A polyphenol-rich dietary pattern improves intestinal permeability, evaluated as serum zonulin levels, in older subjects: the MaPLE randomised controlled trial

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ABSTRACT

Background & aim: Increased intestinal permeability (IP) can occur in older people and contribute to the activation of the immune system and inflammation. Dietary interventions may represent a potential strategy to reduce IP. In this regard, specific food bioactives such as polyphenols have been proposed as potential IP modulator due to their ability to affect several critical targets and pathways that control IP.

The trial aimed to test the hypothesis that a polyphenol-rich dietary pattern can decrease serum zonulin levels, an IP surrogate marker involved in tight junction modulation, and can beneficially alter the intestinal microbiota, and IP-associated biochemical and clinical markers in older subjects.

Methods: A randomised, controlled, cross-over intervention trial was performed. Sixty-six subjects (aged ≥ 60 y) with increased IP based on serum zonulin levels, were randomly allocated to one of the two arms of the intervention consisting of a control diet (C-diet) *vs. a* polyphenol-rich diet (PR-diet). Each intervention was 8-week long and separated by an 8-week wash out period. At the beginning and at the end of each intervention period, serum samples were collected for the quantification of zonulin and other biological markers. Faecal samples were also collected to investigate the intestinal microbial ecosystem. In addition, anthropometrical/physical/biochemical parameters and food intake were evaluated.

Results: Fifty-one subjects successfully completed the intervention and a high compliance to the dietary protocols was demonstrated. Overall, polyphenol intake significantly increased from a mean of 812 mg/day in the C diet to 1391 mg/day in the PR-diet. Two-way analysis of variance showed a significant effect of treatment ($p = 0.008$) and treatment *x* time interaction ($p = 0.025$) on serum zonulin levels, which decreased after the 8-week PR-diet. In addition, a treatment *x* time interaction was observed showing a reduction of diastolic blood pressure ($p = 0.028$) following the PR-diet, which was strongest in those not using antihypertensive drugs. A decrease in both diastolic ($p = 0.043$) and systolic blood pressure ($p = 0.042$) was observed in women.

A trend towards a reduction of total cholesterol was observed (time effect, $p = 0.039$) following both interventions. Interestingly, a significant increase in fiber-fermenting and butyrate-producing bacteria such as the family Ruminococcaceae and members of the genus *Faecalibacterium* was observed following the PR intervention.

The efficacy of this dietary intervention was greater in subjects with higher serum zonulin at baseline, who showed more pronounced alterations in the markers under study. Furthermore, zonulin reduction was also stronger among subjects with higher body mass index and with insulin resistance at baseline, thus demonstrating the close interplay between IP and metabolic features.

Conclusions: These data show, for the first time, that a PR-diet can reduce serum zonulin levels, an indirect marker of IP. These findings may represent an initial breakthrough for further intervention studies evaluating possible dietary treatments for the management of IP in different target populations. This study was registered at www.isrctn.org as ISRCTN10214981

Keywords: Leaky gut; gut barrier function; inflammation; flavonoids; phenolics; ageing

1. Introduction

The integrity of the intestinal barrier is fundamental for gut and human health. This barrier is maintained thanks to the active involvement of “tight junctions”, in which multiprotein complexes serve to seal the junctions between epithelial cells. Tight junctions control mucosal permeability and act as intermediates/transducers in cell signalling cascades [1]. The layer of epithelial cells represents a physical barrier against external factors, including microbial factors, while maintaining a controlled crosstalk with commensal bacteria [2]. The disruption of the junctions between epithelial cells results in increased intestinal permeability (IP), also known as “leaky gut”. It enables the translocation of microorganisms and/or microbial factors from the intestinal lumen to the blood stream, leading to the activation of immune function and inflammation [3]. Increased IP has been proposed as a potential contributor to a wide range of intestinal disorders such as irritable bowel syndrome, and inflammatory bowel and coeliac diseases. More recently, increased IP has also been proposed as a potential cause of age-related conditions [4]. In fact, age has reported as an independent risk factor for altered IP [5], and some studies have shown an increased IP over the age of 50 y due to a potential progressive process of deterioration in the functions and integrity of the intestinal barrier [5]. During aging, an increased IP may contribute to the onset of chronic low-grade inflammation, also known as inflamm-aging [6,7], responsible for the higher risk of several age-related diseases including metabolic syndrome, obesity, diabetes and cardiovascular diseases. Gut microbiota seems to play a central role in driving inflamm-aging, as it can release several inflammatory factors, and contribute to IP (dys)regulation [8,9]. For example, gut microorganisms may act directly on IP by affecting tight junction functionality and/or indirectly by modulating inflammation [4]. Consequently, the manipulation of gut microbiota has been proposed as a potential novel strategy to improve IP. Dietary patterns and specific food bioactives are considered important factors capable to manipulate and shape gut microbiota, which can positively or negatively affect IP. Recent studies discussed the role of several macro and micronutrients in the modulation of IP. The results highlighted that an excessive energy intake, high-fat, high-sugar and high-animal protein consumption, as well as alcohol intake are associated with an alteration of the

intestinal microbial ecosystem and an increased IP [10-13]. Moreover, inadequate nutrient intake (e.g. low protein intake) that often occurs in older subjects can contribute to increase IP [4]. Conversely, diets rich in low-energy dense foods (e.g. fruits and vegetables) and fibres have been associated with a healthier gut microbiota and a reduced IP [14]. In the context of a diet-microbiota-IP axis, several food bioactives, including polyphenols, may represent a potential strategy to positively affect microbiota composition and to improve IP and related conditions [15]. Polyphenol biological functions include antioxidant and anti-inflammatory properties, and immunomodulatory activity at both intestinal and systemic levels [2]. Although the exact molecular mechanisms are not completely understood, polyphenols may directly and/or indirectly act at different levels of the intestinal barrier by regulating tight junction function, the production of numerous inflammatory cytokines and the activation of antioxidant genes [2]. Furthermore, polyphenols undergo extensive modifications by the gut microbiota and, consequently, affect the intestinal microbial ecosystem. For such reasons, polyphenols are promising candidates for developing dietary intervention strategies to counteract the detrimental effects of elevated IP.

The evaluation of IP in human subjects is challenging. Zonulin, also known as prehaptoglobin-2, has been suggested as a candidate marker; it is a 47-kDa protein produced mainly by epithelial cells (e.g. in the gut) that is able to reversibly modulate paracellular permeability [16]. In fact, zonulin is a fundamental regulator of intercellular junctions since it can bind the epidermal growth factor receptor through the activation of protease-activated receptor 2. The derived complex induces the signalling pathway causing tight junction disassembly (induced by the phosphorylation of zonula occludens proteins) thus enabling the paracellular passage of factors between the luminal environment and the inner part of the mucosa. For this reason, zonulin has been considered as a good surrogate marker of impaired intestinal barrier function and increased IP, and has been shown to be involved in different physiological and pathological conditions [17]. Moreover, several studies have reported correlations between the results obtained from the most common and validated IP test (based on lactulose/mannitol urine excretion evaluation following standardised sugar intake; the ‘multi-sugar assay’) and serum

zonulin levels [18-20]; while such correlation was not reported considering lactulose/rhamnose urine excrection in healthy adults [21].

To the best of our knowledge, human intervention studies aimed at investigating the role of polyphenols in the modulation of IP are still lacking. Within this context, the MaPLE (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly) randomised, controlled, crossover trial was designed to assess whether a high intake of polyphenol-rich foods in older subjects would modulate the intestinal microbial ecosystem, reduce serum zonulin levels as indirect marker of IP and improve markers of inflammation, oxidative stress and vascular function.

2. Materials and Methods

2.1 Setting and subjects' recruitment

The MaPLE trial was carried out at Civitas Vitae (OIC Foundation, Padua, Italy), an institution including residential care and independent residences for older subjects. The setting was selected in order to enable a significant control of most of the experimental variables affecting dietary intervention studies as previously described [22]. Subjects selection was performed in collaboration with physicians and staff at OIC Foundation, based on medical examination and the evaluation of drug therapies. The final eligibility was defined according to the inclusion and exclusion criteria reported below.

To be included in the trial, the subjects had to be ≥ 60 years old, with an adequate nutritional status, a good cognitive status, good functional autonomy, and with an increased IP evaluated as serum zonulin level concentrations by considering reference values and other literature as previously detailed [17-18; 22-23]. Exclusion criteria included: having Celiac disease, advanced stage of chronic diseases such as cirrhosis, renal insufficiency (dialysis), severe Chronic Obstructive Pulmonary Disease (COPD) or severe cardiovascular disease (heart failure class III or IV NYHA - New York Heart Association). Moreover, subjects with malignant tumours that required treatment in the previous 2 years were excluded as well as those treated with antibiotics in the last month before the intervention period.

The entire process of subject selection and randomization within the clinical trial is reported in **Figure 1**. The study protocol complied with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the University of Milan, Italy (ref: 6/16/CE_15.02.16_Verbale_All-7). All participants were informed about the study protocol and they signed an informed consent before the enrolment. The trial was registered under ISRCTN.com (ISRCTN10214981).

2.2 Definition and set up of the dietary intervention

The dietary intervention protocol was developed following an initial evaluation of the nutrient composition (through MetaDieta® software by Me.Te.Da S.r.l., San Benedetto del Tronto, Italy) and total polyphenol content (mainly through Phenol-Explorer.eu database) of the daily menu provided by OIC Foundation to the host. The development of the polyphenol-rich (PR) dietary pattern was designed by the substitution of some low-polyphenol products in the control diet (C-diet) with other comparable PR-products (e.g. foods used for snack or breakfast) and maintaining as much as possible the overall energy and nutrient composition. Specifically, subjects consumed three small portions per day of the following selected PR-foods: berries and related products, blood orange and juice, pomegranate juice, green tea, Renetta apple and purée, and dark chocolate (callets and cocoa powder-based drink), which provided a mean of 724 mg/day of total polyphenols estimated by Folin-Ciocalteu analysis [24]. Thus, the total polyphenol intake in the intervention diet, i.e. including the menu plus the PR-foods, was roughly doubled compared to the C-diet.

A schematic plan of the type and serving sizes of PR-foods consumed daily within the intervention has been reported previously [22].

2.3 Experimental design

The trial consisted of an 8-week, randomised, repeated measure cross-over intervention study (i.e. PR-diet *vs* C-diet). Volunteers were randomly allocated in one of the two arms of the intervention starting with PR-diet or C-diet according to a computerized randomization protocol [22]. Subjects assigned to the PR-diet received the 3-daily portions of selected PR-products described before. During the C-diet

period, subjects followed the regular menus provided by the nursing home that were previously evaluated for their nutritional composition. After a wash-out period (8 weeks) performed to avoid any carry-over effect, the groups were switched to the other treatment.

At the beginning and at the end of each intervention periods all participants underwent to physical and general condition examinations (i.e. height, weight, blood pressure and clinical signs). In addition, biological samples (blood and faeces) were collected for the analysis of metabolic and functional markers and microbial ecosystem.

2.4 Compliance

To ensure adequate compliance to the dietary intervention protocol PR-rich foods, that were in part or completely not consumed, were registered at the end of each day. In addition, weighted food diaries were filled in during the trial to assess the adherence to both dietary treatments (PR- and C- diet) [21].

2.5 Anthropometrical and physical evaluations

Height and weight were measured according to Lohman et al international guidelines [25]; body mass index (BMI) was calculated according to the formula – weight (kg)/height (m^2). Reference scores were defined according to international guidelines [25]. Blood pressure was obtained in resting, seated position following the JNC 7 guidelines [26].

2.6 Blood sampling and analysis

After an overnight fast, blood samples were drawn in Vacutainer tubes containing silicon gel for serum and maintained at room temperature for at least 30 min. Serum was then obtained by tube centrifugation (1400 g x 15 min, 4°C), splitted in small aliquots into specific vials and stored at -80°C until analysis. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood by density gradient centrifugation with Histopaque 1077 [27] and cryopreserved at -80°C in a media (50% fetal bovin

serum, 40% RPMI-1640, 10% DMSO) until analysis. Samples were used for the evaluation of several metabolic and functional parameters [22].

In particular, glucose, insulin, lipid profile (total cholesterol, triglycerides), liver and renal function (i.e. aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, creatinine) were analysed using a standardized routine-use automatic biochemical analyser (ILAB 650, Instrumentation Laboratory, Lexington, MA). Serum concentration of low-density lipoprotein cholesterol (LDL-C) and non-high-density lipoprotein cholesterol (HDL-C) were estimated by using the Friedewald formula [28] and by subtracting HDL-C from total cholesterol (TC), respectively. In addition, the homeostasis model assessment of insulin resistance (HOMA-IR) was performed, and values > 3 were considered as a criterion for insulin resistance [29]. The Cockroft-Gault (C-G) index based on creatinine clearance was calculated according to the formula previously defined in literature [30,31].

2.7 Evaluation of IP

Serum samples for IP evaluation (at recruitment and at each time point of intervention) were defrosted at room temperature and the serum zonulin levels were quantified using the Immunodiagnostik® ELISA kit (Bensheim, Germany). The assay, based on a competitive Elisa method, entails the addition to each sample (including standard and control samples) of a biotinylate zonulin tracer and the subsequent use of a pre-coated 96-well plate with polyclonal anti-zonulin antibody. Peroxidase-labelled streptavidin addition was used to bind the biotinylate zonulin tracer and after the reaction, a plate reader (TECAN Infinite F200, Tecan Group Ltd. Mannedorf, Switzerland) was used to read the fluorescence at 450 nm. Serum zonulin concentrations were quantified using a standard curve calculated by a 4-parameter algorithm as reported by the manufacturer.

2.8 Evaluation of inflammatory markers

C-reactive protein (CRP), tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) levels were quantified in serum at the beginning and the end of each intervention period using specific ELISA kits (DCRP00, HSTA00E, and HS600B, respectively; R&D Systems, Biotechne, Abingdon, UK).

2.9 Evaluation of vascular markers

Serum samples at each time point were used to quantify vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) using an ELISA kit (Booster® from Vinci Biochem S.r.l., Vinci, Italy). After competitive treatment with antibodies and fluorophore, fluorescence was quantified using a TECAN Infinite F200 plate reader. A 4-parameter algorithm was used to create the standard curve and to calculate serum concentrations.

2.10 Evaluation of oxidative stress markers

The levels of endogenous and oxidatively-induced DNA damage were evaluated in PBMCs using the comet assay. Endogenous DNA damage was determined by using a specific endonuclease (formamidopyrimidine DNA glycosylase, FPG sensitive sites) able to detect 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases, while the levels of oxidatively-induced DNA damage were measured by exposing cells to hydrogen peroxide and measuring their capacity to counteract an oxidative insult. The full protocols of comet assay have been previously published [27].

2.11 Evaluation of faecal bacterial community structure

The intestinal microbiota of volunteers was assessed by 16S rRNA gene profiling. In brief, DNA isolation and amplicon sequencing were carried out as previously described [22]. In brief, DNA was isolated from faeces resuspended in Lysing Matrix E bead beating tubes (MPBio, Santa Ana, CA, USA) through the FastDNA™ SPIN Kit for Soil (MPBio) according to the manufacturer's protocol. Then, the V3-V4 region of the 16S rRNA gene was amplified with panbacterial primers 16S 341F (5'–

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3') and 16S 806R (5'–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC–3'). Finally, amplicons were sequenced using a 600 cycle MiSeq v3 reagent kit (Illumina, San Diego, CA, USA).

Subsequently, sequencing reads were subjected to pairing, filtering, taxonomic assignment, and biodiversity analyses by means of the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) 2 version 2020.6 [32] through the Devisive Amplicon Denoising Algorithm (DADA2) using the Greengenes database (version 13_5). Illumina sequencing generated 4,030,722 filtered paired-end reads (median of 19,328 reads per sample). After merging and denoising by DADA2 the final sequences were 1,076,356 (mean=5,021, SD=3,306). The sequence length statistics in bp showed: min= 240, max=457, median=433, standard deviation=25. Overall, 7,729 unique amplicon sequence variants (ASVs) were identified.

2.12 Statistical analysis

Sample size was calculated based on previous published data [26,33]. It was estimated that 50 subjects were needed to detect a 30% decrease in plasma zonulin with 80% power and alpha=0.05 with an estimated drop-out rate of 15%.

Differences between treatments were computed by ANOVA for repeated measures design (using the least significant difference (LSD) test as a post hoc analysis to evaluate differences among means). In addition, although a relatively high zonulin level was used as an inclusion criterion [22], we were interested to determine whether the response to dietary treatments would be different in subjects stratified with respect to median serum zonulin levels at baseline, as it was also reported in recent publications [9,10]. Specifically, subjects were stratified in two groups: LSZ group (lower serum zonulin levels; i.e. ≤ median value) and HSZ group (higher serum zonulin levels; i.e. > median level). The regression and correlation analyses (Spearman and Kendal test) were carried out to highlight

associations between zonulin levels (HSZ *vs* LSZ) and physiological and biochemical parameters. In addition, a further statistical analysis in which subjects were stratified in two groups based on median values for BMI and HOMA-index was performed in order to investigate the contribution of metabolic characteristics on IP and related markers. Potential gender differences were also considered in the analyses.

To identify the bacterial taxa that significantly changed over the trial, taxonomic abundance data were normalized with the negative binomial distribution method (R/Bioconductor DESeq2 package) and statistically analysed using ANOVA for repeated measures design.

Significance was set at $p \leq 0.05$. P values in the range $0.05 < p < 0.10$ were considered as trends. All analyses were performed using the R statistic software version 3.4.2.

3. Results

3.1 Recruitment phase workflow

Of the initial 491 older subjects considered, 349 were excluded after evaluation by OIC physicians since they did not meet the inclusion criteria and 70 subjects declared not to be interested into participate for personal reasons. A total of 72 subjects were further screened and 3 subjects were excluded for low serum zonulin levels. Difficulty in drawing blood was the reason for excluding others 3 subjects.

Finally, 66 subjects (27 men, 39 women) were enrolled in the trial, but only 51 subjects completed the entire intervention study. A schematic flowchart of the protocol, reporting all the information from the recruitment until the end of the study, is shown in **Figure 1**.

3.2 Baseline characteristics of the participants

The main characteristics at baseline of the 51 subjects who completed the study protocol are provided in **Table 1**, while this data stratified by sex is shown in **Table 1A** (supplementary material). Participant ages ranged between 60 and 98 years with a median value of 77 years. Overall, a high inter-individual variability was observed for several markers and in particular BMI (IQR: 22.5;30.7), glucose (IQR: 86;113) and total cholesterol levels (IQR 167;242). Age distribution and data obtained for most of the variables under evaluation were comparable in men and women. HDL-C and insulin levels were significantly higher in women with respect to men ($p = 0.03$ and $p = 0.007$ respectively).

3.3 Correlation analysis of subjects' characteristics based on HSZ or LSZ at baseline

Serum zonulin levels were positively correlated with creatinine ($p = 0.033$) and triglycerides ($p = 0.004$) considering all participants (**Figure 2A**). However, the correlation of zonulin with creatinine clearance (C-G index) was not significant. A positive correlation was observed among inflammatory markers (i.e. IL-6, TNF-α, CRP); in addition, a positive correlation emerged between CRP levels and BMI ($p = 0.021$), and between TNF-α and TG ($p = 0.0009$) (**Figure 2A**).

When subjects were stratified according to high *versus* low serum zonulin levels at baseline, the HSZ group showed a positive correlation between zonulin and HOMA index ($p = 0.037$), and creatinine ($p = 0.025$) (**Figure 2B**). This last correlation was not confirmed when C-G index was used. Regarding LSZ subjects, no significant correlation was observed between serum zonulin levels and the other markers under study (**Figure 2C**).

3.4 Compliance to the dietary intervention

The nutrient composition of the diet consumed by participants during both treatment periods is reported in **Table 2**. A comparable pattern of food consumption was evidenced, except for the PR-products provided in the PR-diet. Energy and overall composition of the diet did not differ in the two periods of intervention (PR-diet *vs* C-diet). Following the PR-diet a small decrease in animal proteins and lipids and an increase in carbohydrates and fibre intake (less than 1 g as a mean) was observed with respect

to the C-diet. Overall, a high adherence to the dietary protocol was registered: the subjects accepted and easily consumed all the PR-products provided daily and no adverse effects were reported. On average, subjects increased their total polyphenol intake by approximately 70% during the PR-diet compared to the C-diet (**Table 2**).

3.5 Effect of dietary interventions on markers under study

Table 3 shows the results concerning anthropometrical and physical characteristics, biochemical, inflammatory, vascular and oxidative stress markers evaluated before and after each treatment.

A treatment *x* time interaction was observed for diastolic blood pressure ($p = 0.024$) and uric acid levels ($p = 0.034$). Post hoc analysis highlighted a decrease of uric acid by 5.2% following the C-diet and a significant reduction in DBP of 3.8% (-2.9 mmHg) following the PR-diet intervention. It is noteworthy that the analysis of data stratified based on anti-hypertensive treatment showed a significant effect of the PR-diet only in the group not taking drugs ($p<0.05$).

Overall, body weight and BMI measured along the study resulted different in the two treatment periods ($p = 0.023$ and $p = 0.017$, respectively) being slightly lower (-0.4 %) during C-diet intervention. Finally, a time effect ($p = 0.039$) was observed for TC with a trend towards reduction following both interventions (-2.8 % and -1.8 % respectively; -5.4 and -3.5 mg/dL). No significant effect was found for all the remaining variables.

Considering sex (**Table 3A and 3B, supplementary material**), a significant time effect was observed within men for TC ($p = 0.003$), LDL-C ($p = 0.020$) and the ratio TC/HDL-C ($p = 0.039$), LSD test showed a significant reduction after the PR-diet (by about -7.5 %, -7.6 % and -6 % respectively; i.e. -14, -8.9 and -0.26 mg/dL) but not the control diet. A significant treatment effect was found for AST ($p = 0.042$) and CRP ($p = 0.032$) that showed a trend towards a reduction following both interventions.

Regarding women, a treatment *x* time interaction was evidenced for SBP ($p = 0.042$) and DBP ($p = 0.043$) showing a reduction after the PR-diet (-3.8 % and -3.9 % i.e. -4.8 and -2.9 mmHg, respectively), but not after the C-diet. A significant effect of treatment was observed for triglycerides ($p = 0.030$).

3.6 Effect of intervention on IP and related markers

In **Table 3** are reported the results on serum zonulin levels before and after each treatment. A significant treatment ($p = 0.008$) and treatment *x* time interaction ($p = 0.025$) was observed showing a decrease in serum zonulin levels (-6.9%) after the PR-diet. After stratifying by gender (**Table 3A and 3B, supplementary material)**, significant treatment and treatment *x* time interaction ($p = 0.004$ and $p = 0.010$ respectively) were detected for women but not for men.

The analysis of data based on HSZ or LSZ highlighted the importance of baseline zonulin level as a significant contributor to the impact of the dietary intervention. In fact, HSZ subjects were those with the higher IP reduction ($p = 0.026$) following PR-diet (i.e. -14%) and a significant decrease of DBP ($p = 0.01$; i.e. -4.6%), glucose levels ($p = 0.049$; i.e. -10.9%) and a trend towards a reduction of IL-6 ($p = 0.097$; i.e. -19%); conversely a significant reduction ($p = 0.03$; i.e. -8.1%) in uric acid levels was found after C-diet (data not shown).

After stratifying subjects by BMI, a significant reduction of zonulin levels ($p = 0.007$) and DBP ($p = 0.024$) was observed after PR diet in the group with BMI higher than the median value. Additionally, a significant increase in IL-6 serum levels ($p = 0.049$) and decrease in uric acid levels ($p = 0.027$) was found during the C-diet (data not shown).

Similarly, by considering HOMA-index (i.e. higher *vs.* lower depending on median basal values) as stratification factor, a significant reduction of serum zonulin levels ($p = 0.027$) and DBP ($p = 0.013$) following the PR-diet and a decrease ($p = 0.027$) in uric acid after C-diet was observed (data not shown).

3.7 Effect of intervention on faecal bacterial community structure

The dietary interventions did not significantly affect the α- and β-diversity of the bacterial community structure within the faecal samples (data not shown). On the contrary, repeated measure ANOVA revealed a significant treatment *x* time interaction for 12 taxonomic units. In specific, 8 taxa belonging

to the order Clostridiales (1 family, 3 genera, and 4 ASVs) and two Bacteroidales (1 genus and 1 ASV) were found to be significantly increased after the PR-diet, whereas only two ASVs, ascribed to *Bacteroides uniformis* and *Streptococcus agalactiae* (p = 0.026 and p = 0.034, respectively) were reduced (**Table 4**). Notably, the most abundant bacterial significantly changed during the trial was the Clostridiales family Ruminococcaceae (mean relative abundance of 21 %; p = 0.049). Within this family, the genus of butyrate producing *Butyricicocci* (P=0.049) and *Faecalibacterium prausnitzii* (3 ASVs; p = 0.022, p = 0.035 and p = 0.049, respectively) were found to be significantly increased (**Table 4**). Finally, the relative abundance of two members of the Clostridiales family Lachnospiraceae was found to be significantly increased: the genus *Lactonifactor* (p = 0.040) and an ASV ascribed to the species Anaerobutyricum hallii (p = 0.018). The members of the Ruminococcaceae and Lachnospiraceae families were the most influenced by the dietary intervention also when subjects were stratified according to zonulin levels in both the LSZ and HSZ group (data not shown). On the contrary, after stratification, the Bacteroidetes taxa did not show any significant change with the only exception of the same ASV ascribed to *Bacteroides umilis* mentioned above, which was confirmed to be significantly decreased after the PR-diet in the LSZ group (p = 0.037).

4. Discussion

In this study, we have shown that modifying the diet of older subjects by including small portions of PR-products can reduce serum zonulin concentrations, a widely recognised surrogate marker of IP. Interestingly, greater reductions in serum zonulin concentrations following the PR-diet were observed in the HSZ sub-group, which was accompanied by decreases in diastolic blood pressure, glucose and IL-6 levels (even if the latter was not statistically significant). This supports the notion that the efficacy of PR-diet could depend on the baseline IP condition.

Increased serum zonulin levels and impaired IP condition have been previously found in individuals with metabolic disorders, such as diabetes and obesity [19]. In this regard, we documented a significant association between serum zonulin levels and HOMA index at baseline in subjects classified in the

HSZ group but not in the LSZ group suggesting an important contribution of zonulin in discriminating subjects suffering metabolic dysregulation [19].

Similarly, we observed a more pronounced IP reduction after the PR-diet in subjects with higher BMI and HOMA index at baseline, which supports the hypothesis of a link between IP and metabolic disorders.

Previous studies have also reported that leaky gut can play a significant role in age-related inflammation and frailty. Interestingly, Qi et al [34] found, in a preliminary exploratory study, higher serum zonulin levels in older subjects with respect to young ones. Moreover, a positive association between zonulin levels and markers of inflammation (TNF-α, IL-6) was shown, and an inverse one with physical performance (muscle strength and steps/day). In another study, higher levels of zonulin were associated with gastrointestinal symptoms and psychological distress suggesting the contribution of IP to these signs that are frequently found in the older population [35].

It has been suggested that increased serum zonulin levels also reflect the host response to an inflammatory process, suggesting that a two-way interaction can be present between inflammation and IP [36]. This is also supported by the observation of increased IP in most of the inflammation-related diseases both at intestinal (e.g. inflammatory bowel disease, irritable bowel syndrome, celiac disease) and systemic levels (e.g. obesity, type 2-diabetes) including the age-related low-grade systemic inflammation [37].

The study of the inflammatory state is complex, because each of the available inflammatory markers provide different information on a multifaceted process that is dependent on the triggers and is modulated by both the host and environmental conditions. One of the most used markers is the C-reactive protein (CRP) which is considered a hallmark for inflammation and a sensitive risk factor for cardiovascular diseases. CRP is one of the major acute proteins phase reactants secreted in response to increased levels of inflammatory cytokines such as IL-6, interleukin-1β and TNF-α. High levels of serum CRP, IL-6 and TNF-α have been reported in smokers, obese subjects, diabetics and older adults [38]. In our experimental conditions, we documented that the 8-week intervention with the PR-diet

failed to modulate inflammatory markers, in line with other intervention studies with polyphenol-rich foods both in adults and older individuals [39-44]. The lack of effect we observed could be for a number of reasons: the large inter-individual variability in the concentrations of the inflammatory markers reducing the possibility to demonstrate a significant reduction; the high baseline levels registered for inflammatory markers, or an insufficient modulatory effect of the diet. As far as the latter is concerned, it is not excluded that higher daily intakes of polyphenols and/or longer dietary intervention periods could cause a bigger effect on these biomarkers. In fact, several clinical trials providing tart cherry juice, supplements of resveratrol, freeze-dried strawberries, purée and dried bilberries or juice for different time periods (from 4 to 26 weeks of intervention) observed an effect on inflammation strictly dependent on the markers analysed, the trial characteristics and the target subjects considered [45-50]. It is well known that oxidative stress increases with age and some reported meta-analyses have shown an association between age and DNA damage in humans [51]. High polyphenol intake has been inversely associated with a reduced risk of oxidative stress, cardiovascular events and mortality [52], possibly by decreasing the levels of reactive oxygen species, adhesion molecules or by inducing the production of vasodilators [53]. In the present study, we could not demonstrate an effect of the PR dietary pattern on DNA damage evaluated both as FPG sensitive sites and protection from H_2O_2-induced strand breaks. These results are in contrast with our previous observations [39] and with the findings reported in other human trials, and summarized in a recent systematic review [54], showing a reduction of endogenous and oxidatively-induced DNA damage following polyphenols and polyphenol-rich food intervention. We may speculate that the different results could be ascribable to the type of target population under study i.e. the older subjects and/or the sample size and the extent and duration of the intervention. The same could be hypothesized for the lack of effect of the dietary intervention on ICAM-1 and VCAM-1 levels and similar results are present in literature [39-41]. As previously reported for inflammatory markers, we found a large inter-individual variability for the vascular markers and this may have precluded the observation of significant effects of the treatment versus control diet. Furthermore, it is noteworthy that consistent with the data reported here, previously

studies have described elevated levels of both VCAM-1 and ICAM-1 in older compared to younger individuals [55,56].

The aging process is not only associated with a physiological alteration of blood vessels and vascular function but also with increasing systolic blood pressure. Therefore, hypertension, in particular systolic hypertension is very common in older subjects representing a major risk factor for cardiovascular disease and strokes [57]. Data from the literature suggest a potential role of polyphenols and polyphenol-rich foods in the modulation of blood pressure [58]. In the present study, most of the subjects showed normal blood pressure levels or a mild hypertension treated with drugs [59]. The PR-diet intervention significantly reduced diastolic blood pressure in both men and women and this effect was strongest in the group of subjects not taking anti-hypertensive drugs (mainly women) but not in those taking anti-hypertensive drugs. However, it is possible that the relatively small number of subjects taking hypertensive drugs (n=19) prevented us from observing a significant treatment effect. In addition, we found a significant reduction in systolic blood pressure in women, but not in men. The impact of gender on the response of blood pressure to treatments has been recently reviewed, and the role of the kidneys, the renin-angiotensin system, relaxin, and developmental programming highlighted as potential contributors to the differences observed [60].

Aging is associated with numerous physiological dysfunctions at cellular and tissue levels, including deregulation of lipids and glucose metabolism. Dietary polyphenols seem to play a role in the regulation of glucose homeostasis, insulin sensitivity and lipid metabolism [52,61,62]. In our study the PR-diet did not modify glucose and lipid parameters, apart from a reduction trend in total and LDL-C. Similar findings were observed for tea and tea extracts [63-65], orange juice/hesperidin [66], pomegranate [61,62] and different fruit juices [67]. On the contrary, beneficial effects were documented following the consumption of cocoa products, dark chocolate, and flavan-3-ols [68-70], berries [71,72] and black cumin [73,74]. Nevertheless, despite the overall lack of significant effect of the PR-diet on metabolic features of the host, the degree of IP at baseline was found to affect the impact of the treatment on glucose levels, which was significantly reduced only in the HSZ group, as

previously discussed. It is also interesting that a decrease in TC and LDL-CHOL was only found in men together with a significant decrease in CRP levels. However, the small sample size may represent a limitation not enabling a strong emphasis on a potential gender specific response to the dietary treatment.

This study also suggested that the intestinal bacterial community structure, which has been reported to change with aging [75] and may influence IP [4], can be positively affected by the PR-diet. In fact, we observed a significant increase of *Anaerobutyricum hallii*, *Butyricicoccus* spp. and *Faecalibacterium prausnitzii*, which are fibre-fermenting human gut commensal bacteria that produce as the main end-product of their catabolism, butyrate, a short chain fatty acid of pivotal importance for intestinal homeostasis, and whose production by the gut microbiota was shown to be lower in older adults [76]. Notably, we found an increase in three taxonomic units belonging to *F. prausnitzii*, a dominant member of the healthy human large intestine, which (a) has been negatively correlated to inflammatory bowel disease and colorectal cancer in numerous reported studies [77], (b) possesses the ability to counteract inflammatory processes [78], and (c) has been proposed as a live biotherapeutic agent to promote intestinal health [79]. Interestingly, after the intervention with the PR-diet, we also observed a significant increase of the genus *Lactonifactor*, a member of the *Lachnospiraceae* family that includes bacteria reported to be involved in the conversion of dietary phytoestrogens such as the plant lignan secoisolariciresinol diglucoside into bioactive forms within the human gut [80,81]. Finally, we also observed an increase of *Alistipes*, a relatively new genus of the phylum Bacteroidetes that includes succinate producing bacteria whose role in human health is unclear due to contradictory outcomes from previously reported studies [82].

The only taxonomic units that were significantly reduced after the PR-diet were ascribed to *Bacteroides uniformis* and *Streptococcus agalactiae*. These changes may be considered potentially beneficial for the intestinal microbiota of the older subjects. In fact, *S. agalactiae* (Group B streptococci) is a common constituent of the intestinal microbiota that possesses highly invasive and inflammatory potential, often reported to cause sepsis in infants and in older people [83]. On the other hand, *B. uniformis* has been

reported to be increased in the gut of breast-fed infants compared to non-breast-fed [84] and one strain of this species (named CECT 7771) has been recently proposed as a potential next generation probiotic [85].

Overall, the main outcome of the MaPLE RCT was to generate evidence that supports the notion that IP reduction can be achieved by increasing the daily intake of polyphenol-rich food sources, and that dietary interventions have real potential as strategies to improve IP status in older populations. It is noteworthy that only limited research has been carried out to investigate the efficacy of dietary treatments in the management of IP [2], and only in one recently published report were both healthy adults and older subjects considered as target populations. In this study, Wilms et al. [86] reported no significant effects of a dietary fibre (sugar beet pectins) on multiple IP parameters, it is tempting to speculate that the effects of the MaPLE treatment diet were due, as hypothesized, to the polyphenols. However, the various other differences between the trials must also be considered, for example the MaPLE trial PR products contained several different sources/types of fiber (not just a single-source pectin), the trial durations and control of diets were different, and there were differences in subject characteristics. Nevertheless, since older subjects are generally low consumers of dietary fibre, and there are frequently reasons why it is difficult to increase their intakes, the evidence reported here that supports the idea that non-fiber dietary interventions can be effective has real potential to be exploited in the development of foods, diets and dietary advice that has beneficial effects on the maintenance of host functional and metabolic homeostasis.

The MaPLE study has several strengths including the well-controlled protocol of intervention including the setting, the daily preparation of products and the continuous interaction with the participants. On the other hand, it has also some limitations related mainly to the relatively small sample size. Furthermore, the evaluation of IP using also the gold standard method (i.e. multi-sugar test, difficult to apply in the population under study) or multiple IP markers could have provided a better insight on the impact of the diet on this condition. This is an important aspect that deserves future investigation in order to support our observation obtained through the analysis of serum zonulin levels.

5. Conclusions

In conclusion, the MaPLE RCT has demonstrated the feasibility and efficacy of a PR dietary pattern, providing approximately 700 mg of total polyphenols daily for 8 weeks, in the reduction of serum zonulin levels, as a marker of IP, and on some associated biomarkers. These results are novel and have potentially important clinical implications. Further intervention studies should be performed aimed at investigating the role of non-pharmacological dietary treatments in the management of IP.

Authors' contributions

PR and SG designed the trial and in collaboration with AC, CAL and PAK optimised the study protocol including the selection of clinical and biochemical markers and the development of the polyphenol-rich diet. CDB contributed to the development of the study protocol and with PR, SG and SB drafted the first version of the manuscript. CDB and SB performed the analysis of zonulin, VCAM-1 and ICAM-1. BK and MSW performed the evaluation of inflammatory markers. BK, GG, SG and PAK carried out the analysis of the faecal microbiota, while MM and LG analysed and elaborated DNA damage supervised by CDB and PR. GG performed the statistical analysis in collaboration with RGD and GP. MP, NHL and RZR contributed to the elaboration of dietary polyphenol intake. All the authors critically revised the draft and approved the final version.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was completed as part of the MAPLE project (Gut and Blood Microbiomics for Studying the Effect of a Polyphenol-Rich Dietary Pattern on Intestinal Permeability in the Elderly) supported within the European Joint Programming Initiative “A Healthy Diet for a Healthy Life” (JPI HDHL, http:// www.healthydietforhealthylife.eu/) granted by Mipaaf (Italy, D.M. 8245/7303/ 2016), MINECO

(Spain, PCIN-2015-238), and BBSRC (U.K., BB/R012512/1). Cristina Andres-Lacueva was also supported by grant 2017SGR1546 from the Generalitat de Catalunya's Agency AGAUR, funds from CIBERFES (co-funded by the FEDER Program from the European Union), and ICREA Academia Award 2018. Additional funding was provided by the Biotechnology and Biological Sciences Research Council (UK) through an Institute Strategic Programme Grant ('Food Innovation and Health'; Grant No. BB/R012512/1 1 and its constituent projects BBS/E/F/000PR10343 (Theme 1, Food Innovation) and BBS/E/F/000PR10346 (Theme 3, Digestion and Fermentation in the Lower GI Tract) to the Quadram Institute Bioscience. Raúl González-Domínguez thanks the "Juan de la Cierva" program from MINECO (FJCI-2015-26590). Raul Zamora-Ros was supported by the "Miguel Servet" program (CP15/00100) from the Institute of Health Carlos III (Co-funded by the European Social Fund (ESF) - ESF investing in your future). Cristian Del Bo' was supported by "Piano di sostegno alla ricerca- Linea 2, azione A-grant number PSR2019- CDELB and PSR2018-CDELB".

We sincerely thank all the volunteers for their valuable contribution and dedication to the study. We are grateful to the nursing and medical staff working at Opera Immacolata Concezione (OIC Foundation, Padua, Italy) for their coordinating activities in the nursing home and for the success of the study. In particular we would like to acknowledge Alberto Fantuzzo, Chiara Cavazzini, Lorella Pinton, Paolo Bergantin, Rosanna Ceccato, Pamela Soranzo, and Silvana Giraldini. We are also grateful to all the physicians (Michela Rigon, Lorena D'Aloise, Antonio Merlo, Elisabetta Bernardinello, Nadia Malacarne, Silvana Bortoli, Fabiola Talato, Agostino Corsini, Maria Licursi, Nicoletta Marcon, Angela Sansone), the nurses and other personnel at OIC who were essential to complete the study successfully. In addition, we would like to thank Giulia Minto and Nicola Fassetta for their help during the intervention period. Finally, the authors are grateful to Barry Callebaut, Indena, Melinda, Oranfrizer, Roberts Berrie, Zuegg for their in-kind contribution of the products.

Supplementary data

Table 3A and 3B are provided as supplementary material.

Figure 1: Consort flow diagram

Figure 2 – Correlations between the different markers at baseline in the whole group of older subjects (A), in HSZ subjects (serum zonulin levels > median) (B) and LSZ subjects (serum zonulin levels ≤ median) (C)

The heatmap represents the R value of Spearman's correlation. Asterisks indicate the Kendall rank correlation: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Legend: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft-Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor –α; IL-6, interleukin-6

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1

Table 1 – Baseline characteristics of subjects selected for the study

Variables	Median (IQR)	Mean (SD)
Age (y)	77 (70;87)	78.0 ± 10.3
Body weight (kg)	73.6 (62;83)	73.1 ± 14.0
BMI (kg/m^2)	25.7 (22.5;30.7)	26.8 ± 5.5
SBP (mm Hg)	125 (120;130)	125.6 ±10.8
DBP (mm Hg)	75 (70;80)	74.5 ± 8.2
Glucose (mg/dL)	95 (86;113)	113.5 ± 67.2
Creatinine (mg/dL)	0.87 (0.62;1.05)	0.9 ± 0.29
Uric Acid (mg/dl)	5.10 (4.20;6.60)	5.5 ± 1.76
TC (mg/dL)	194 (167;242)	196.3 ± 50.1
HDL-C (mg/dL)	45 (37;55)	46.5 ± 14.9
LDL-C (mg/dL)	120 (85;146)	120.5 ± 36.7
TC/HDL-C (ratio)	4.18 (3.54;5.43)	4.45 ± 1.17
LDL/HDL-C (ratio)	2.57 (2.08;3.45)	2.72 ± 0.76
TG (mg/dL)	117 (89;169)	146.1 ± 93.4
AST (U/L)	17 (13;22)	17.8 ± 5.7
ALT (U/L)	11 (8;19)	13.4 ± 7.2
GGT (U/L)	23 (17;46)	38.1 ± 39.0
Insuline uU/mL	6.20 (4.70;9.20)	8.4 ± 6.4
HOMA index	1.55 (1.15;2.50)	2.9 ± 5.4
C-G index	69.4 (53.7;82.5)	74.8 ± 40.5
Zonulin (ng/mL)	40 (34.5;49.2)	42.2 ± 11.8
sVCAM-1 (ng/mL)	967.9 (628.0;1327.1)	1239 ± 1683

sICAM-1 (ng/mL)	51.4 (43.9;65.4)	55.6 ± 20.5
CRP (mg/L)	3.5 (1.6;9.8)	7.02 ± 8.0
TNF-α (pg/mL)	1.2 (1.0;1.8)	1.6 ± 1.2
IL-6 (pg/mL)	3.1 (1.9;5.4)	4.5 ± 4.1

All data are presented as median and interquartile range (IQR) and as mean ± standard deviation (SD). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft-Gault, sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor-alpha; IL-6, interleukin-6

12 **Table 2 – Effect of intervention on nutrient and polyphenol intake**

Variables	PR- diet	C diet	P value
Energy (Kcal)	1537 ± 183	1559 ± 153	0.271
Total carbohydrates (% of energy)	47.2 ± 5.4	45.2 ± 5.2	0.024
Protein (% of energy)	17.7 ± 1.8	18.0 ± 1.9	0.191
Animal proteins (% of energy)	11.7 ± 2.2	12.3 ± 2.2	0.019
Vegetable proteins (% of energy)	4.6 ± 0.9	4.9 ± 0.9	0.005
Total lipids (% of energy)	34.9 ± 4.7	36.9 ± 4.7	0.023
SFA (% of energy)	11.3 ± 2.3	11.8 ± 2.5	0.079
MUFA (% of energy)	15.2 ± 2.8	16.4 ± 2.7	0.020
PUFA (% of energy)	3.2 ± 0.8	4.0 ± 1.5	<0.001
ω-3 (% of energy)	0.6 ± 0.2	0.6 ± 0.2	0.341
ω-6 (% of energy)	2.6 ± 0.7	3.4 ± 1.3	<0.001
Total Fibre (g/1000 kcal)	11.4 ± 1.8	10.5 ± 1.8	0.001
Cholesterol (mg)	216.3 ± 62.2	210.8 ± 67.0	0.468
Total carbohydrates (g)	188.6 ± 24.2	184.2 ± 27.0	0.263
Proteins (g)	66.7 ± 10.5	68.9 ± 8.7	0.040
Animal proteins (g)	45.0 ± 9.8	48.0 ± 8.7	0.002
Vegetable proteins (g)	17.7 ± 3.8	19.3 ± 3.7	0.001
Total lipids (g)	59.1 ± 13.3	63.1 ± 11.3	0.024
SFA (g)	19.2 ± 5.5	20.3 ± 5.3	0.043
MUFA (g)	26.0 ± 5.5	28.6 ± 6.0	0.008
PUFA (g)	5.6 ± 2.0	6.9 ± 2.6	<0.001
Total ω-3 (g)	1.0 ± 0.4	1.1 ± 0.4	0.296
Total ω-6 (g)	4.5 ± 1.7	5.7 ± 2.3	<0.001

Fibre (g/day)	17.4 ± 3.3	16.4 ± 3.2	0.004
Calcium (mg)	736.9 ± 207.7	875.0 ± 233.2	<0.001
Iron (mg)	8.5 ± 1.7	9.2 ± 1.6	0.008
Vitamin B_{12} (µg)	6.2 ± 6.5	5.4 ± 6.3	0.537
Vitamin C (mg)	128.8 ± 47.2	111.7 ± 40.1	0.009
Vitamin E (mg)	8.5 ± 2.2	8.9 ± 2.3	0.341
Vitamin B_1 (mg)	0.9 ± 0.2	0.9 ± 0.2	0.171
Folates (µg)	233.3 ± 66.0	250.8 ± 72.7	0.091
Vitamin B_6 (mg)	1.4 ± 0.3	1.5 ± 0.3	0.130
Total Polyphenols (mg/day)	1391.2 ± 188.1	812.3 ± 193.1	<0.001

All data are expressed as mean ± standard deviation (SD); Data with P<0.05 are significantly different. PR, polyphenol-rich diet; C, control diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids

Table 3- Effect of 8-week intervention with PR-diet and C-diet on anthropometrical, physical, biochemical, functional characteristics, oxidative stress markers and serum zonulin levels in the whole group of subjects

Variables (n = 51)	Before PR-diet	After PR-diet	Before C diet	After C diet	P for T	P for t	P for T *x* t
Body weight (kg)	73.4 ± 14.5	73.7 ± 14.6	72.8 ± 13.7	72.6 ± 13.9	0.023	0.779	0.126
BMI (kg/m^2)	26.9 ± 5.7	27.0 ± 5.7	26.7 ± 5.4	26.6 ± 5.6	0.017	0.677	0.090
SBP (mmHg)	127.2 ± 12.7	124.5 ± 14.6	126.5 ± 9.8	126.2 ± 10.4	0.749	0.107	0.234
DBP (mmHg)	76.7 ± 8.6	73.8 ± 9.4	75.5 ± 6.8	76.9 ± 7.5	0.345	0.285	0.024
Glucose (mg/dL)	114.4 ± 68.2	107.4 ± 42.8	108.6 ± 42.3	105.7 ± 38.2	0.163	0.096	0.360
Creatinine (mg/dL)	0.89 ± 0.29	0.89 ± 0.32	0.89 ± 0.35	0.87 ± 0.31	0.386	0.220	0.422
Uric Acid (mg/dL)	5.6 ± 1.8	5.7 ± 1.7	5.8 ± 1.9	5.5 ± 1.7	0.793	0.361	0.034
TC (mg/dL)	194.9 ± 51.1	189.5 ± 49.7	191.6 ± 49.2	188.1 ± 50.9	0.411	0.039	0.700
HDL (mg/dL)	47.1 ± 14.6	46.6 ± 14.0	47.0 ± 14.9	46.9 ± 15.6	0.876	0.607	0.695
LDL (mg/dL)	119.3 ± 36.6	115.4 ± 33.9	116.4 ± 35.3	114.1 ± 36.9	0.321	0.054	0.646
TC/HDL (ratio)	4.3 ± 1.2	4.2 ± 1.0	4.3 ± 1.1	4.2 ± 1.1	0.610	0.107	0.511
LDL/HDL-C (ratio)	2.6 ± 0.7	2.6 ± 0.7	2.6 ± 0.7	2.6 ± 0.7	0.426	0.238	0.775

TG (mg/dL)	140.2 ± 86.9	136.9 ± 76.3	141.6 ±91.7	135.6 ± 92.9	0.992	0.285	0.781
AST (U/L)	17.7 ± 5.4	17.4 ± 5.2	17.7 ± 5.3	17.9 ± 5.3	0.632	0.840	0.509
ALT (U/L)	13.7 ± 7.2	13.2 ± 6.6	13.5 ± 6.8	13.9 ± 6.5	0.656	0.831	0.382
GGT (U/L)	38.7 ± 31.9	37.1 ± 30.7	38.8 ± 39.6	36.8 ± 29.0	0.954	0.354	0.903
Insuline (uU/mL)	8.3 ± 6.6	7.2 ± 3.6	8.4 ± 6.7	7.3 ± 4.4	0.467	0.068	0.639
HOMA index	2.9 ± 5.5	2.0 ± 1.9	2.7 ± 4.6	2.1 ± 2.2	0.153	0.145	0.810
C-G index	72.8 ± 36.0	74.8 ± 40.5	74.3 ± 40.8	74.6 ± 38.7	0.494	0.189	0.449
sVCAM-1 (ng/mL)	980.4 ± 527.8	1037.4 ± 683.9	1319.9 ± 1713.2	1094.4 ± 703.0	0.095	0.462	0.197
sICAM-1 (ng/mL)	54.9 ± 20.5	59.9 ± 28.8	57.9 ± 23.8	55.7 ± 22.8	0.665	0.352	0.600
CRP (mg/L)	6.8 ± 8.7	5.9 ± 7.6	5.0 ± 5.6	6.3 ± 7.7	0.364	0.846	0.158
TNF-α (pg/mL)	1.5 ± 1.1	1.4 ± 0.6	1.4 ± 0.7	1.4 ± 0.6	0.148	0.376	0.562
IL-6 (pg/mL)	4.5 ± 3.7	4.3 ± 5.1	4.2 ± 3.8	5.3 ± 9.3	0.500	0.628	0.189
net-H_2O_2-induced DNA damage (% DNA in tail)	29.1 ± 11.5	30.2 ± 9.7	28.2 ± 9.0	28.5 ± 9.5	0.235	0.507	0.650
net-FPG sensitive sites (% DNA in tail)	20.2 ± 11.3	21.5 ± 10.6	19.9 ± 10.4	22.4 ± 10.8	0.765	0.030	0.577

Zonulin (ng/mL)	41.9 ± 10.4	39.0 ± 8.9	42.8 ± 10.9	44.3 ± 12.5	0.008	0.462	0.025

All data are expressed as mean ± standard deviation (SD). Data with $P<0.05$ are significantly different. T: treatment effect; t: time effect; T *x* t: treatment *x* time interaction.

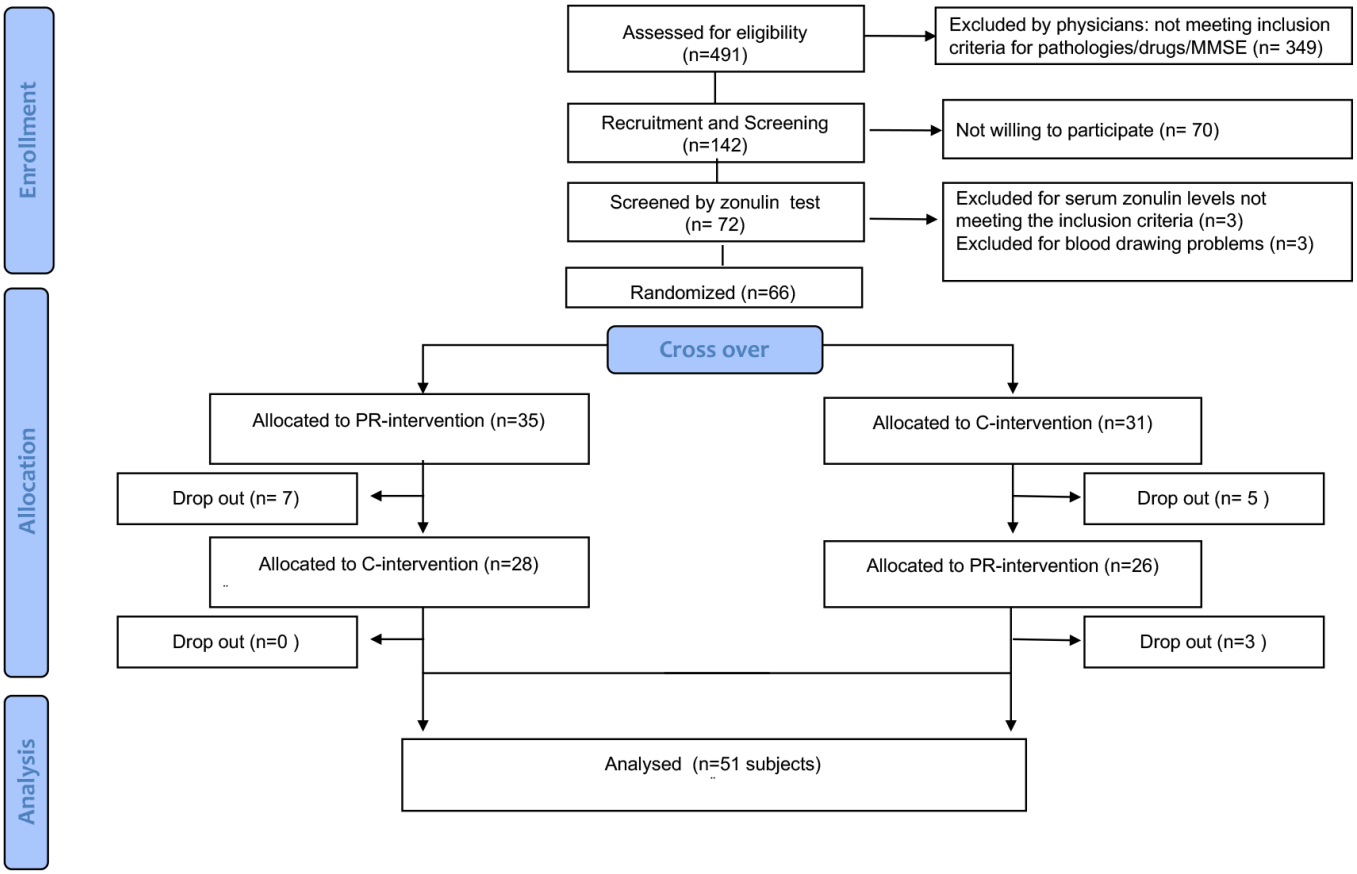
PR, polyphenol-rich diet; C, control diet; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft-Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor –α; IL-6, interleukin-6; H_2O_2, hydrogen peroxide; FPG, formamidopyrimidine DNA glycosilase.

Table 4. **Bacterial taxa in faecal samples that significantly changed their relative abundance during the MaPLE dietary intervention trial.**

			PR-diet		C diet	
ASV nr.	**Taxonomy**	***p***	**Before**	**After**	**Before**	**After**
Dada_235	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_uniformis**	0.026	0.16	0.09	0.04	0.11
	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_Alistipes	0.048	0.93	1.31	1.35	1.17
Dada_247	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_Alistipes;s_onderdonkii*	0.043	0.04	0.16	0.15	0.04
Dada_199	*p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus;s_agalactiae*	0.034	0.18	0.08	0.08	0.12
Dada_184	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Anaerobutyricum;s_hallii**	0.018	0.03	0.16	0.18	0.06
	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Lactonifactor	0.040	0.22	0.30	0.41	0.21
	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae	0.049	20.2	22.0	20.8	20.1
	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_*	0.048	1.89	2.36	2.09	1.76
	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Butyricicoccus	0.049	0.47	0.73	0.45	0.44
Dada_128	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii**	0.049	0.07	0.16	0.15	0.09
Dada_212	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii**	0.022	0.03	0.13	0.08	0.07
Dada_89	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii**	0.035	0.09	0.22	0.17	0.13

Significant differences were determined according to repeated measure ANOVA (*p*). The taxonomic lineage of each taxon is p: phylum; c: class; o: order; f: family; g: genus; s: species. ASV, amplicon sequence variant. C, control diet; PR, polyphenol-rich diet. Data with P<0.05 are significantly different. *, the taxonomic identification of the species level has been carried out manually by means of a BLAST search within the 16S ribosomal RNA database of the GenBank, setting a similarity limit of 98% for the taxonomic assignment.

40 Figure 1



41

Figure 2 A

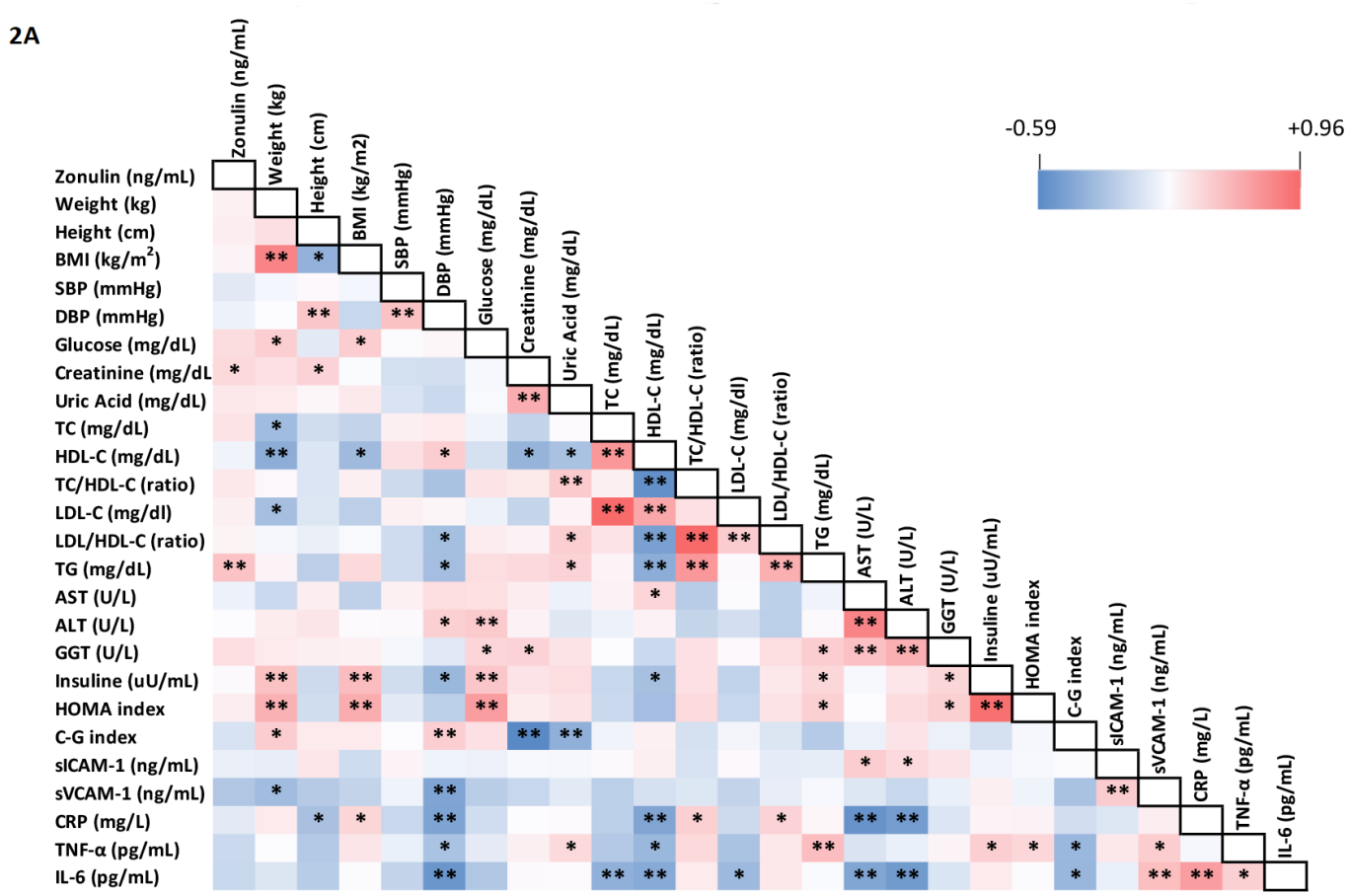

2A
-0.59
+0.96
Zonulin (ng/mL)
Weight (kg)
Height (cm)
BMI (kg/m2)
SBP (mmHg)
DBP (mmHg)
Glucose (mg/dL)
Creatinine (mg/dL)
Uric Acid (mg/dL)
TC (mg/dL)
HDL-C (mg/dL)
TC/HDL-C (ratio)
LDL-C (mg/dl)
LDL/HDL-C (ratio)
TG (mg/dL)
AST (U/L)
ALT (U/L)
GGT (U/L)
Insuline (uU/mL)
HOMA index
C-G index
sICAM-1 (ng/mL)
sVCAM-1 (ng/mL)
CRP (mg/L)
TNF-α (pg/mL)
IL-6 (pg/mL)

Figure 2B

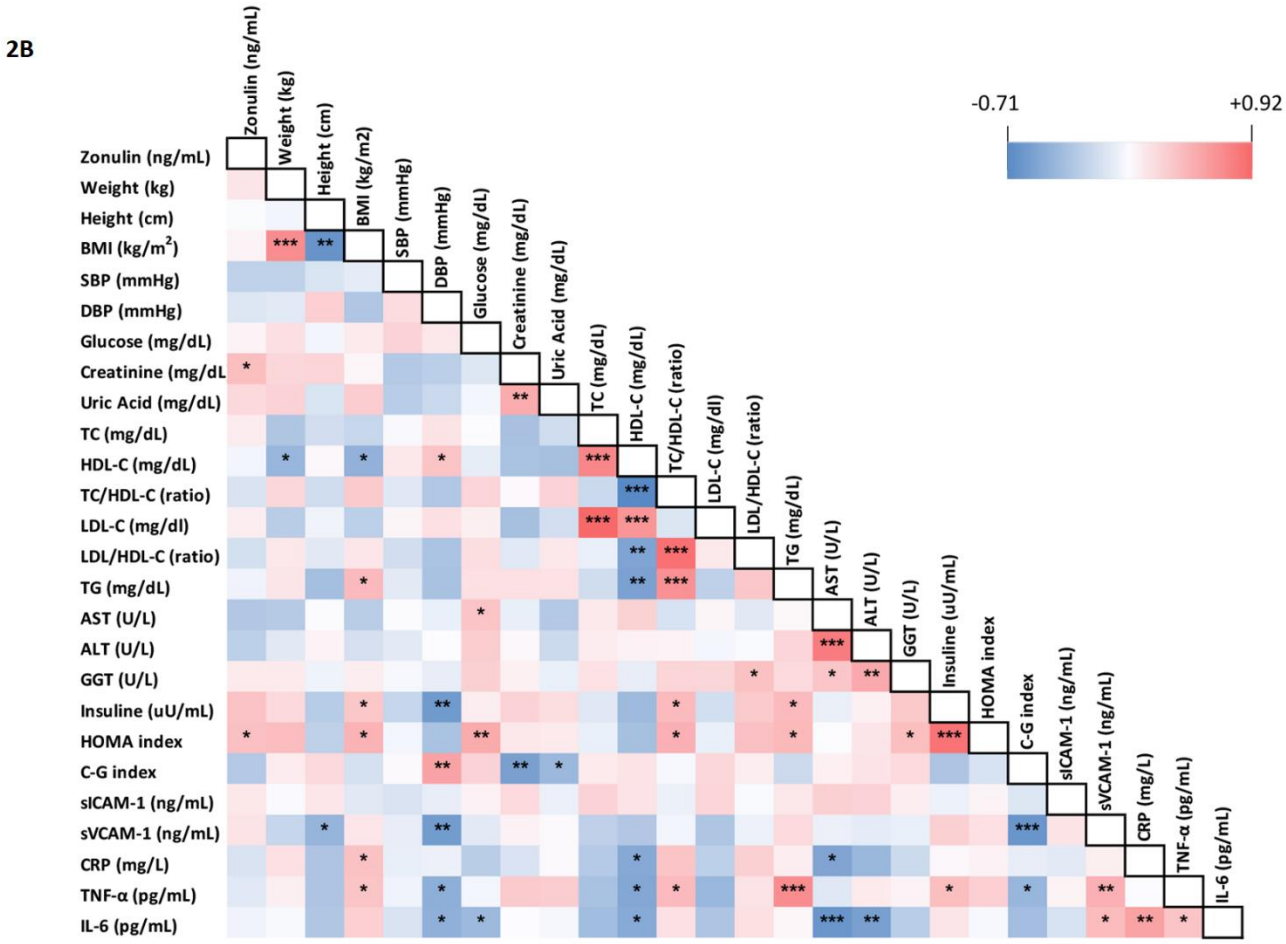
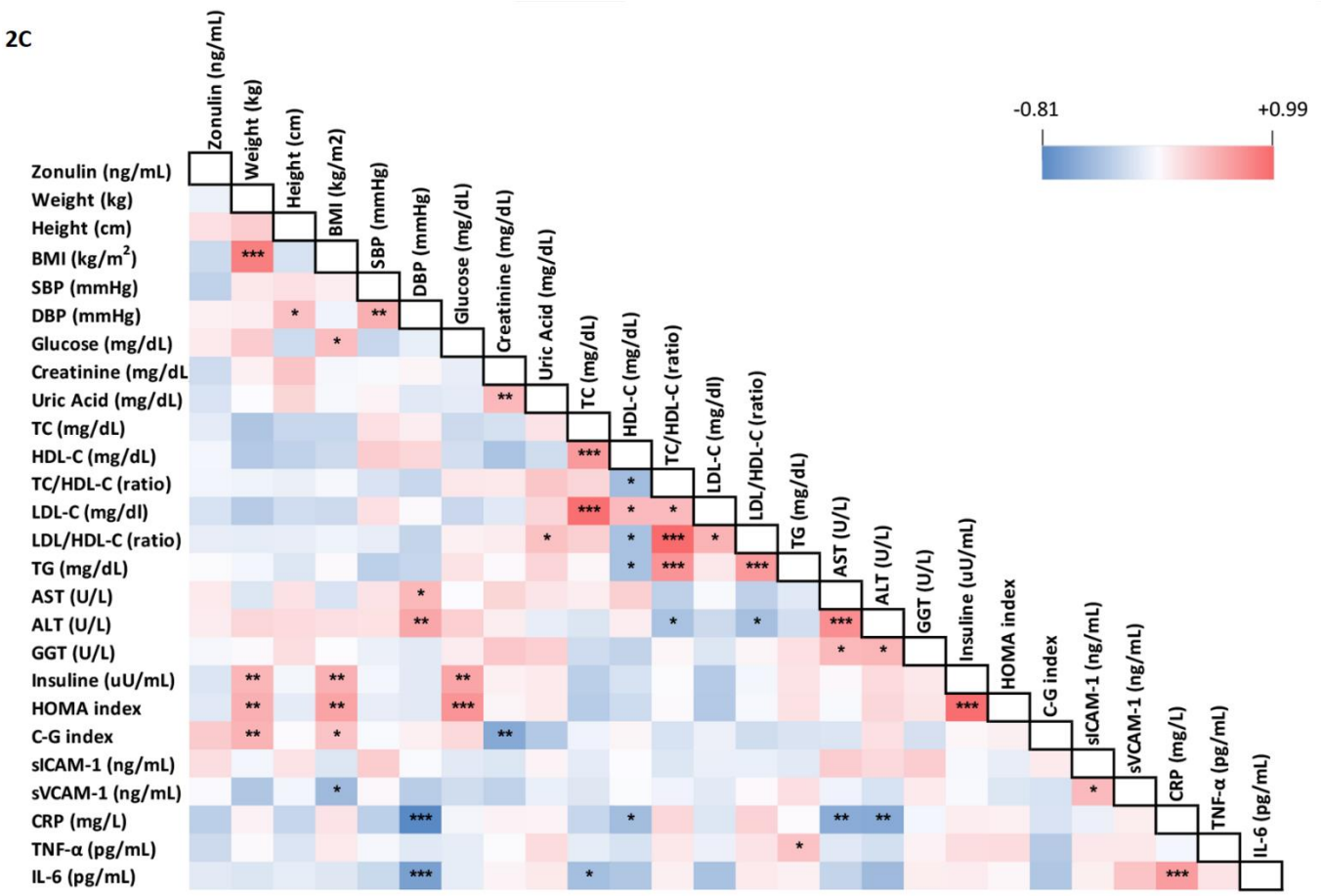

2B
-0.71
+0.92
Zonulin (ng/mL)
Weight (kg)
Height (cm)
BMI (kg/m2)
SBP (mmHg)
DBP (mmHg)
Glucose (mg/dL)
Creatinine (mg/dL)
Uric Acid (mg/dL)
TC (mg/dL)
HDL-C (mg/dL)
TC/HDL-C (ratio)
LDL-C (mg/dl)
LDL/HDL-C (ratio)
TG (mg/dL)
AST (U/L)
ALT (U/L)
GGT (U/L)
Insuline (uU/mL)
HOMA index
C-G index
sICAM-1 (ng/mL)
sVCAM-1 (ng/mL)
CRP (mg/L)
TNF-α (pg/mL)
IL-6 (pg/mL)

Figure 2C


2C
-0.81
+0.99
Zonulin (ng/mL)
Weight (kg)
Height (cm)
BMI (kg/m2)
SBP (mmHg)
DBP (mmHg)
Glucose (mg/dL)
Creatinine (mg/dL)
Uric Acid (mg/dL)
TC (mg/dL)
HDL-C (mg/dL)
TC/HDL-C (ratio)
LDL-C (mg/dl)
LDL/HDL-C (ratio)
TG (mg/dL)
AST (U/L)
ALT (U/L)
GGT (U/L)
Insuline (uU/mL)
HOMA index
C-G index
sICAM-1 (ng/mL)
sVCAM-1 (ng/mL)
CRP (mg/L)
TNF-α (pg/mL)
IL-6 (pg/mL)