Effect of a wild blueberry (*Vaccinium angustifolium)* drink intervention on markers of oxidative stress, inflammation and endothelial function in humans with cardiovascular risk factors

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Abbreviations: AACC, American Association for Clinical Chemistry; **ACNs**, anthocyanins; **AI**, augmentation index; **AI@75**, augmentation index standardized for heart rate of 75 bpm; **ANOVA**, analysis of variance; **AOAC**, Association of Official Analytical Chemists; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BMI**, body mass index; **CI**, confidence interval; **CRP**, C-reactive protein; **CVD**, cardiovascular disease; **FMD**, flow mediated dilation; **FPG**, formamidopyrimidine DNA glycosylase; **FRHI**, Framingham reactive hyperemia index; **GGT**, gamma-glutamyltransferase; **GSH**, reduced glutathione; **GSH-Px**, glutathione peroxidase; **GSSG**, oxidized glutathione; **GST**, glutathione S-transferase; **HDL**, high density lipoprotein; **HPLC**, High Performance Liquid Chromatography; **IL-6**, interleukin-6; **LC-DAD-MS/MS**; Liquid Chromatography-diode array detector-mass spectrometry; **LDL**, low density lipoprotein; **LSD**, least significant difference; **MNBC**, mononuclear blood cell; **NO**, nitric oxide; **PBS**, phosphate buffer saline; **PL**, placebo; **RH**, reactive hyperemia; **RHI**, reactive hyperemia index; **SD**, standard deviation; **SOD**, superoxide dismutase; **SPE**, solid phase extraction; **sVCAM-1**, soluble vascular adhesion molecule-1; **TNF-*α***, tumor necrosis factor alpha; **TFA**, trifluoroacetic acid; **UHPLC-MS/MS**, Ultra High Pressure Liquid Chromatography-mass spectrometry; **WB**, wild blueberry.

Abstract

Purpose Wild blueberries (WB) (*Vaccinium angustifolium*) are rich sources of polyphenols such as flavonols, phenolic acids and anthocyanins (ACNs) suggested to decrease the risk of cardiovascular and degenerative diseases. This study investigated the effect of regular consumption of a WB drink or a placebo (PL) drink on markers of oxidative stress, inflammation and endothelial function in subjects with risk factors for cardiovascular diseases.

Methods Eighteen male volunteers (ages 47.8 ± 9.7 years; body mass index 24.8 ± 2.6 kg/m^2) received according to a cross-over design, a WB (25 g freeze-dried providing 375mg of ACNs) or PL drink for six weeks, spaced out by a 6 week wash-out. Endogenous and oxidatively induced DNA damage in mononuclear blood cells, serum interleukin levels, reactive hyperemia index, nitric oxide, soluble vascular adhesion molecule concentration and other variables were analyzed.

Results Wild blueberry drink intake significantly reduced the levels of endogenously oxidized DNA bases (from 12.5 ± 5.6% to 9.6 ± 3.5%, $p \leq 0.01$) and the levels of H_2O_2-induced DNA damage (from 45.8 ± 7.9% to 37.2 ± 9.1%, $p \leq 0.01$), while no effect was found after the PL drink. No significant differences were detected for markers of endothelial function and the other variables under study.

Conclusions In conclusion, the consumption of the WB drink for six weeks significantly reduced the levels of oxidized DNA bases and increased the resistance to oxidatively induced DNA damage. Future studies should address in greater detail the role of WB on endothelial function. This study was registered at www.isrctn.org as ISRCTN47732406.

KEY WORDS: Wild blueberry; endothelial function; DNA damage; blood lipids; cardiovascular risk.

Introduction

Dietary habits and lifestyle in general are major risk and/or protective factors in the development and progression of degenerative diseases. The incidence of cardiovascular disease (CVD) as well as the prevalence of the metabolic syndrome is increasing world-wide [1]. As a consequence, costs for health care are rising and there is a strong demand for preventive strategies that can be easily implemented by the majority of the population. Diets rich in fruits and vegetables are among the recommended lifestyle modifications to decrease the risk of CVD, but they can also reduce the complications associated with aberrant metabolic states or already established disorders [2]. There is increased interest in the nutritional and functional aspects of berries (e.g. blackberry, bilberry, blackcurrant, cranberry, strawberry and blueberry) that can be consumed as fresh or processed foods [3-5]. They contain high amounts of flavonoids and phenolic acids that may be important components for the biological processes [6-7]. Wild or lowbush blueberries (*Vaccinium angustifolium*) are a rich source of anthocyanins (ACNs) that have high antioxidant capacity as demonstrated *in vitro*, in the animal model and in humans [8-10]. ACNs have been demonstrated to positively affect inflammation, hyperglycemia, lipid metabolism, fat deposition, endothelial function, and to decrease the oxidative damage of macromolecules [11-18]. However, the study of the mechanisms by which wild blueberries contribute to the beneficial health effects remains quite difficult since ACNs are poorly absorbed (within 1-3h), and are rapidly metabolized and excreted (within 12-24 h) after consumption [19].

The protective effect of wild blueberry intake has been studied in several animal models in our laboratories [12; 20-25]. In particular, we demonstrated that the intake of wild blueberries (24 mg ACNs per day) can improve vasomotor tone in the rat aorta and lymphocyte resistance against oxidatively induced DNA damage [21-22]. However, it is important to validate the observations from animal experimental models with controlled human interventions to determine the protective effect of wild blueberries on a wide range of biomarkers especially in subjects with CVD risk. To our knowledge there is a paucity of studies that have evaluated the *in vivo* effect of blueberry consumption in humans with risk factors for CVD [16; 26]. The aim of the present human intervention study was to investigate the hypothesis that regular intake of a wild blueberry drink for 6 weeks could improve peripheral endothelial function (measured by reactive hyperemia arterial tonometry), biomarkers of oxidative stress (e.g. DNA damage and repair), lipid profile, and inflammatory markers in subjects with at least one risk factor for CVD.

Materials and Methods

Recruitment of subjects

Subjects involved in the study were recruited from the staff of the University of Milan and other Institutes through advertisement on bulletin boards. Only male subjects were included in the study in order to avoid effects related to hormonal fluctuation during the various phases of the menstrual cycle [27]. Inclusion criteria were: healthy subjects with at least one risk factor for CVD (based on American Heart Association guidelines [28]) such as: pre-hypertension (systolic pressure 120-139 mm Hg and diastolic pressure between 80-89 mm Hg), high serum cholesterol (≥5.17 mmol/L), low high density lipoprotein (HDL)-cholesterol (<1.03 mmol/L), high low density lipoprotein (LDL)-cholesterol (≥3.36 mmol/L), high triglycerides (≥1.69 mmol/L) overweight (BMI ≥25 kg/m^2) and smoking (>10 cigarettes/day). Individuals with secondary hypertension or obesity (BMI ≥30) were excluded. A medical history questionnaire was used to exclude subjects with clinical diseases such as diabetes, renal insufficiency, known food allergic reactions, chronic constipation, diarrhea or any other gastrointestinal problem or disease. Subjects were specifically excluded from the study if they were taking drugs, supplements, specific probiotics or medications during the last month before the beginning of the experiment. Weight and height were assessed and subjects were selected on the basis of an interview to evaluate their dietary habits and ensure that they were as homogeneous as possible, in particular for fruit and vegetables consumption. This was obtained by means of a food frequency questionnaire previously published and specifically revised to focus on food sources rich in antioxidants [29]. Other exclusion criteria were: high (> 5 portions/day) or low (<2 portions/day) intake of fruit and vegetables and habitual alcohol consumption (< 3 drinks per week were tolerated). Volunteers who followed a specific diet such as vegetarian, vegan or macrobiotic, and those who had a specific aversion to blueberries or their products were excluded. Twenty male subjects, ages 47.8 ± 9.7 years with body mass index (BMI) 24.8 ± 2.6 kg/m^2 were selected. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan. All participants signed informed consent form.

Blueberry and placebo drink preparation

The wild blueberry (WB) drink was prepared by suspending 25 g of WB freeze-dried powder (i.e. a composite from Wayman's (Cherryfield, ME), standardized at 1.5% total ACNs by FutureCeuticals, (Momence, IL, USA)) in 250 ml of water to give an amount of WB equivalent to 1 cup of raw fruits (148 g, providing approximately 375 mg of ACNs). The nutritional composition of the soluble WB powder used in the study is reported in **Table 1**. The placebo (PL) drink was prepared to have sensory characteristics similar to the WB drink but it did not

contain polyphenols. The PL drink consisted of 250 mL water, 7.5 g fructose, 7 g glucose, 0.5 g citric acid, and 0.03 g blueberry flavor (Kerry Ingredients & Flavours Italia S.p.A., Bergamo, Italy). In order to reach the same color, small amounts of food colors typically used by food industry for the production of sweetened soft drinks were added to the PL drink. The microbiological and chemical stability of the two drinks was evaluated. Microbiological stability was ensured for 72 h. Analysis of ACNs documented that their content in the WB drink decreased by about 20% after 24 h and by about 35% after 48 h at 4°C.

Experimental design

A randomized, repeated measures crossover design was utilized. Subjects were randomly divided (by using a computer random number generator) into two groups of ten subjects each: group 1 was assigned to the sequence WB drink/wash-out/PL drink, whereas group 2 followed the sequence PL drink/wash-out/WB drink. WB and PL drinks were consumed daily for 6 weeks; the two treatments were separated by a 6 week wash-out period. Subjects received each morning the freshly prepared WB or PL drink in appropriate iceboxes. Participants were instructed to keep the drink under refrigeration and to avoid exposing it to a heat source or light and consume the drink within the morning. Additionally participants were asked to mix the beverage very well before drinking, rinse out each bottle with water and drink it to ensure complete intake of the WB powder. Every Friday, subjects received the drinks for the week-end and kept them refrigerated. Subjects were instructed to maintain their normal dietary and lifestyle habits (as declared before enrollment) but to abstain from consuming berries and other ACN-rich food sources. For this reason, during the experimental period, each subject received a complete list of foods high in ACNs to be avoided. A 24 h record of food consumption was kept by each volunteer one day before blood collection to check compliance to the dietary instructions. Moreover a 3-day food record and a weekly direct interview were scheduled randomly during the two experimental periods. At the beginning and at the end of each treatment period, fasting venous blood samples were collected early in the morning after an overnight fast.

Variables

The improvement of endothelium-dependent vasodilation (measured by a non-invasive plethysmographic method), the reduction of oxidized purines and the improvement of cell resistance to H_2O_2-induced DNA damage (evaluated in mononuclear blood cells by the comet assay) were considered as the primary endpoints. The other variables under study were: nutritional biomarkers (ACNs, vitamin C, folate, vitamin B_{12}, reduced glutathione (GSH), oxidized glutathione (GSSG)), lipid profile (triglycerides, total cholesterol, LDL and HDL-cholesterol), glucose, markers of inflammation (interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive

protein (CRP), soluble vascular adhesion molecule (s-VCAM-1), total nitric oxide (NO), augmentation index (AI), augmentation index standardized for heart rate of 75 bpm (AI@75), blood pressure, DNA repair activity, enzymes activity (glutathione S-transferase activity (GST), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), serum creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT).

Anthocyanin and phenolic acids extraction and analysis in the wild blueberry powder by LC-DAD-MS/MS

The Liquid Chromatography-diode array detector-mass spectrometry (LC-DAD-MS/MS) analysis of ACNs in the WB powder was performed in accordance to a procedure previously published [20]. Briefly, the WB powder (250 g) was suspended in 1% trifluoroacetic acid (TFA) aqueous solution, and extraction was performed through sonication. After centrifugation the supernatant was injected in the LC system for the analysis. Phenolic acids were extracted as previously described. The High Performance Liquid Chromatography (HPLC) analysis was performed with a system equipped with an Alliance mod 2695 (Waters, Milford, MI, USA) and a mod 2996 photo diode array detector (Waters). Chromatographic data were acquired using an Empower workstation (Waters). The analytical column was a 5µm C_{18} Vydac 201TP54 (250mm×4.6mm, i.d.; Esperia, CA, USA) with biocompatible frits. The eluents were (A) phosphoric acid (0.1%) and (B) methanol/acetonitrile/water/phosphoric acid (22.5/22.5/55/0.1) at a flow rate of 1.5 mL/min. The calibration curves were in the range of 1-10 µg/mL. Visible detection was achieved at 320 nm.

Soluble and insoluble fiber analysis in the wild blueberry powder.

Soluble and insoluble dietary fiber in the WB powder was determined by the AOAC (Association of Official Analytical Chemists) International method 991.43; an AACC (American Association for Clinical Chemistry) 46.13 for the protein in the residue was used [30].

Sample collection and separation

Blood was collected early in the morning by a phlebotomist. Samples were drawn into evacuated tubes with or without heparin. Plasma was separated within 30 min after collection while serum within 1 h, by centrifugation for 15 min at 2300 x g at 4°C. Mononuclear blood cells (MNBC) were obtained from whole blood by density gradient centrifugation with Histopaque 1077 (Sigma Chemicals Co., St. Louis, MO). Mononuclear blood cells

were removed from the gradient, washed with Phosphate Buffer Saline (PBS) and used for the determination of endogenous and cell resistance to H_2O_2-induced DNA damage. An aliquot of MNBC was isolated and dissolved into an appropriate medium (90% RPMI media, 10% Dimethyl sulphoxide, Sigma) and stored at -80°C until evaluation of repair activity. Red blood cells were obtained from whole blood by centrifugation at 2300 x g for 10 min and were washed with an equal volume of PBS solution. Red blood cells (1 mL) were diluted with iced water (1:4) and centrifuged at 10000 x g for 10 min. All samples were then aliquoted, stored at -80°C until determination of hemoglobin and evaluation of SOD and GSH-Px activities. Red blood cells (500 µL) used for glutathione analysis were acidified with an equal solution of metaphosphoric acid (10%) before storage.

Analysis of biochemical parameters

A general laboratory biochemical assessment was performed including evaluation of hepatic and renal function (serum creatinine, AST, ALT and GGT), lipid profile (triglycerides, total cholesterol, and HDL-cholesterol) and glucose [31]. All these parameters were determined using standard laboratory methods. LDL cholesterol was calculated using the Friedewald's method. Plasma IL-6, TNF-α, CRP and s-VCAM 1 concentrations were measured by Quantikine human colorimetric sandwich ELISA immunoassay kits (R&D Systems, Inc. Minneapolis, MN). Plasma concentration of total NO was calculated by measuring the products of oxidation (nitrate and nitrite) by a Fluorometric Assay Kit (Cayman Chemical, Ann Arbor, MI). Lymphocyte GST activity analysis was performed in accordance to the procedure described previously [32]. GST activity was standardized to milligrams of protein and determined using a BCATM Protein Assay Kit (Pierce, Rockford, IL, USA). SOD and GSH-Px activities were measured in erythrocytes using commercial kits (Cayman chemical, Ann Arbor, USA). Enzyme activities were standardized to milligrams of hemoglobin determined by the use of the Drabkins reagent (Sigma Diagnostic, Co., St. Louis, MO). Vitamin C was determined in plasma by HPLC analysis as previously published [18]. Vitamin B_{12} and total folate were measured by competitive immunoassay using direct chemiluminescence [31]. Analysis of GSH and GSSG in the erythrocyte lysate was performed following the instructions reported in a commercially available kit (Cayman chemical, Ann Arbor, USA).

Anthocyanin extraction and analysis in plasma

Two aliquots of plasma (1 mL) were acidified with TFA (1%), vortexed, and centrifuged for 1 min at 4500 x g and the supernatant was stored at -80°C until analysis. Anthocyanins were extracted from plasma using a Micro-Plate solid phase extraction (SPE) HLB Oasys Cartridge preactivated with methanol (500 µL) and washed with 500 µL acidified water (1% TFA). Plasma (400 µL) was diluted with 140 µL of acidified water (1% TFA) and 60

μL of water containing the Internal Standard (50 ng/ml of CydG). Plasma was vortexed, centrifuged and loaded onto the cartridge. The samples were drained under gravity and the cartridge washed with acidified water (100 μL; 1% TFA) and 100 μL acidified MetOH (20% TFA). The ACNs were eluted from the cartridge using 50 μL of methanol (70%) containing TFA. The filtered sample was injected into UHPLC MS/MS system for analysis according to a method previously published [20].

Evaluation of peripheral vasoreactivity

Evaluation of peripheral vasoreactivity was performed before blood drawing to avoid possible side effects on blood pressure and vasoactivity. Endothelial-dependent vasodilation in the small finger arteries was assessed by a non-invasive plethysmographic method (Endo-PAT2000, Itamar Medical Ltd., Caesarea, Israel) based on the registration of pulsatile blood volume in the fingertips of both hands. The Endo-PAT equipment consists of two finger-mounted probes, which include a system of inflatable latex air-cushions within a rigid external case; pulsatile volume changes of the fingertip are sensed by a pressure transducer, located at the end of each probe, and transferred to a personal computer where the signal is band pass-filtered (0.3 to 30 Hz), amplified, displayed, and stored. The Endo-PAT studies were performed with the patient in the supine position and both hands on the same level in a comfortable, thermoneutral environment. Arterial systolic and diastolic blood pressure and heart rate frequency were measured before starting the test. A blood pressure cuff was placed on one upper arm (study arm), while the contralateral arm served as a control (control arm). After a 10-min equilibration period, the blood pressure cuff on the study arm was inflated to 60 mmHg above systolic pressure for 5 min. The cuff was then deflated to induce reactive hyperemia (RH) whereas the signals from both PAT channels (Probe 1 and Probe 2) were recorded by a computer. The Reactive Hyperemia Index (RHI), an index of the endothelial-dependent flow-mediated dilation (FMD), was derived automatically in an operator independent manner, as the ratio of the average pulse wave amplitude during hyperaemia (60 to 120 s of the post-occlusion period) to the average pulse wave amplitude during baseline in the occluded hand divided by the same values in the control hand and then multiplied by a baseline correction factor. A RHI value of 1.67 provides a sensitivity of 82% and a specificity of 77% to diagnosing endothelial dysfunction [33]. RHI has been shown to correlate with the ischemia-induced FMD in the larger brachial artery measured by high resolution ultrasound and with the gold standard method (the acetylcholine infusion in coronary arteries) for the endothelial function assessment [34-35].

In addition to the RHI we have also reported in our paper the Framingham RHI (FRHI), which was automatically calculated using, however, a different post-occlusion hyperaemia period (90 to 120 s) without baseline correction

factor. The FRHI, that has been shown to correlate with other CVD risk markers [36, 37], was expressed as natural log of the resulting ratio.

Finally, the EndoPAT device also generates the Augmentation Index (AI), a measure of vascular stiffness (pulse wave reflection) that is calculated from the shape of the pulse wave recorded by the probes during baseline. AI can be adjusted to a heart rate of 75 beats/min to correct for the independent effect of heart rate on this measure [38].

Evaluation of DNA damage, repair activity and cell resistance against H_2O_2-induced DNA damage

The level of oxidized bases was determined as formamidopyrimidine DNA glycosylase (FPG) sensitive sites in MNBCs by the comet assay as described previously [39]. The FPG protein detects 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases. This analysis of oxidatively damaged DNA has been thoroughly validated in inter-laboratory validation trials and shows generally good concentration-response relationship with agents that preferentially generate 8-oxodG [40-41]. The repair activity toward oxidatively damaged DNA was measured by the comet assay in substrate A549 cells treated with 1 μM Ro19-8022/white light, which generates 8-oxo-7,8-dihydroguanine. Cell extracts were incubated with substrate cells for 20 min at 37°C [42]. We measured the cell resistance against oxidatively-generated DNA damage (i.e. strand breaks and alkali labile sites) in freshly isolated MNBCs treated with H_2O_2 (500 μmol/L in PBS) for 5 min as described previously [43]. The levels of DNA damage were calculated as percentage of DNA in tail. For each sample, the percentage DNA in tail of control cells (not treated with H_2O_2 or not incubated with FPG) was subtracted from the percentage DNA in tail of H_2O_2-treated or FPG incubated cells, respectively.

Statistical analysis

Sample size has been calculated taking into account the expected variation in the primary endpoints considered. In particular twenty subjects were calculated to be sufficient to evaluate a difference of RHI after WB drink of 0.28 (standard deviation 0.40), with alpha=0.05 and a statistical power of 80%. Moreover, the "repeated measure" experimental design in which each subject acts as its own control, allows to conduct experiment more efficiently reducing the error variance.

Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). The Shapiro-Wilk test was applied to verify the normal distribution of the variables. Data obtained were examined by analysis of variance (ANOVA) with the treatment sequence (WB drink vs PL drink or vice versa) as the

independent factor in order to exclude the presence of carry-over effects. As none was detected, data were analyzed by ANOVA for repeated measures design. ANOVA with treatment (WB drink vs PL drink) and time (before and after each treatment) as dependent factors was applied to evaluate the effect of the WB drink on the variables under study. The difference in responses to the WB and PL drink periods was evaluated by statistically significant P-values for the interaction between treatment and time in the overall repeated ANOVA. Moreover, ANOVA with type of treatment as the dependent factor was used to evaluate percentage change (i.e. [after treatment-before treatment]/ before treatment *100) at the different endpoints of peripheral arterial function. The mean changes are described as mean with 95% confidence interval (CI). Differences are considered significant at $p \leq 0.05$; post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as level of statistical significance. Finally, a statistical analysis of data on peripheral arterial function and DNA damage was performed by considering a stratification according to the number of risk factors in the subjects enrolled. In particular two classes were considered: class 1 (subjects with ≤ 2 risk factors) and class 2 (subjects with >2 risk factors).

RESULTS

Composition and characteristics of the wild blueberry powder

The nutritional composition of the WB powder used for the study expressed for 100 g of product and 25 g (one portion) are reported in **Table 1**. One portion of the WB powder provided about 102 kcal, 1.5 g of lipids, 1 g of proteins, 17 g of sugars (glucose and fructose) and 4.2 g of total fiber. The soluble and insoluble fiber was 0.6 g and 3.6 g, respectively. Chlorogenic acid (127.5 mg / 25 g) was the main phenolic acid detected in the WB powder. The total ACN concentration was 375 mg / 25 g with peonidin-3-glucoside, malvidin-3-galactoside, delphinidin-3-glucoside and delphinidin-3-galactoside as the dominant ACN compounds which represented about 35% of the total amount of ACNs as previously reported [20].

Baseline characteristics of the subjects

Twenty people began the study and eighteen completed the whole protocol. Two subjects withdrew from the study due to personal reasons. Baseline anthropometric and clinical characteristics of the remaining 18 subjects are available in **Table 2**. Blood pressure, lipid profile and inflammatory markers were within the range of normality. According to the guidelines of the American Heart Association (28), 8 subjects were classified as overweight (BMI > 25 kg/m^2), 7 had high levels of total cholesterol (≥6.2 mmol/L) and 7 borderline high (5.17-6.18 mmol/L). Six subjects had high levels of LDL-cholesterol (≥4.13 mmol/L) and 7 were borderline high

(3.36-4.11 mmol/L). Ten subjects were pre-hypertensive (systolic pressure 120-139 mm Hg and diastolic pressure between 80-89 mm Hg). Nine subjects were smokers/ex-smokers. Finally eight subjects had below normal endothelium-dependent vasodilation with a RHI value ≤ 1.67.

Effect of intervention on dietary markers

The effect of the 6 week intervention with the WB vs the PL drink on dietary markers evaluated in plasma is reported in **Table 3.** The intervention resulted in no significant effect on vitamin C, vitamin B_{12}, folate, GSH and GSSG concentrations and GSH/GSSG ratio. No ACNs were detected in the plasma after the WB or PL drink treatment after 6 weeks of intervention.

Effect of intervention on vascular function and blood pressure

The values of RHI, FRHI, AI, AI@75, NO, sVCAM-1 and blood pressure are reported in **Table 4**. One subject was omitted from the analysis due to abnormal values registered in 2 different time points. Vascular function of the subjects measured by Endo-PAT2000 did not improve significantly, according to the repeated measures ANOVA, after the WB and PL drinks (P=0.452 for the interaction between treatment and time). However, an apparent decrease in the RHI was observed during consumption of the PL drink with respect to WB drink. The mean percent change between the pre-to-post intervention was +4.9% (95%CI: -11%, +20.8%) after the WB drink and -4.9% (95%CI: -12.6%, +3.9%) after the PL drink. On the whole a high inter-individual variability was observed in the percent change of RHI (**Figure 1**) with about half of the subjects (53%; 9 out of 17) having an improvement following the intervention with the WB drink while the other half experienced (47%; 8 out of 17) a decrease. In the placebo group we observed that few subjects (23%; 4 out of 17) had an improvement following the intervention with the PL drink, while more than a half of the subjects (59%; 10 out of 17) showed a decrease. The remaining subjects (18%; 3 out of 17) did not show any effect. No significant changes in FRHI, AI, AI@75, blood pressure (systolic and diastolic), total plasma NO and sVCAM-1 were documented after the 6-week intervention with the WB drink or the PL drink.

Effect of intervention on DNA damage and repair activity in blood mononuclear cells

Results on the levels of DNA damage and repair activity are shown in **Table 5**. There was a significant different treatment effect between the WB and PL drink periods (P=0.039 for the interaction between treatment and time). Estimation of oxidized purines in MNBC DNA through quantification with the formamidopyrimidine DNA glycosylase (FPG)-sensitive sites indicated a statistically significant decrease following the WB drink (from 12.5

± 5.6% to 9.6 ± 3.5%, p≤0.01) with respect to the PL drink (from 12.0 ± 4.5% to 11.9 ± 4.4%, p=0.89). Moreover, there was also a different treatment effect between the WB and PL drink periods (P=0.037 for the interaction between treatment and time). The intake of the WB drink significantly decreased the levels of H_2O_2-induced DNA damage (from 45.8 ± 7.9% to 37.2 ± 9.1%, p≤0.01), while no effect was observed after the PL drink (from 44.9 ± 12.2% to 44.4 ± 8.4%, p=0.84). Additionally, no significant effect was observed in DNA repair activity following the two treatments (WB vs PL drink).

Effect of intervention on anthropometric measures and biochemical parameters

Body weight, BMI and biochemical markers before and after the WB or PL drink are presented in **Table 6**. The intervention had no significant effect on body weight and BMI, glucose, creatinine and enzyme activity (AST, ALT and GGT) in liver and SOD and GSH-Px activity in erythrocytes, and GST activity in lymphocytes) of the subjects under study. No significant differences in lipid profile (triglycerides, total cholesterol, LDL and HDL cholesterol) were observed following WB drink consumption.

Effect of intervention on biomarkers of inflammation

The plasma levels of IL-6, TNF-α and CRP of the subjects before and after the WB and PL drink are reported in **Table 7.** No significant difference in inflammatory markers was detected as a result of the intervention.

Effect of risk factor exposure on peripheral arterial function and levels of DNA damage

Statistical analysis did not show differences in peripheral arterial function and levels of DNA damage before and after WB and PL drink depending on the number of risk factors identified in the subjects: class 1 (subjects with ≤ 2 risk factors) and class 2 (subjects with >2 risk factors). When the subjects were classified as smokers/ex-smokers vs. nonsmokers, we observed that the reduction in the levels of H_2O_2-induced DNA damage after the WB intervention was higher: smokers/ex-smokers (44.4 ± 7.7% vs 33.7 ± 5.9%, p≤0.01) with respect to the nonsmoker group (47.2 ± 8.3% vs 40.7 ± 10.7%, p=0.08) as indicated by the LSD test (data not shown).

DISCUSSION

Most of the beneficial evidence on the modulation of endothelial function derives from experimental *in vitro* and *ex vivo* studies in which the vasoreactivity has been evaluated in the aorta of animals with invasive methods [12, 21-25, 44]. The evaluation of the peripheral arterial function, as a marker of CVD risk, is a relatively new approach and only few human studies investigated the impact of polyphenol-rich foods on this parameter [45-48]. Some studies have demonstrated an improvement in peripheral arterial function, through Endo-PAT 2000, after the ingestion of a single dose of polyphenol-rich foods, in particular flavonol-rich foods/beverages such as cocoa [45-47] and chocolate [48]. The exploitation of Endo-PAT device to evaluate the long term effect of dietary interventions on vascular function merits further attention. To our knowledge, only one human intervention study investigated the effect of ACN-rich food on the modulation of endothelial function in a medium/long term intervention. Recently, Dohadwala et al. [49] demonstrated that 4 weeks of supplementation with cranberry juice (480 ml/day, providing 94 mg ACNs and 835 mg total polyphenols) had no effect on vascular function (measured by flow-mediated dilation in the brachial artery) in subjects with coronary artery disease. In the present study we have demonstrated no significant difference in the peripheral arterial function in subjects with CVD risk factors, with about half of the subjects exhibiting an improvement following the intervention with the WB drink. No significant difference was also observed in blood pressure, s-VCAM-1 and total NO. The non-significant effect of the WB drink on the modulation of peripheral arterial function may be attributed to the kinetics of wild blueberry ACNs and/or other polyphenols. It is widely recognized that ACNs are rapidly cleared from plasma after an oral dose of berry fruits with maximum bioavailability between 1 and 3h [19]. In our study, ACNs were non-detectable in plasma after WB consumption, since blood samples were taken 12h after the intake of the drink, when possibly ACNs were cleared from the circulation. However, many other factors may have affected our results such as: the large inter individual variation, individual differences in CVD risk factors, the duration of the intervention, and the amount and form of the WB product. We provided our subjects with a dietary daily amount of ACNs through the WB drink since we were interested in studying a realistic condition. Future studies with larger numbers of subjects with homogenous and/or more pronounced CVD risk factors or established endothelial dysfunction may show beneficial effect of blueberry intervention.

The effects of berries on oxidative stress have been investigated in different human intervention studies, but results are still inconclusive. Results from intervention studies with berries (single berry items or mixture of different berries) have shown a reduction of the levels of oxidatively damaged DNA evaluated by means of the

comet assay [50-51], whereas there are studies showing no effect after ingestion of ACN-rich blackcurrant juice or an ACN drink [52]. Under our experimental conditions, the WB drink reduced the level of oxidized DNA bases and increased the protection from the *ex vivo* H_2O_2 induced DNA damage. These results were particularly evident in the group of smokers/ex-smoker subjects with respect to nonsmokers. This effect on DNA damage may be dependent on phytochemicals present in WB drink that can exert a direct protective effect against oxidative insult by scavenging reactive oxygen species in blood. Moreover, ACNs may act through upregulation of the expression of different antioxidant genes involved in the detoxification of molecules such as hydrogen peroxide [53]. Other processes that may have been modulated by the WB drink could be: alkylation or formation of ACN-DNA complexes, and/or modulation of DNA repair genes [54-55]. In this regard, it has been reported that a flavonoid-rich diet could affect the expression of DNA repair genes belonging to different repair pathways like nucleotide excision repair, base excision repair and double strand breaks repair [55]. In our study, 6 weeks of WB drink consumption did not show an increase in DNA repair activity. In addition no effect on SOD, GSH-Px or GST activity was observed suggesting that upregulation of antioxidant enzymes is not the mechanism for the increased resistance toward H_2O_2-induced DNA damage.

Studies on ingestion of polyphenol bioactive compounds have shown results on lipid profile data that are not univocal. Bilberries, blackcurrants, cranberry juice or extracts have shown a favorable effect on the lipid profile in dyslipidemic, hypercholesterolemic or metabolic syndrome subjects from improving HDL-cholesterol to lowering LDL-cholesterol and total triglycerides [56-59]. On the contrary Basu et al. [16] reported that 8 weeks of supplementation with a freeze dried blueberry beverage (50 g of powder, providing 742 mg of ACNs per day) did not change the plasma lipid profile in subjects with the metabolic syndrome. Similarly Stull et al. [26] found that the consumption of two blueberry smoothies per day (45 g of freeze dried blueberry powder, providing 668 mg of ACNs) did not affect the lipid profile in obese subjects after 6 weeks of intervention. The above studies are in agreement with the present study, since the WB drink intervention did not alter the plasma lipid profile in subjects with risk factors for CVDs.

The role of berries in decreasing markers of inflammation has not been well investigated and very few observations have shown an antinflammatory effect [26, 60]. Our results do not show a significant effect of the WB drink intake on the plasma levels of IL-6, TNF-α and CRP. These results are in accordance with other observations reported in literature [16, 26, 60]. It should be noted that the inflammatory markers in our

volunteers were in the normal physiologic concentration ranges at the onset of the study thus the potential of WB to affect such biomarkers maybe more appropriately evaluated on subjects with high initial levels.

CONCLUSIONS

In conclusion, consumption of a WB drink, providing 375 mg of ACNs, for 6 weeks reduced the level of oxidized purines and improved the resistance against H_2O_2-induced DNA damage in MNBCs. This indicates that regular consumption of wild blueberries can reduce oxidative stress in a relevant cell population in the blood, even though peripheral arterial function and the other variables studied here were not significantly affected. Future, larger studies are necessary to understand the duration of exposure to ACNs, the dose and/or the form of the WB product that may be effective in modulating endothelial function and the other parameters studied.

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Conflict of interest

The authors declare that they have not conflict of interest.

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588

Legend

Fig 1. Individual values of reactive hyperemia index (RHI) measured by Endo-PAT2000 (Itamar Medical Ltd., Caesarea, Israel) and registered before and after a six weeks WB drink and PL drink intake in the group of volunteers (n = 17). WB, wild blueberry drink; PL, placebo drink

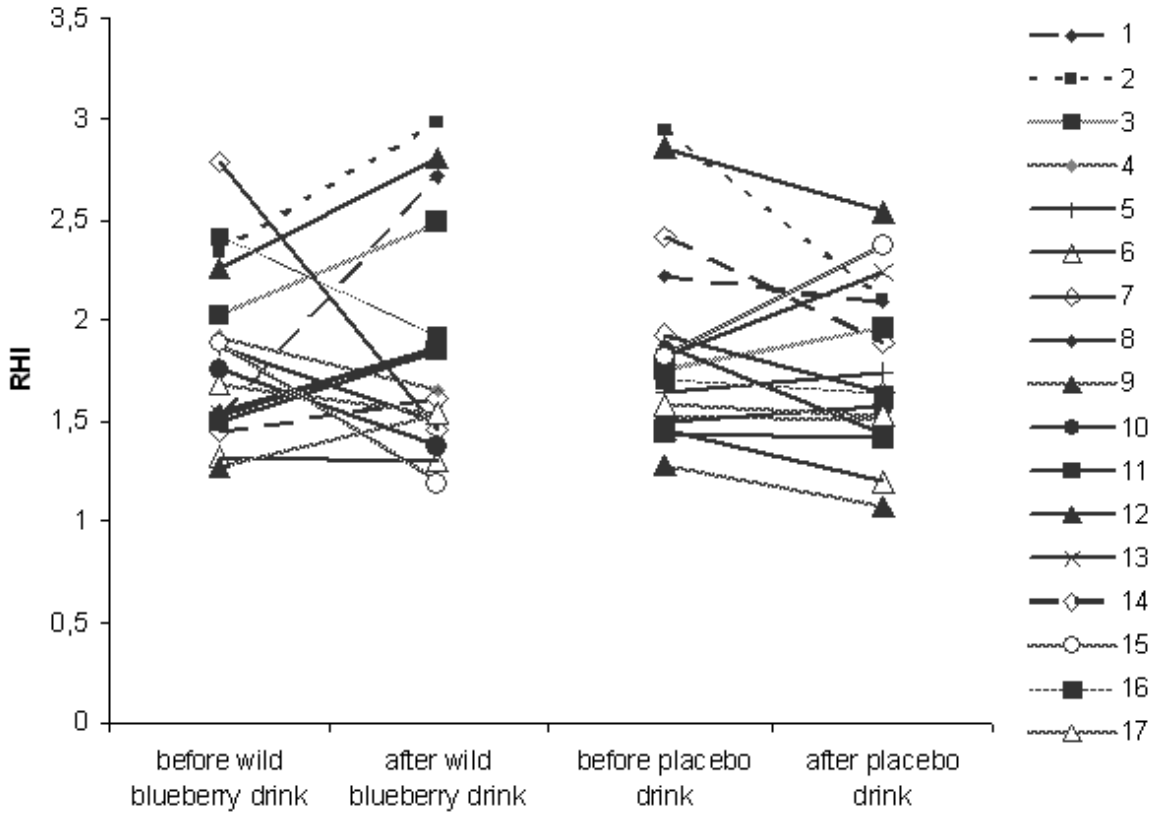

Table 1 Composition of the wild blueberry powder drink.

Nutritional composition	One portion (25 g)
Energy (kcal)	101.8
Total fat (g)	1.4
Saturated fat (g)	0.2
Monounsatured fat (g)	0.3
Polyunsatured fat (g)	0.6
Cholesterol (mg)	0
Protein (g)	0.7
Sugars (g)	17.1
Fiber (g)*	4.2
Soluble	0.6
Insoluble	3.6
Anthocyanins (g)*	0.4
Chlorogenic acid*	127.5
Vitamin C (mg)	4.2
Folate (mg)	11.1

Nutritional information was provided by FutureCeuticals Company.

*The analysis was performed at our laboratory.

612

613 **TABLE 2** Subject characteristics at the beginning of the study (n=18)[1]

Variables	Value	Variables	Value
Age (y)	47.8 ± 9.7	NO (μmol/L)	45.3 ± 7.3
Body weight (kg)	75.7 ± 8.7	sVCAM-1 (ng/mL)	402 ± 116
BMI (kg/m^2)	24.8 ± 2.6	CRP (mg/dL)	0.19 ± 0.22
Glucose (mmol/L)	4.9 ± 0.5	IL-6 (pg/mL)	2.1 ± 1.1
Triglycerides (mmol/L)	1.2 ± 0.5	TNF-α (pg/mL)	3.4 ± 1.5
Total cholesterol (mmol/L)	5.8 ± 1.2	Diastolic pressure (mm Hg)	79.4 ± 8.7
LDL-cholesterol (mmol/L)	3.8 ± 1.0	Systolic pressure (mm Hg)	121 ± 16
HDL-cholesterol (mmol/L)	1.4 ± 0.3	RHI	1.84 ± 0.46
AST (U/L)	22.8 ± 4.8	FRHI	0.32 ± 0.27
ALT (U/L)	26.7 ± 1.5	AI	5.22 ± 18.5
GGT (U/L)	26.8 ± 10.7	AI@75	0.0 ± 17.4
Creatinine (mmol/L)	79.6 ± 8.8	H_2O_2-induced DNA damage (% DNA in tail)	45.6 ± 10.1
Anthocyanins (μmol/L)	nd	FPG sensitive sites (% DNA in tail)	13.4 ± 5.6
Vitamin C (μmol/L)	63.1 ± 17.3	DNA repair activity (%DNA in tail)	6.5 ± 1.7
Folate (nmol/L)	16.5 ± 4.5		
Vitamin B_{12} (pmol/L)	312 ± 110		
GSH (μmol/g Hb)	5.8 ± 1.0		
GSSG (μmol/g Hb)	0.65 ± 0.42		
GSH/GSSG ratio	11.2 ± 4.53		
SOD activity (U/mg Hb)	4.7 ± 0.5		
GST activity (nmol/min/mg proteins)	35.1 ± 17.4		
GSH-Px activity (μmol /min/mg proteins)	17.4 ± 7.3		

614
615 [1]Data are expressed as mean ± SD. BMI, body mass index; nd, not detectable; AST, aspartate aminotransferase;
616 ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; GSH, reduced glutathione; GSSG, oxidized
617 glutathione; SOD, superoxide dismutase; GST, glutathione S-transferase; GSH-Px, glutathione peroxidase; NO,
618 nitric oxide; sVCAM-1, vascular cell adhesion molecule-1; CRP, C-reactive protein; IL-6, interleukin-6; TNF-α,

tumor necrosis factor alpha; RHI, reactive hyperemia index; FRHI, Framingham reactive hyperemia index; AI, augmentation index; AI@75, augmentation index standardized for heart rate of 75 bpm.

TABLE 3 Effect of a 6-week intervention with the wild blueberry (WB) drink or placebo (PL) drink on nutritional biomarkers (n=18)[1]

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
Anthocyanins (µmol/L)	nd	nd	nd	nd	-
Vitamin C (µmol/L)	61.5 ± 16.8	61.7 ± 20.2	64.1 ± 15.8	67.5 ± 16.5	0.488
Folate (nmol/L)	16.3 ± 5.0	15.0 ± 4.8	17.2 ± 4.8	15.9 ± 4.1	0.930
Vitamin B_{12} (pmol/L)	308 ± 92	298 ± 99	314 ± 107	322 ± 105	0.392
GSH (µmol/g Hb)	6.8 ± 2.5	6.0 ± 0.9	6.2 ± 1.2	6.6 ± 1.5	0.140
GSSG (µmol/g Hb)	0.77 ± 0.44	0.74 ± 0.27	0.74 ± 0.40	0.83 ± 0.42	0.574
GSH/GSSG ratio	11.25 ± 6.58	8.99 ± 2.98	9.03 ± 3.74	9.45 ± 4.62	0.216

[1] Data are expressed as mean ± SD. WB, wild blueberry; PL, placebo; nd, not detectable; GSH, reduced glutathione; GSSG, oxidized glutathione

[2]P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).

633 **TABLE 4** Effect of a 6-week intervention with the wild blueberry (WB) drink or placebo (PL) drink on

634 background and H_2O_2 induced strand breaks, FPG sensitive sites, DNA repair activity (n=18)[1]

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
Background SBs (% DNA in tail, EB)	6.3 ± 1.6	6.7 ± 1.2	6.2 ± 1.4	6.2 ± 1.5	0.201
Net FPG-sensitive sites (% DNA in tail)	12.5 ± 5.6	9.6 ± 3.5*	12.0 ± 4.5	11.9 ± 4.4	0.039
Background SBs (% DNA in tail, PBS)	5.9 ± 1.1	6.5 ± 1.2	6.2 ± 1.4	6.8 ± 2.0	0.594
Net H_2O_2-induced DNA damage (%DNA in tail)	45.8 ± 7.9	37.2 ± 9.1*	44.9 ± 12.2	44.4 ± 8.4	0.037
DNA repair activity (% DNA in tail)	6.4 ± 1.7	6.7 ± 1.9	6.6 ± 1.5	6.4 ± 1.8	0.425

635

636 [1]Data are expressed as mean±SD. WB, wild blueberry; PL, placebo; SBs, strand breaks; PBS, phosphate buffer

637 saline; EB, endonuclease buffer. *Significantly different from each other point, P≤0.05

638 [2]P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa,
639 OK, US).

640

641

TABLE 5 Effect of a 6-week intervention with wild blueberry (WB) drink or placebo (PL) drink on vascular function, blood pressure, plasma total nitric oxide and soluble vascular cell adhesion molecule-1 (n=18)[1]

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
RHI	1.83 ± 0.43	1.86 ± 0.55	1.87 ± 0.48	1.76 ± 0.41	0.452
FRHI	0.35 ± 0.28	0.29 ± 0.33	0.34 ± 0.31	0.30 ± 0.25	0.838
AI	3.57 ± 15.6	3.18 ± 14.9	6.94 ± 17.5	4.71 ± 16.5	0.407
AI@75	-1.71 ± 15.6	-3.76 ± 14.1	0.00 ± 17.9	0.47 ± 16.3	0.458
Diastolic pressure (mm Hg)	81.6 ± 8.8	81.4 ± 7.2	78.3 ± 8.2	78.8 ± 8.5	0.740
Systolic pressure (mm Hg)	123 ± 16	122 ± 16	122 ± 16	120 ± 16	0.878
Total NO (µmol/L)	48.5 ± 24.1	49.8 ± 23.6	45.9 ± 16.9	43.7 ± 20.2	0.666
sVCAM-1 (ng/mL)	410 ± 103	576 ± 364	417 ± 129	540 ± 232	0.762

[1]Data are expressed as mean±SD; (n=17 vascular function). WB, wild blueberry; PL, placebo; RHI, reactive hyperemia index; FRHI, Framingham reactive hyperemia index; AI, augmentation index; AI@75, augmentation index standardized for heart rate of 75 bpm; Total NO, total nitric oxide; sVCAM-1, vascular cell adhesion molecules-1.

[2]P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).

TABLE 6 Effect of a 6-week intervention with the wild blueberry (WB) drink or placebo (PL) drink on anthropometric and biochemical characteristics of volunteers (n=18)[1]

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
Body weight (kg)	75.9 ± 8.4	75.8 ± 8.7	76.2 ± 9.3	76.1 ± 9.3	0.977
BMI (kg/m^2)	24.9 ± 2.5	24.8 ± 2.6	24.9 ± 2.7	24.9 ± 2.6	0.977
Glucose (mmol/L)	5.0 ± 0.5	5.0 ± 0.3	4.9 ± 0.6	5.0 ± 0.6	0.357
Triglycerides (mmol/L)	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.6	1.4 ± 0.6	0.279
Total cholesterol (mmol/L)	5.9 ± 1.2	5.7 ± 1.3	5.9 ± 1.2	5.7 ± 1.3	0.774
LDL-cholesterol (mmol/L)	3.9 ± 0.9	3.7 ± 1.0	3.8 ± 1.2	3.6 ± 1.0	0.726
HDL- cholesterol (mmol/L)	1.4 ± 0.3	1.3 ± 0.2	1.4 ± 0.3	1.3 ± 0.3	0.241
AST (U/L)	23.8 ± 8.1	23.3 ± 6.2	23.1 ± 4.8	23.8 ± 6.9	0.644
ALT (U/L)	26.4 ± 10.7	27.5 ± 12.8	27.2 ± 12.1	27.5 ± 10.8	0.679
GGT (U/L)	27.2 ± 11.0	26.5 ± 11.4	27.0 ± 10.7	25.3 ± 11.0	0.217
Creatinine (mmol/L)	81.3 ± 11.5	82.2 ± 13.3	80.4 ± 10.7	80.4 ± 13.3	0.734
SOD activity (U/mg Hb)	4.7 ± 1.3	4.4 ± 1.3	4.8 ± 1.3	4.5 ± 1.4	0.870
GSH-Px activity (µmol/min/ml/g Hb)	18.6 ± 9.5	18.0 ± 5.6	16.5 ± 5.6	19.4 ± 7.0	0.121
GST activity (nmol/min/mg proteins)	25.9 ± 13.3	25.8 ± 9.6	31.4 ± 17.9	28.5 ± 11.0	0.672

[1]Data are expressed as mean±SD. WB, wild blueberry; PL, placebo; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidise; GST, glutathione S-transferase.

[2]P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).

TABLE 7 Effect of a 6-week intervention with the wild blueberry (WB) drink or placebo (PL) drink on circulating levels of interleukin 6, Tumor Necrosis Factor-α and C-reactive protein (n=18)[1]

Variables	Before WB Drink	After WB Drink	Before PL Drink	After PL Drink	P value[2]
IL-6 (pg/mL)	2.1 ± 1.4	2.1 ± 0.9	2.4 ± 1.3	1.9 ± 1.0	0.303
TNF-α (pg/mL)	3.6 ± 2.1	3.1 ± 1.0	3.4 ± 2.1	3.5 ± 1.9	0.438
CRP (mg/dL)	0.18 ± 0.20	0.20 ± 0.15	0.16 ± 0.13	0.22 ± 0.21	0.717

[1]Data are expressed as mean±SD. WB, wild blueberry; PL, placebo; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; CRP, C-reactive protein.

[2]P-values correspond to the interaction between treatment and time in the overall ANOVA (Statsoft Inc., Tulsa, OK, US).