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Effect of apple polyphenols on vascular oxidative stress and endothelium function: A translational study

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**Effect of apple polyphenols on vascular oxidative stress and endothelium function: a translational study**

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**Abbreviations** - ABP= Aortic Blood Pressure; AIX= Augmentation Index; ALT= alanine transaminases; AP= Augmentation Pressure; apoA1= apolipoprotein A1; apoB= apolipoprotein B; APP= Aortic Pulse Pressure; AST= aspartate transaminases; CI= Cardiac

Index; CO= Cardiac Output; CPK= creatinine phosphokinase; Cr= Creatinine; CVD= CardioVascular Disease; ER= Endothelial Reactivity; FPG= Fasting Plasma Glucose; HUVECs= living endothelial cells; MAP= Mean Arterial Pressure; PV= Pulse Volume; PWA= Pulse Wave Analysis; SUA= Serum Uric Acid; SV = Stroke Volume; TC= Total Cholesterol; TG= triglycerides; TPR= Total Peripheral Resistance; WC= Waist Circumference; XO= Xantine Oxidoreductase;

**Keywords:** apple polyphenols; endothelial reactivity; fasting plasma glucose; serum uric acid; xanthine oxidase

## **Abstract**

**SCOPE:** We aimed examining apple polyphenols' effect on uricemia and endothelial function in a sample of overweight subjects.

**METHODS AND RESULTS:** This was a two-phases study. *In vitro* experiment aimed to evaluate apple polyphenols' ability to lower uric acid in comparison with allopurinol. *In vivo* study consisted in a randomized, double-blind, parallel placebo-controlled clinical trial involving 62 overweight volunteers with suboptimal values of fasting plasma glucose ( $100\text{mg/dL} \leq \text{FPG} \leq 125\text{mg/dL}$ ), randomized to 300mg apple polyphenols or placebo for 8 weeks.

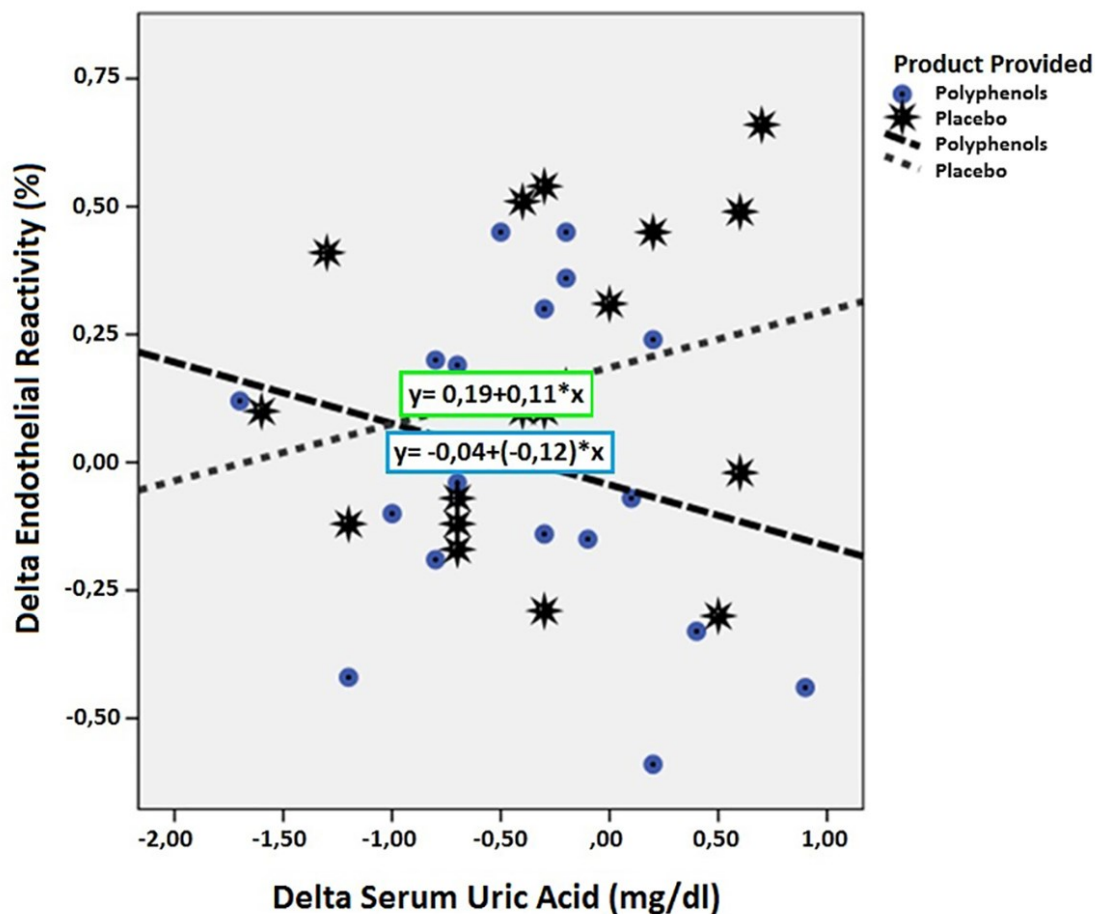
Apple polyphenols extract inhibited xanthine oxidase activity, with an  $\text{IC}_{50} = 130 \pm 30 \text{ ng/mL}$ ; reducing uric acid production with an  $\text{IC}_{50} = 154 \pm 28 \text{ ng/mL}$ . During the trial, after the first 4 weeks of treatment, FPG decreased in the active treated group ( $-6,1\%$ ,  $P < 0,05$ ), while no significant changes were observed regarding the other hematochemistry parameters. After 4 more weeks of treatment, active-treated patients had an improvement in FPG compared to baseline ( $-10,3\%$ ,  $P < 0,001$ ) and the placebo group ( $P < 0,001$ ). Uric acid ( $-14,0\%$ ,  $P < 0,05$  vs baseline;  $P < 0,05$  vs placebo) and endothelial reactivity ( $0,24 \pm 0,09$ ,  $P = 0,009$  vs baseline;  $P < 0,05$  vs placebo) significantly improved too.

**CONCLUSION:** *In vivo*, apple polyphenols extract has a positive effect on vascular oxidative stress and endothelium function and reduce FPG and uric acid by inhibiting xanthine oxidase, as our *in vitro* experiment attests.

## GRAPHICAL ABSTRACT

Apple's polyphenols including phloridzin, chlorogenic acid and quercetin work synergistically to modulate the glucose absorption, lower postprandial glycaemic load and regulate insulinaemic levels. Moreover, they have just been tested as uric acid lowering agents and seem to have vasorelaxant properties, even for low doses.

Our *in vivo* findings reflect the *in vitro* ones, opening up new possibilities as adjunct therapy in lifestyle interventions to treat patients with both mild hyperuricemia and hyperglycemia. If confirmed in larger clinical trials, our results support the use of apple polyphenols among the potentially useful nutraceuticals for the management of the metabolic syndrome.



## 1. INTRODUCTION

Several epidemiological and experimental studies support a beneficial role of dietary polyphenols in modulating different functions responsible for cardiovascular diseases risk factor. Their antioxidant effect seems mainly involved, positively affecting the cardio-metabolic processes and in a pleiotropic way. Therefore, an antioxidant-rich diet including a large consumption of vegetables and fruits, such as apples (which are rich source of polyphenols) (1,2), is recommended to mitigate the chronic disease, slow age and maintain a healthy lifestyle in general (3).

Apple polyphenols include flavonols (kaempferol, quercetin glycoside and rutin), dihydrochalcones (phloridzin and phloretin glycoside), flavan-3-ols (epicatechin and procyanidins) and phenolic acids (caffeic and coumaric acid), chlorogenic acid which have recognized anti-diabetic, anti-inflammatory and anti-proliferative properties and anti-cancer and cardio-protective effects (4). More importantly, these compounds are all safe and not harmful at the average dietary levels (~1 g per day), which also remain about 120-fold below the toxic threshold (5).

Apple's phloridzin, chlorogenic acid and quercetin work synergistically to modulate the glucose absorption, lower postprandial glycaemic load and regulate insulinaemic levels (6). In vivo, quercetin and phloridzine competitively inhibit the intestinal and renal glucose transport mediated by SGLT1 and SGLT2 (7-9). Moreover, phloridzine also decreases SGLT4 availability in the muscle and delays the hepatic glucose release (6). Lastly, chlorogenic acid inhibits the glucose-6-phosphatase, which is involved in the neoglucogenesis and hepatic glyconenolysis (10).

In the past, a number of studies focused on the importance of the insulin resistance for hyperuricemia, bringing as link a fructose intake excess (11-13). Up to date, the apple polyphenols as a whole have rarely been tested as uric acid lowering agents, though recent

studies attribute to quercetin the inhibition of xanthine oxidoreductase [XO] (14) besides vasorelaxant properties, even for low doses (15).

In this context, in order to assess the apple polyphenols' ability to lower uric acid by xanthine oxidase inhibition, a cell-based assay has been performed in order to compare apple polyphenols' activity with the allopurinol one. Then we aimed to evaluate if a dietary supplementation with standardized apple polyphenols could positively affect serum uric acid (SUA) and endothelial reactivity (ER) in a sample of overweight subjects with suboptimal blood glucose levels.

## 2. MATERIALS AND METHODS

### XANTHINE OXIDASE ACTIVITY CHEMILUMINESCENT ASSAY

**Reaction cocktail.** The reaction mixture was constituted of 100  $\mu\text{M}$  EDTA, 10  $\mu\text{M}$   $\text{FeCl}_2$ , 40  $\mu\text{M}$  sodium perborate, 0.1 mM luminol sodium salt in borate buffer, 0.1 mol/L, pH 10.3. Briefly, the experimental procedure was adapted from the one previously described by Roda and Caliceti et al. (16,17) by adding 0.5 mL of a  $\text{Fe}^{2+}$ -EDTA solution (116 mg Na-EDTA and 2.5 mg  $\text{FeCl}_2$  in 50 mL of distilled water), 0.05 mL of a luminol solution (20 mg luminol in 1 mL of borate buffer, 0.1 mol/L, pH 10.3), 0.1 mL Na-perborate solution (30 mg sodium perborate in 20 mL borate buffer, 0.1 mol/L, pH 10.3) in 50 mL of borate buffer (0.1 mol/L pH 10.3). The solution is stable for months, at 25°C but stored in the dark.

Xanthine oxidase from bovine milk, luminol sodium salt, xanthine, oxypurinol, allopurinol, PBS tabs, Na-EDTA salt, gelatin from bovine skin, penicillin/streptomycin, Trypsin-EDTA were purchased from Sigma Aldrich (St Louis, MO, USA). Sodium perborate, Boric acid, NaOH,  $\text{FeCl}_2$  were from Carlo Erba (Milan, Italy). M200 medium, Low Serum Growth Supplements and Fetal Bovine Serum were purchased from Thermo Fischer Scientific



(Waltham, MA USA). All the other chemicals and solvents were of the highest analytical grade.

**Cell Culture.** Different batches (each batch made of cells pooled from 22 donors) of Human Umbilical Vein Endothelial cells [HUVEC]s purchased from Thermo Fischer Scientific (Waltham, MA USA) were utilized for the experiments. Briefly HUVECs ( $5 \times 10^3$  cells/well) were plated on gelatin-coated tissue culture dishes and maintained in phenol red-free basal medium M200 (Thermo Fischer Scientific) containing 10% FBS and growth factors (LSGS, Thermo Fischer Scientific) at 37°C with 5% CO<sub>2</sub>. Cells from passages 3 to 7 were actively proliferating (70–90% confluent) when samples were analyzed (18). Briefly,  $5 \times 10^3$  cells/well were plated in a 96-black wells microtiter plate; the day after, cells were incubated at 37°C with CL reaction cocktail solution and the CL emission was monitored for 20 minutes; CL reaction cocktail in absence of cells was used as control to normalize the CL signals. All the experiments were performed at 37°C and the viability of cells was observed by trypan blue - exclusion test in all the conditions tested.

**Inhibition of intracellular xanthine oxidase activity in HUVECs.** We developed a sensitive method able to measure only the intracellular xanthine oxidase activity and its inhibition caused by drugs that are effectively able to cross the cell membrane and we reported that the xanthine oxidase activity in living endothelial cells (HUVECs) was  $(6 \pm 1) \times 10^{-7}$  mU/ml/cell and the IC<sub>50</sub> of oxypurinol, the active metabolite of allopurinol, was  $152 \pm 76$  ng/mL. 10 mg of apple polyphenols preparation (®SelectSIEVE Apple PCQ, kindly provided by Amitalia Srl, Solara, MI, Italy) were solubilized in 10 ml of borate buffer, 0.1 mol/ L, pH 10.3 to obtain a final concentration of 1.0 mg/ml (1% w/w). Subsequent dilutions in reaction mixture (containing 2.0 mM xanthine) were performed to prepare the inhibition curve, range  $1 \times 10^{-3}$ - $1 \times 10^{-6}$  mg/mL. The chemiluminescent cocktail was stored in the dark for 20 min, then cells

were incubated with the reaction mixture at 37°C and the light output from all wells was measured and monitored for 20 min using the Luminoskan<sup>TM</sup> Ascent luminometer automatic plate reader (Thermo Fisher Scientific, Roskilde, Denmark). Reaction mixture without xanthine oxidase was used as the negative control and reaction mixture with xanthine oxidase was used as the positive control (17).

**Method validation.** GraphPad Prism v. 5.04 (GraphPad Software, Inc., La Jolla, CA) was used to perform statistical analysis, to plot the CL signal as a function of xanthine oxidase activity or oxypurinol concentrations and for least-squares fitting of calibration curves. CL kinetic profiles were shown as the mean of three replicates of each concentration of samples. The CL values used for graph data were shown as the mean value of three replicates together with standard deviation of each sample (at 95% confidence interval). The IC<sub>50</sub> obtained from cell-based assays was calculated as the mean  $\pm$  SD (n=3, at 95% confidence interval), normalized for  $5 \times 10^3$  cells (mean cell population for each well). In particular, the IC<sub>50</sub> of apple polyphenols was  $130 \pm 30$  ng/mL.

**Determination of uric acid production through UV-Vis spectroscopy in cell-free system.** To exclude that the inhibitory activity of the preparation was due to its direct antioxidant activity in respect with the hydrogen peroxide produced during the enzymatic reaction of xanthine oxidase, the uric acid production was also calculated according to the increasing absorbance at 290 nm, thus the enzymatic catalysed uric acid production was monitored in time by ultraviolet–visible (UV-vis) spectroscopy according to the increasing absorbance at 290 nm (19) using a Varian Cary 50 UV-Vis spectrophotometer (Agilent Technologies, USA) with a 1.0 cm quartz cell. Briefly, a series of assay solutions were prepared in borate buffer, 0.1 mol/L, pH 10.3 by adding a constant concentration of xanthine (final concentration 50  $\mu$ mol/L) and various amounts of inhibitor solutions. The final assay concentration of the

tested preparation ranging from  $2 \times 10^{-3}$  to  $1 \times 10^{-4}$  mg/mL. The results obtained by this dose-response curve were compared with the results obtained by a dose-response curve related to a reference standard inhibitor allopurinol (final concentration ranging from 1.36 to 136.11 ng/mL). Allopurinol and the tested preparation were prepared in a borate buffer, 0.1 mol/L, pH 10.3 just before the assay. Then the assay was initiated by adding the xanthine oxidase (final concentration was 5.0 mU/assay) to the 1.0 mL reaction mixture. The absorbance of the mixtures was measured at 290 nm every 5 min, for a period of 20 min, at room temperature. The effect of inhibition of the tested preparation and reference standard allopurinol on xanthine oxidase activity was valued by the slope of linear plot fitting the absorbance change at 290 nm vs time interval ( $\Delta t = 5$  min). To a minor slope is related a greater inhibition degree.

#### TRIAL DESIGN AND PARTICIPANTS

The present study was a randomized, double-blind, parallel placebo-controlled clinical trial conducted in a group of 62 overweight volunteers ( $BMI > 25 \text{ kg/m}^2$ ) with suboptimal values of fasting plasma glucose ( $100 \text{ mg/dL} \leq \text{FPG} \leq 125 \text{ mg/dL}$ ) and aged 18-70 years. Patients with obesity (body mass index [BMI]  $< 30 \text{ Kg/m}^2$ ), diabetes mellitus, personal history of atherosclerosis-related cardiovascular diseases (coronary artery disease, cerebrovascular disease, ultrasound diagnosed carotid atherosclerosis), absorption disease, myopathy, renal failure or chronic liver disease, or serious or invalidating other diseases reducing the subjects' ability to comply with the full protocol were excluded from the study as well as subjects with a known apple intolerance.

The study included a 2-week run-in period of diet standardization and an 8-week treatment period. At enrolment, patients were given standard behavioural and qualitative dietary suggestions to correct their unhealthy habits. In particular, subjects were strongly

recommended to follow the general indications of a Mediterranean diet, avoiding an excessive intake of dairy and red meat derived products and reducing the dietary excesses, in order to maintain an overall balanced diet for the entire duration of the study. A specialist physician suggested them provide around 50% of calories from carbohydrates, 30% from fat (6% saturated), and 20% from proteins, with a maximum cholesterol content of 300 mg/day and 35 g/day of fiber. Individuals were also encouraged to increase their physical activity by walking briskly or cycling from 3 to 5 times for week, at least 20 minutes every time.

The study fully complied with the ethical guidelines of the Declaration of Helsinki. The study protocol was approved by the Ethical Committee of the University of Bologna (clinicaltrial.gov ID: NCT03053986). All patients signed a written informed consent to participate.

## TREATMENT

After 2 weeks of diet standardization, the enrolled subjects were allocated to the treatment with an indistinguishable pill of placebo or an active product, containing 300 mg of apple extract (*Malus Domestica*) with glycosilated polyphenols ( $\geq 90\%$ ) including glycosilated phlorizin (15-30%), chlorogenic acid (10-25%) and quercetin (15-25%) (kindly provided by Amitalia Srl, Solara, MI, Italy). Patients were asked to take the pills regularly, every day early in the morning. The amount of glycosilated polyphenols was assessed spectrophotometrically at 280 nm, using synthetic catechin as standard. Glycosilated phlorizin, chlorogenic acid and quercetin were measured by HPCL-MS [high performance liquid chromatography-mass spectrometry].

The randomization was performed 1:1 ratio and the blocks were stratified by sex and age. An alphabetical code was assigned to each lot code (corresponding to treatment or placebo) impressed on the dose box. The study staffs and the investigators, as well as all of the

volunteers, were blinded to the group assignment. Codes were kept in a sealed envelope, which was not opened until the end of the trial. Dose boxes were mixed and a blinded dose box was assigned to each enrolled patient. Participants' compliance was assessed by counting the number of pills returned at the time of specified clinic visits. At the end of the study all unused pills were retrieved for inventory.

Patients were evaluated anamnestically and by the execution of a physical examination and laboratory analyses at the baseline, in the middle and at the end of the trial.

The hemodynamic variables recorded (endothelial function, arterial stiffness and related parameters) were investigated before and after the intervention period. All the instrumental measurements were carried out following standardized protocols by specially trained staff.

## ASSESSMENTS

***Clinical data and anthropometric measurements.*** The patients' personal history was evaluated taking particular attention to cardiovascular disease and other diseases, dietary and smoking habits assessment (both evaluated with validated semiquantitative questionnaires) (20), physical activity and pharmacological treatment. Height, weight and waist circumference [WC] were measured by standard procedure.

***Clinical chemistry analyses.*** The biochemical analyses were carried out on venous blood, withdrawn early in the morning from the basilica vein. Subjects were fasted for at least 12 hours at the time of sampling. All the hematochemical measurements were centrally performed in our department's laboratory. Serum used was centrifuged at 3000 RPM for 15 minutes at ambient temperature. All of the laboratory analyses were performed by trained personnel immediately after centrifugation, in accordance with standardized methods largely described elsewhere (21). The hematochemistry variables investigated were: total cholesterol [TC], high-density lipoprotein-cholesterol [HDL-C], triglycerides [TG], low-density

lipoprotein cholesterol [LDL-C], apolipoprotein B [apoB], apolipoprotein A1 [apoA1], fasting glucose [FPG], creatinine [Cr], alanine transaminase [ALT], aspartate transaminase [AST] and creatinine phosphokinase [CPK].

***Augmentation index and relative measures.*** The Augmentation Index [AIx] was noninvasively evaluated by the use of the Vicorder® apparatus (Skidmore Medical Ltd, Bristol, UK), which is a validated cuff-based oscillometric device deriving the central blood pressure curve using a brachial-to-aortic transfer function. The Pulse Wave Analysis [PWA], from which AIx was obtained, was recorded in each subject supine and at rest, with a cuff applied at the right upper arm. AIx was calculated as the ratio of the pressure increment caused by the reflected wave (augmented pressure) to the pulse pressure (22). It represents, as the pulse wave velocity, a measure of the arterial stiffness and is a marker of cardiovascular risk (23). The intensity of the wave reflection depends from the diameter and the elasticity of the small arteries and arterioles (24) and is inversely related to the body height (25) and heart rate (26,27). The Vicorder System® guarantees an excellent intra- and inter- operator reliability (28). It displays the central aortic pulse wave in real time and documents the systolic and diastolic blood pressure, deriving even the mean arterial pressure [MAP] value. Vicorder® also estimates the stroke volume and the cardiac output. The stroke volume is calculated from the difference between the estimated end-diastolic volume and the end-systolic volume. The cardiac output results from the multiplication of the stroke volume and the heart rate (29,30).

***Endothelial reactivity.*** ER was evaluated, according to the published guidelines (31) by the use of the Endocheck® (BC Biomedical Laboratories Ltd, Vancouver, BC, Canada), which is embedded in the Vicorder® System. The test was carried out before the morning drug intake, with the patients in a supine position and in abstinence from cigarette smoking and

caffeinated beverage for at least 12 hours. The brachial pulse volume (PV) waveforms were recorded for 10 seconds at baseline and, then, during reactive hyperaemia, which was provoked through PV displacement, obtained by inflating up to 200 mmHg for 5 minutes a cuff positioned distally around the forearm. When the cuff was released, the PV waveforms were recorded again for 3 minutes. The percent PV displacement was calculated as the percentage change from baseline to peak dilatation (32). All of the hemodynamic measurements were performed by a trained physician who was blinded to the treatment groups.

## STATISTICAL ANALYSES

Sample size was calculated for both primary outcomes (SUA decrease and ER improvement). Considering a Type I error of 0.05 and a power of 0.80 and expecting a minimum SUA reduction of 0.5 mg/dL with a SD of 0.7, and considering a 20% drop-out rate, we calculated to enrol 62 patients (31 per arm). This calculation was also valid to detect significant changes in ER. Data were analysed using intention to treat by means of the Statistical Package for Social Science (SPSS) version 21.0 (IBM Corporation, Armonk, NY, USA) for Windows. The normality distribution of the tested parameters was evaluated by the Kolmogorov-Smirnov test. The baseline characteristics of the population were described by the independent T-test and the  $\chi^2$  test, followed by Fisher's exact test for the categorical variables. Every continuous parameter was compared by repeated-measures analysis of variance (ANOVA). The intervention effects were adjusted for all of the considered potential confounders by the analysis of covariance (ANCOVA). ANOVA was performed in order to assess the significance within and between groups. The statistical significance of the independent effects of treatments on the other variables was determined by the use of the ANCOVA. A one-sample T-test was used to compare the values obtained before and after the

treatment administration; 2-sample T-test were used for between-group comparisons. The Tukey's correction was carried out for multiple comparisons.

All data were expressed as mean  $\pm$  standard deviation (SD). Every test was two-tailed. *P*-values < 0.05 were always regarded as statistically significant.

### 3. RESULTS

***In vitro tests.*** Cells were incubated for 20 minutes with CL cocktail containing different amount of apple polyphenols preparation, ranging from  $1 \times 10^{-3}$  to  $1 \times 10^{-6}$  mg/mL, and as it is shown in figure 1A, the CL emission was related to the concentration of the preparation. The intracellular IC<sub>50</sub> of the preparation ( $130 \pm 30$  ng/mL) was calculated by a dose-response curve (Figure 1B) after 20 minutes of incubation. Simultaneously, we monitored for 1 hour the viability of cells during the experimental procedures with trypan blue- exclusion test, using cells grown in standard cell medium as control; we did not observe any significant differences in cell viability according to the different treatments and buffers employed (data not shown). This cell-based assay utilising whole cells takes into consideration also the bioavailability of the compound, especially the ability to cross cell plasma membranes, so it is more representative and predictive to human situation. However, antioxidant molecules that can interfere with the emission of the CL signal can give false positives, so it is required the use of a complementary method to monitor the production of uric acid, the final product of the oxidation of purines catalysed by xanthine oxidase activity.

Using UV-Vis spectroscopy, we were able to directly monitor over time (0-20 minutes) the increased concentration of uric acid, the product of the enzymatic reaction between the enzyme xanthine oxidase and its substrate xanthine. The presence of known dilutions of the xanthine oxidase inhibitor allopurinol is able to influence the kinetics of the reaction, decreasing the rate of formation of uric acid (Figure 2 A and B). Considering therefore the



variations of absorbance during 20 minutes as the rate of formation of the product of xanthine oxidase enzymatic reaction, it was possible to assess the degree of inhibition of xanthine oxidase by the apple polyphenols ( $IC_{50} = 154 \pm 28$  ng/mL) and of the standard allopurinol ( $IC_{50} = 2.65 \pm 0.88$  ng/mL) (Figure 2 C and D). *In vitro* tests do not give informations on the pharmacokinetic profile of the compound and a human study is required to confirm the preclinical data.

**Clinical trial.** Into the trial, we overall enrolled 62 subjects (31 men and 31 women), who were randomly assigned to the active treatment or the placebo group. Nine patients failed to complete the study: six patients were not complying with the treatment and three were lost to follow-up. Subjects who dropped out were all excluded from the analysis because of the lack of the end-of-period data. All of the other volunteers ( $n = 53$ ; men: 27, women: 26) completed the trial according to the study design.

The final distribution between men and women considering the assumed products did not show any significant difference ( $\chi^2 = 2,29$ ,  $P > 0,05$ ). The two study-groups were well matched for all of the considered variables at baseline ( $P > 0,05$  always) (Table 1). No patient experienced any kind of subjective or laboratory adverse events and active treatment was as well tolerated as the placebo.

From the randomization visit until the end of the study, the enrolled subjects maintaining similar dietary habits. After the first four weeks of treatment, the FPG decreased in the active treated group ( $-6,1\%$ ,  $P < 0,05$ ), while no significant changes were observed in both groups of treatment regarding the other hematochemistry parameters or the blood pressure values and the anthropometric characteristics ( $P > 0,05$  always) (Table 2). At the end of the eighth week of treatment, the active-treated patients experienced an improvement in the FPG compared to the baseline ( $-10,3\%$ ,  $P < 0,001$ ) and the placebo group ( $P < 0,001$ ) (Table 3). SUA significantly improved too ( $-14,0\%$ ,  $P < 0,05$  vs baseline;  $P < 0,05$  vs placebo).

Regarding the hemodynamic parameters, the endothelial reactivity increased in the actively treated subjects in comparison with the baseline ( $0,24\pm 0,09$ ,  $P=0,009$ ) and the placebo-treated group ( $P<0,05$ ). In the apple polyphenols treated group, the ER improvement was proportional to SUA decrease (Figure 3). No significant change was observed concerning the considered stiffness-related parameters (Table 4).

#### **4. DISCUSSION**

In the present study, we firstly evaluated the anti-hyperuricemic effects of the dietary supplementation with apple polyphenols extract *in vitro* in human umbilical vein endothelial cells (HUVECs) and then we confirm previous data in a clinical trial enrolling a group of overall healthy overweight subjects with suboptimal fasting plasma glucose levels.

In addition to a classical method which rely on the direct quantification of the concentration of uric acid in biological samples using Vis-UV spectroscopy, we also utilized a cell-based assay in which uric acid production was quantified by the evaluation of xanthine oxidase activity and the inhibitory effects done by of oxypurinol (the active metabolite of allopurinol) (17).

In literature are described two cell-based assays to monitor xanthine oxidase activity: one is based on the conversion of the non-fluorescent molecule pterin into the fluorescent oxidized isoxanthopterin, in presence of reactive oxygen species (ROS) produced by xanthine oxidase. The limit of this method consists in the use of lysed/dead cells to monitor weak fluorimetric signal due to low intracellular enzyme activity (33). To overcome the limits connected to the weak fluorescence signal and, especially, to the not viable cell status, in the present study it was utilized a method previously described by Caliceti et al.; the detection method is based on an enhanced chemiluminescence (CL) emission between luminol and  $H_2O_2$  produced by xanthine oxidase in presence of Fe-EDTA complex catalyst in whole living HUVECs (17).

CL, despite the low light emission is characterized by a very high signal/noise ratio thus allowing a quantitative analysis. This *in vitro* cell-based assay do not give information on the pharmacokinetic profile of the compound but, utilizing whole cells, this method takes into consideration the bioavailability of the compound, especially the ability to cross cell plasma membranes, allowing to be more predictive to human situation. Moreover, just few of the assays proposed in literature compared the inhibitory action of drugs or natural substances (flavonoids, etc.) to oxypurinol, thus making our study more representative.

High serum levels of uric acid have been suggested as a predictor biomarker of cardiovascular disease (CVD) mortality linked to endothelial dysfunction, obesity, insulin resistance and metabolic syndrome; increasing evidence suggests that among patients with established CVD uric acid is a biomarker of disease state and prognosis. Accordingly, xanthine oxidase activity inhibition has been shown to improve a range of surrogate markers in hyperuricemic patients with CVD (34).

The tested phenolic compounds at the considered doses were sufficient to produce the recorded changes and provided a more practical and standardized form of consuming naturally-derived glycosilated phlorizin, quercetin and chlorogenic acid, obviously without showing any adverse event. Of course, these clinical findings are supported by the present *in vitro* experiment's ones, which highlight the apple polyphenols' ability to inhibit also the xanthine oxidase activity, ultimately reducing the uric acid production with a mechanism of action which is confirmed to be similar to the allopurinol's one (35).

In our study, after a 4-week period, as expected, the active-treated subjects already improved significantly their glycemetic control versus the baseline. After 4 more weeks of treatment, in the polyphenols-treated group not only FPG but also SUA decreased when compared both to the baseline and the placebo-treated group. Moreover, the endothelial function also improved, while no significant change was observed regarding the other hemodynamic and stiffness-

related parameters. These latter observations are not entirely in agreement with previous studies' ones, which in some cases open instead to the possibility quercetin have some hypotensive effect, in particular in hypertensive subjects (36,37). On the contrary, the endothelial function improvement had just been observed by Dower et al. (38) in patients actively treated with tea's quercetin.

Certainly, the present study has some limitations. Firstly, participants in the clinical trial were not frankly hyperuricemic patients. On the other hand, the tested product does not aim to replace a drug treatment and it is not expected to be useful in manifestly pathological situations but rather for pre-hyperuricemic subjects. Moreover, what we could suppose before to carry out the preclinical test was that apple polyphenols could improve SUA mainly by improving insulin-resistance, a common condition among overweight subjects with impaired fasting glucose. Secondly, the sample size was relatively small. However, the study power was adequate for the primary endpoints, as previously declared. Moreover, up to now, apple polyphenols extract had ever been tested as SUA lower agents or vasoactive drugs in smaller sample with well-defined characteristics (37). Finally, the relatively short duration of the clinical trial and the reduced observation time of the considered parameters' changes do not clarify on the possible occurrence of adaptation phenomena, which have never been documented for apple polyphenols. However, our findings certainly open up new possibilities as adjunct therapy in diet or lifestyle interventions to treat patients affected by mild hyperuricemia, besides slightly hyperglycemic. However, if confirmed in larger clinical trials, our results support the use of apple polyphenols among the potentially useful nutraceuticals for the management of the metabolic syndrome (39).

In conclusion, apples polyphenols are strong xanthine oxidase inhibitors in vitro and exert a mild but significant SUA lowering effect in humans. This effect, associated to the decrease of

FPG, is probably the cause of the observed improvement in endothelial reactivity in the studied subjects.

#### **AUTHOR CONTRIBUTIONS**

A.F.G. analyzed data and wrote the article; CC and FF collected and assembled the data and wrote the article; A.C. critically revised the article; D.C. and M.G. collected the data and performed the laboratory analyses; M.V. and A.R. critically revised the article; C.B. coordinated the research group and critically revised the article. All authors read and finally approved the paper.

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#### **CONFLICT OF INTEREST STATEMENT**

None of the authors has any conflict of interest in the publication of this paper.

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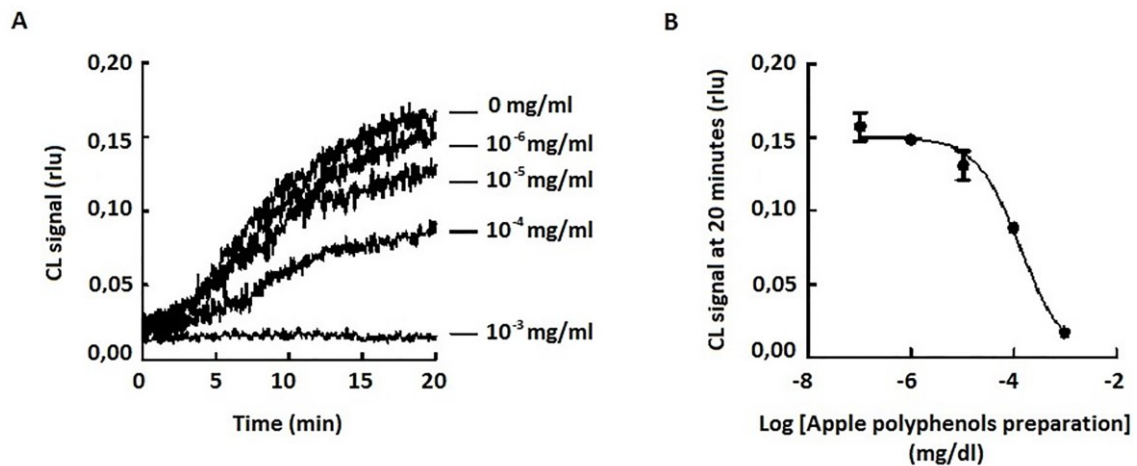


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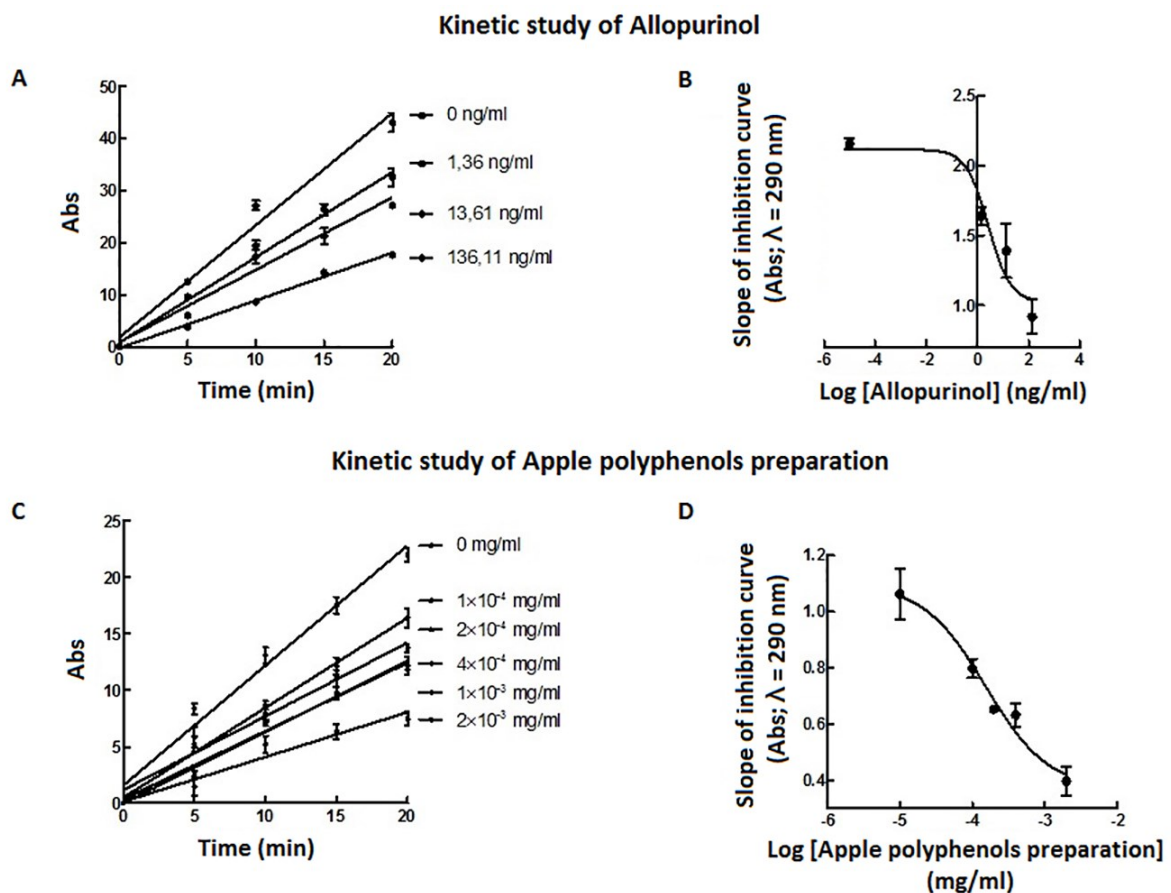
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## FIGURE LEGENDS

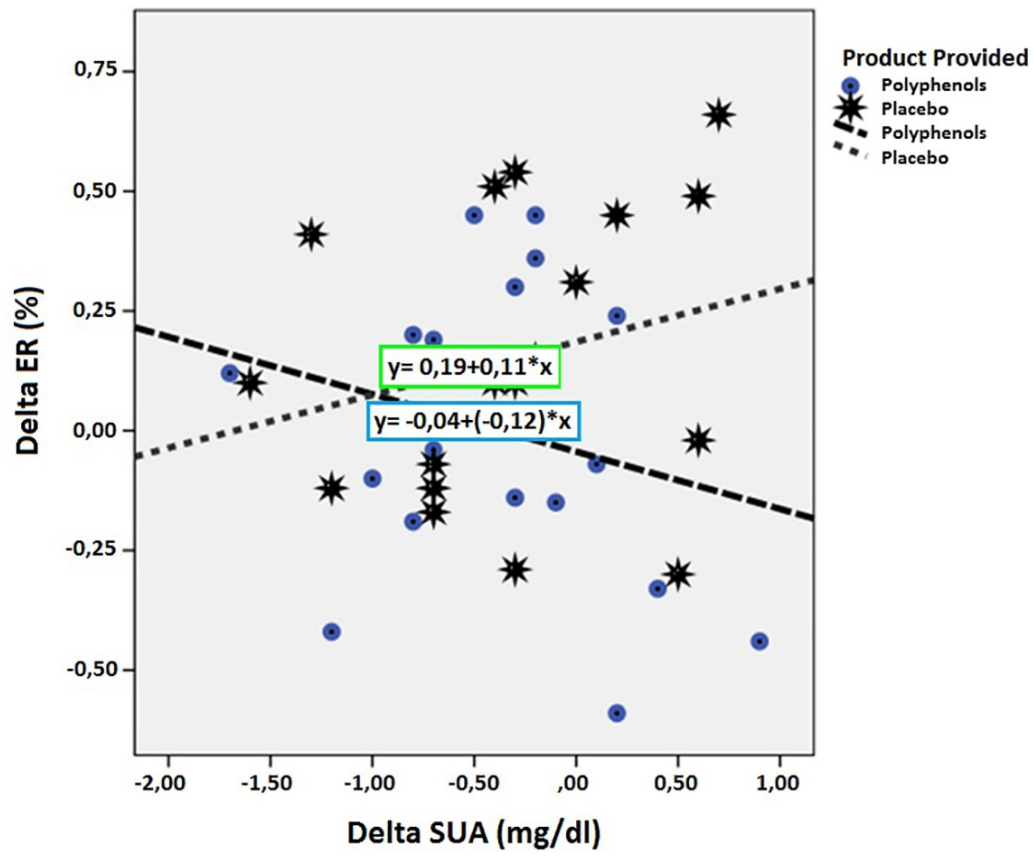
**Figure 1. Chemiluminescent cell-based assay.** **A** CL kinetic profiles of the intracellular light emission (HUVECs,  $5 \times 10^3$  cells/well) using Fe-EDTA-luminol reaction cocktail in borate buffer (pH 10.3) at 37 °C for 20 min in the presence of different concentrations of apple polyphenols preparation (range  $1 \times 10^{-3}$  -  $1 \times 10^{-6}$  mg/mL). **B** Concentration-response plot of intracellular XO inhibition obtained by analyzing CL signals after 20 min of incubation with Fe-EDTA-luminol reaction cocktail in borate buffer (pH 10.3) at 37 °C for 20 min in the presence of different concentrations of apple polyphenols preparation (range  $1 \times 10^{-3}$  -  $1 \times 10^{-6}$  mg/mL). Values are means of  $n = 3$  with standard deviation (SD) indicated by vertical bars.



**Figure 2. Vis-UV spectroscopic assay.** **A.** Kinetic study of uric acid production in presence of different concentrations of allopurinol (from 136,11-1,36 ng/ml) in borate buffer (pH 10.3) at 37 °C for 20 min. The production of uric acid was monitored in time (0-20 minutes) by ultraviolet–visible (UV-vis) spectroscopy according to the increasing absorbance at 290 nm. Values are means of  $n = 3$  with standard deviation (SD) indicated by vertical bars. **B.** Dose-response curve obtained plotting kinetic curves slope vs log [allopurinol] in ng/ml. **C.** Kinetic study of uric acid production in presence of different concentrations of apple polyphenols preparation (from  $2 \times 10^{-3}$  to  $1 \times 10^{-4}$  mg/mL) in borate buffer (pH 10.3) at 37 °C for 20 min. The production of uric acid was monitored in time (0-20 minutes) by ultraviolet–visible (UV-vis) spectroscopy according to the increasing absorbance at 290 nm. Values are means of  $n = 3$  with standard deviation (SD) indicated by vertical bars. **D.** Dose-response curve obtained plotting kinetic curves slope vs log [apple polyphenols] in mg/ml.



**Figure 3.** Relationship between SUA change and endothelial reactivity change in subjects treated with apple polyphenols and placebo.



**Table 1** – Baseline main clinical, hematochemistry and hemodynamic characteristics of the subjects enrolled in the trial and randomized to the different treatments (active or placebo), expressed as mean  $\pm$  standard deviation. No significant difference has been observed between the groups.

<i>Parameters</i>	<i>Active</i>		<i>Parameters</i>	<i>Active</i>	
	<i>treatment</i> <i>group</i> (N=31)	<i>Placebo group</i> (N= 31)		<i>treatment</i> <i>group</i> (N=31)	<i>Placebo</i> <i>group</i> (N=31)
Age (years)	63,3 $\pm$ 7,5	63,2 $\pm$ 11,6	Alanine aminotransferase (U/L)	19,2 $\pm$ 4,3	23,3 $\pm$ 10,6
Weight (Kg)	79,0 $\pm$ 16,1	82,3 $\pm$ 14,7	Gamma-glutamyltransferase (U/L)	22,4 $\pm$ 11,5	31,1 $\pm$ 26,2
Waist circumference (cm)	95,6 $\pm$ 13,0	96,9 $\pm$ 12,9	Creatine phosphokinase (U/L)	127,8 $\pm$ 70,9	122,2 $\pm$ 85,8
Total energy intake (kcal)	2375,6 $\pm$ 160,8	2352,8 $\pm$ 142,4	Creatinine (mg/dL)	1,0 $\pm$ 0,2	1,0 $\pm$ 0,2
Fat dietary intake (% on total energy)	29,3 $\pm$ 2,1	28,4 $\pm$ 3,1	eGFR (ml/min)	89,9 $\pm$ 4,3	91,2 $\pm$ 4,8
Saturated fat intake (fat dietary intake % on total energy)	10,3 $\pm$ 0,8	10,2 $\pm$ 1,1	Serum uric acid (mg/dL)	5,0 $\pm$ 1,0	5,1 $\pm$ 1,2
Protein intake (% on total energy)	17,3 $\pm$ 1,4	18,0 $\pm$ 1,2	Systolic blood pressure (mmHg)	144,3 $\pm$ 17,8	145,15 $\pm$ 14,1
Carbohydrate intake (% on total energy)	42,3 $\pm$ 3,4	43,7 $\pm$ 3,6	Diastolic blood pressure (mmHg)	75,9 $\pm$ 8,4	75,0 $\pm$ 8,3

Cholesterol intake (mg)	189,7 ± 9,4	191,2 ± 6,4	Pulse pressure (mmHg)	68,5 ± 13,5	69,8 ± 11,5
Total cholesterol (mg/dL)	206,7 ± 33,9	191,0 ± 33,1	MAP (mmHg)	104,1 ± 11,9	104,4 ± 9,7
Triglycerides (mg/dL)	105,5 ± 41,9	119,0 ± 60,0	Heart rate (bpm)	66,7 ± 12,5	65,2 ± 11,8
HDL-cholesterol (mg/dL)	51,2 ± 9,4	46,6 ± 11,5	Aortic blood pressure (mmHg)	141,6 ± 17,0	142,0 ± 14,3
LDL-cholesterol (mg/dL)	134,3 ± 31,0	120,5 ± 29,5	Aortic pulse pressure (mmHg)	66,0 ± 13,2	67,5 ± 11,8
VLDL-cholesterol (mg/dL)	21,1 ± 8,4	23,8 ± 12,0	Augmentation pressure (mmHg)	18,1 ± 4,4	20,3 ± 9,2
Apolipoprotein A1 (mg/dL)	153,8 ± 21,6	148,6 ± 26,0	Augmentation Index (%)	28,0 ± 6,1	29,7 ± 9,9
Apolipoprotein B (mg/dL)	87,2 ± 18,4	82,5 ± 13,5	Cardiac output (L/min)	7,4 ± 2,2	7,2 ± 1,7
Fasting plasma glucose (mg/dL)	107,4 ± 8,7	103,1 ± 11,0	Peripheral resistance (PRU)	0,9 ± 0,2	0,9 ± 0,2
Aspartate aminotransferase (U/L)	20,7 ± 3,4	22,8 ± 9,3	Stroke volume (mL)	111,3 ± 23,1	112,2 ± 24,5

**Table 2** – Changes in the investigated parameters after 4 weeks of treatment.

<i>Parameters</i>	<i>Placebo treatment</i>				<i>Active treatment</i>			
	<i>Mean</i>	<i>95 % Confidence Interval</i>		<i>P vs</i> <i>baseline</i>	<i>Mean</i>	<i>95 % Confidence Interval</i>		<i>P vs</i> <i>baseline</i>
	<i>delta vs</i> <i>baseline</i>	<i>Lower</i>	<i>Upper</i>		<i>delta vs</i> <i>baseline</i>	<i>Lower</i>	<i>Upper</i>	
Weight (Kg)	-1,0	-10,5	8,6	0,969	-0,5	-10,9	9,9	0,993
Waist circumference (cm)	-0,5	-9,0	8,0	0,989	-0,2	-8,6	8,1	0,998
Total cholesterol (mg/dL)	-7,1	-29,7	15,4	0,732	-6,7	-28,9	15,4	0,748
Triglycerides (mg/dL)	-0,7	-39,4	38,0	0,999	12,6	-19,9	45,2	0,625
HDL-cholesterol (mg/dL)	-0,5	-8,4	7,4	0,988	-3,2	-9,5	3,1	0,452
LDL-cholesterol (mg/dL)	-2,5	-22,0	17,1	0,951	-6,1	-26,5	14,3	0,756
VLDL-cholesterol (mg/dL)	-0,1	-7,9	7,6	0,999	2,5	-4,0	9,0	0,625
Apolipoprotein A1 (mg/dL)	-2,9	-19,3	13,5	0,907	-0,6	-14,0	12,8	0,994
Apolipoprotein B (mg/dL)	-0,5	-14,7	13,6	0,563	1,7	-13,1	16,5	0,957
Fasting plasma glucose (mg/dL)	-0,08	-7,6	7,4	1,000	-6,6	-12,5	-0,6	0,027
Alanine aminotransferase (U/L)	0,8	-6,4	8,1	0,958	-0,2	-3,9	3,5	0,989
Aspartate aminotransferase (U/L)	0,6	-5,6	6,7	0,973	-0,4	-3,0	2,2	0,924
Gamma-glutamyltransferase (U/L)	-1,4	-18,0	15,2	0,978	-0,9	-7,7	5,8	0,943
Creatine phosphokinase (U/L)	4,5	-63,9	72,8	0,987	-0,0	-58,7	58,6	1,000
Creatinine (mg/dL)	-0,0	-0,2	0,1	0,903	-0,0	-0,1	0,1	0,758
Serum uric acid (mg/dL)	-0,1	-0,9	0,7	0,937	-0,0	-0,7	0,6	0,993
Systolic blood pressure (mmHg)	-8,3	-18,0	1,3	0,103	-4,5	-15,8	6,8	0,611
Diastolic blood pressure (mmHg)	4,8	-3,4	13,0	0,349	0,2	-5,6	6,1	0,995
Pulse pressure (mmHg)	-12,8	-24,9	-0,8	0,035	-4,7	-13,9	4,5	0,444



**Table 3** – Changes in the main parameters evaluated at the end of the intervention period (after 8 weeks of placebo or active treatment).

<i>Parameters</i>	<i>Placebo treatment</i>				<i>Active Treatment</i>				
	<i>Mean delta vs baseline</i>	<i>95 % Confidence Interval</i>		<i>P vs baseline</i>	<i>Mean delta vs baseline</i>	<i>95 % Confidence Interval</i>		<i>P vs baseline</i>	<i>P vs placebo</i>
		<i>Lower</i>	<i>Upper</i>			<i>Lower</i>	<i>Upper</i>		
Weight (Kg)	-1,5	-11,1	8,1	0,923	-1,0	-11,4	9,3	0,969	0,972
Waist circumference (cm)	-0,8	-9,3	7,6	0,972	-0,7	-9,1	7,8	0,980	0,982
Total cholesterol (mg/dL)	-5,9	-28,4	16,7	0,807	-3,4	-25,5	18,8	0,930	0,769
Triglycerides (mg/dL)	-4,3	-43,0	34,3	0,961	10,2	-22,4	42,7	0,736	0,618
HDL-cholesterol (mg/dL)	-1,9	-9,9	6,0	0,832	-2,9	-9,2	3,5	0,528	0,418
LDL-cholesterol (mg/dL)	-3,1	-22,6	16,4	0,924	-2,6	-22,9	17,8	0,952	0,774
VLDL-cholesterol (mg/dL)	-0,9	-8,6	6,9	0,961	2,0	-4,5	8,5	0,736	0,618
Apolipoprotein A1 (mg/dL)	-10,5	-27,3	6,2	0,296	-2,9	-16,9	11,1	0,873	0,874
Apolipoprotein B (mg/dL)	5,5	-8,6	19,7	0,620	8,2	-6,6	23,0	0,388	0,384
Fasting plasma glucose (mg/dL)	-0,7	-8,1	6,8	0,976	-11,1	-17,0	-5,1	<b>&lt;0,001</b>	<b>&lt;0,001</b>
Alanine aminotransferase (U/L)	-0,8	-8,1	6,5	0,962	-0,5	-4,2	3,2	0,940	0,945
Aspartate aminotransferase (U/L)	-0,6	-6,7	5,6	0,973	-0,8	-3,4	1,8	0,751	0,771
Gamma-glutamyltransferase (U/L)	-1,6	-18,2	15,0	0,971	-0,7	-7,5	6,1	0,966	0,943
Creatine phosphokinase (U/L)	17,7	-50,7	86,0	0,811	-6,8	-65,5	51,9	0,959	0,951
Creatinine (mg/dL)	-0,0	-0,1	0,1	0,932	-0,1	-0,2	0,1	0,466	0,495
Serum uric acid (mg/dL)	-0,4	-1,2	0,4	0,541	-0,7	-1,3	-0,1	<b>0,042</b>	<b>0,025</b>
Systolic blood pressure (mmHg)	-1,8	-11,4	7,9	0,900	-2,3	-12,6	9,0	0,878	0,639

Diastolic blood pressure (mmHg)	0,5	-7,7	8,7	3,422	-2,3	-8,1	3,5	0,616	0,523
Pulse pressure (mmHg)	-2,0	-14,0	10,1	0,918	-0,1	-9,3	9,1	1,000	0,380

**Table 4** – Changes in the hemodynamic parameters at the end of the intervention period (after 8 weeks of active treatment or placebo).

Parameters	Placebo treatment				Active Treatment			
	Mean delta vs baseline	95 % Confidence Interval		P vs baseline	Mean delta vs baseline	95 % Confidence Interval		P vs baseline
		Lower	Upper			Lower	Upper	
Heart rate (bpm)	-1,5	-8,2	5,3	0,666	-1,2	-7,6	4,3	0,781
ABP (mmHg)	1,2	-6,8	9,2	0,767	0,9	-8,5	10,3	0,843
APP (mmHg)	3,6	-2,7	9,8	0,257	-2,3	-9,5	5,0	0,537
AP (mmHg)	2,2	-2,5	6,9	0,342	-2,2	-5,0	-0,7	0,133
AI (%)	2,0	-3,2	7,3	0,443	-2,0	-5,8	1,7	0,282
CO (L/min)	-0,2	-1,1	0,7	0,643	-0,3	-1,6	0,9	0,578
TPR (PRU)	0,0	-0,1	0,2	0,486	0	-0,1	0,2	0,778
SV (mL)	-4,3	-20,4	11,8	0,595	-6,9	-20,8	7,0	0,321
CI (%)	-1,4	-23,8	21,0	0,901	12,1	-38,3	14,0	0,353

ABP= Aortic Blood Pressure; AIX= Augmentation index; AP= Augmentation pressure; APP= Aortic pulse pressure; CI= Cardiac Index; CO= Cardiac output; SV = Stroke Volume; TPR= Total Peripheral Resistance