specifically attributed to any single
281 treatment.
282 It is widely recognized that smoking strongly influences the levels of the sulphur-containing aminoacids
283 glutamylcysteine, cysteinylglycine and GSH (29). Gamma-glutamyl transpeptidase, the only enzyme of the cycle
284 located on the outer surface of plasma membrane, plays a key role in GSH homeostasis by catabolizing extracellular
285 GSH and providing cysteine for its synthesis (28). Observational studies have documented an association between
286 tobacco smoke exposure and a decrease of plasma cysteine levels (30), while others did not show this association (31).
287 Moriarty *et al.* (29) reported that smokers have low levels of GSH and cysteine compared to nonsmokers. In the present

288 study, the levels of aminothiols were in the normal range and comparable with those of nonsmokers probably due to the
289 young age of the volunteers. The effect of acute cigarette smoke on plasma cysteine levels was observed only in one
290 study. Tsuchiya *et al.* (32) documented a significant decrease in plasma cysteine levels, 5 min after cigarette smoke;
291 however, this reduction was only transient and the levels of cysteine returned to baseline within 60 min from smoking.
292 Regarding GSH, it is well recognized its role in the storage and transportation of nitric oxide (vasodilator agent) and in
293 the maintenance of the endothelial cell barrier function (28). We theorized that the consumption of blueberry could
294 increase GSH levels and explain, at least in part, the improvement in the endothelial function previously observed (19).
295 In fact, evidence from *in vitro* and *in vivo* studies seems to support a beneficial effect of berries (e.g blueberries,
296 cranberries) in the modulation of GSH levels (33-37). Spormann *et al.* (36) showed that 4-week red fruit juice intake
297 (200 mL/day, containing a mix of berries) increased plasma circulating levels of GSH and decreased GSSG in a group
298 of hemodialysis patients. Weisel *et al.* (37) reported that 4-week intervention with fruit/berries juice (700 mL/day)
299 increased blood levels of GSH in healthy subjects. Unfortunately, neither acute cigarette smoking nor blueberry intake
300 affected GSH plasma levels, while an increase of cysteine_pt and cysteine-glycine_pt levels was observed following
301 smoking treatment. We may hypothesize that this increase might be due to a mechanism of cell protection against
302 oxidative stress induced by smoking. On the contrary, the reduced forms of aminothiols and homocysteine did not vary
303 significantly following the three treatments.
304 In relation to antioxidant compounds and vitamin status, the evidence consistently supports that compared with
305 nonsmokers, smokers have lower circulating concentrations of vitamin C, carotenoids and folate (9-11). We
306 documented that the concentrations of vitamins were in the normal range in our group of volunteers, with the exception
307 of some subjects that reported low serum levels of folate. The effects of acute smoking on concentrations of vitamins
308 and antioxidants have been poorly investigated. Tsuchiya *et al.* (32) documented a significant reduction in the levels of
309 ascorbic acid and uric acid in the first 5 min after smoking but this reduction was only transient and the concentrations
310 returned to baseline levels within 60 min. Dietary intervention studies with berries have failed to positively affect
311 plasma/serum concentrations of carotenoids, folate and vitamin $B_{12}$ (38, 39), while a significant increase has been
312 reported for the levels of vitamin C (36, 40-41). In the present experimental conditions, we observed an increase of
313 ascorbic acid, folate and vitamin $B_{12}$ along time for each treatment, while no significant effect was observed for uric
314 acid. These results are surprising and in contrast to observations reported in the literature (32). However, one study has
315 reported an increase of the cell antioxidant capacity, measured as Trolox equivalent, after acute cigarette smoking (42).
316 We may theorize that the increase of vitamins, in particular vitamin C and folate, might be due to an antioxidant
317 protection/repair mechanism against oxidative stress induced by smoking.

318 Several observational studies from Italy, Turkey, Greece, France, Poland and Scotland have shown that chronic
319 smoking is associated with high levels of DNA damage (43-48). Few studies examined the effect of acute cigarette
320 smoking on the levels of DNA damage (49). Most of the data reported in the literature refer to markers of lipid
321 peroxidation and degradation products of extracellular matrix proteins (50). Concerning the effect of acute cigarette
322 smoking on oxidative DNA damage, Kiwosawa *et al.* (49) documented that smoking two consecutive cigarettes in 10
323 min increased the levels of 8-hydroxy-2'-deoxyguanosine in healthy male volunteers. The role of berries in the
324 modulation of endogenous and oxidatively-induced DNA damage has been poorly investigated (51). We previously
325 found a significant reduction in the levels of FPG-sensitive sites and $H_2O_2$-induced DNA damage following 6-week
326 wild blueberry intake (38). On the contrary, Duthie *et al.* (39) reported a lack of protective effect on Endo III-sensitive
327 sites and DNA oxidative damage after 2-week cranberry intervention. Wilms *et al.* (41) documented no significant
328 effect on the levels of $H_2O_2$-induced DNA damage after 4-week consumption of blueberry/apple juice, while Møller *et*
329 *al.* (40) observed a significant increase in FPG-sensitive sites after 3-week blackcurrant intervention. In the present
330 study, we documented that acute cigarette smoking did not increase the levels of FPG-sensitive sites and $H_2O_2$-induced
331 DNA damage in our subjects at 20 min from smoking, while at the same time a decreased endothelial function was
332 previously registered (19). The lack of effect of smoking on DNA damage could be related to: the short time of
333 exposure to toxic compounds, the insufficient dose of cytotoxic substances able to induce DNA oxidative damage (the
334 stressor used was only one cigarette), or the compensatory increase in DNA repair mechanisms. The consumption of the
335 portion of blueberries provided to the smokers did not affect the levels of FPG sensitive sites and $H_2O_2$-induced DNA
336 damage. These latter results are in contrast with a previous study in which the same portion of blueberry (300 g) was
337 able to improve DNA resistance to oxidative damage in a group of nonsmoker subjects (52).

338 Very few studies have examined the relationship between smoking and liver damage evaluated through GGT,
339 ALT and AST activity. Most of these studies have shown no positive association between smoking and ALT or AST
340 (53), while a significant association between smoking and GGT activity has been reported (54). High levels of GGT are
341 associated with an inflammatory and oxidative stress status (55, 56). Some *in vitro* and *in vivo* studies reported the
342 protective effects of berries against liver damage (57-59). In our experimental conditions, no effect on liver (evaluated
343 through GGT, ALT and AST activity) was observed after one cigarette smoking and the consumption of a portion of
344 blueberries supporting data of the literature (53) and data from our previous finding (38).

345 **Limitations of the study**

346 A first limitation of the study is the lack of a control group consisting in non-smoker subjects. Thus, we cannot exclude
347 that the fluctuations of concentrations of the different biomarkers analyzed over the day are unrelated to smoking
348 treatment but dependent to other factors (e.g. diurnal physiological changes). Another possible limitation of the study is

349 the lack of a proper control treatment. In fact, since an actual placebo is not feasible we selected as control condition a
350 product providing the same amount of sugars present in the blueberry portion but devoid of other bioactive compounds.
351 Finally, it cannot be excluded that the number of subjects enrolled could be insufficient to highlight a modulation of
352 some of the biomarkers under study.

353 **Conclusions**

354 In conclusion, we previously reported a significant effect of acute cigarette smoking in inducing endothelial dysfunction
355 and the ability of blueberry intake to counteract this impairment. Here, we did not document an increase of antioxidant
356 defense and a reduction of oxidative stress markers following the consumption of a single blueberry portion possibly
357 suggesting these markers are not implicated in the modulation of endothelial function. Although, smoking cessation is
358 the only safe and reliable approach to minimize cigarette smoking damage, further studies are necessary to confirm this
359 finding and help clarifying the mechanisms of protection of blueberry against endothelial dysfunction.

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362

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372

373 **Conflict of interest statement**

374 The authors declare that the research was conducted in the absence of any commercial or financial relationships that
375 could be construed as potential conflicts of interest.

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377

378 **Figure 1- Effect of smoking (S), blueberry + smoking (BS) and control + smoking (CS) treatment on plasma**
379 **aminothiols concentration (N= 14)[1]**
380
381 Figure Legend: [1]Data are expressed as mean±SE.
382 S, smoking treatment; BS, blueberry treatment + smoking; CS, control treatment + smoking; Cys_pt, cysteine total;
383 Cys-Gly_pt, cysteine-glycine total.
384
385 **Figure 2- Effect of smoking (S), blueberry + smoking (BS) and control + smoking (CS) treatment on vitamin C,**
386 **folate and vitamin $B_{12}$ concentration in plasma and serum (N= 14)[1]**
387
388 Figure Legend: [1]Data are expressed as mean±SE.
389 S, smoking treatment; BS, blueberry treatment + smoking; CS, control treatment + smoking
390
391

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