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

A polyphenol-rich dietary pattern improves intestinal permeability in older subjects: the MaPLE randomised controlled trial

Cristian Del Bo'[1§], Stefano Bernardi[1§], Antonio Cherubini[2], Marisa Porrini[1], Giorgio Gargari[1], Nicole Hidalgo-Liberona[3,4], Raúl González-Domínguez[3,4], Raul Zamora-Ros[3,5], Gregorio Peron[3,4], Mark S Winterbone[6], Benjamin Kirkup[6], Paul A Kroon[6], Cristina Andres-Lacueva[3,4], Simone Guglielmetti[1], Patrizia Riso[1*]

Affiliations

[1]Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences (DeFENS), 20133 Milan, Italy; [2]Geriatria, Accettazione Geriatrica e Centro di Ricerca per l'Invecchiamento, IRCCS INRCA, 60127 Ancona, Italy; [3]Biomarkers and Nutrimetabolomics Laboratory, Department of Nutrition, Food Sciences and Gastronomy, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; [4]CIBER de Fragilidad y Envejecimiento Saludable (CIBERfes), Instituto de Salud Carlos III, 08028 Barcelona, Spain; [5]Unit of Nutrition and Cancer, Cancer Epidemiology Research Programme, Catalan Institute of Oncology (ICO), Bellvitge Biomedical Research Institute (IDIBELL), Spain; [6]Quadram Institute Bioscience, Norwich Research Park, Norwich NR4 7UQ, United Kingdom

§equally contributed as first author

*Corresponding author: patrizia.riso@unimi.it

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 IP and beneficially alter 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. 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, that was underlined in women ($p = 0.043$) showing also a decrease of systolic blood pressure ($p = 0.042$).

A trend towards a reduction of total cholesterol was observed (time effect, $p = 0.039$) following both interventions. The efficacy of this dietary intervention was higher 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 insulin resistance at baseline, thus demonstrating the close interplay between IP and metabolic features.

Conclusions: These data show, for the first time, that PR-diet can reduce IP evaluated as serum zonulin levels. 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: zonulin; leaky gut; inflammation; flavonoids; phenolics; aging

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 symbiosis 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 derived factors from the intestinal lumen to the blood stream, leading to the activation of immune function and inflammation [3]. An 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. In addition, 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 of 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

86 associated with an alteration of the intestinal microbial ecosystem and an increased IP [10-12].
87 Moreover, an inadequate nutrient intake (e.g. low protein intake) that often occurs in older subjects can
88 contribute to increase IP [4]. Conversely, diets rich in low-energy dense foods (e.g. fruits and
89 vegetables) and fibres have been associated with a healthier gut microbiota and a reduced IP [13]. In
90 the context of a diet-microbiota-IP axis, several food bioactives, including polyphenols, may represent
91 a potential strategy to positively affect microbiota composition and to improve IP and related
92 conditions [14]. Polyphenol biological functions include antioxidant and anti-inflammatory properties,
93 and immunomodulatory activity at both intestinal and systemic levels [2]. Despite the exact molecular
94 mechanisms are not completely understood, polyphenols may directly and/or indirectly act at different
95 levels of the intestinal barrier by regulating tight junction function, the production of numerous
96 inflammatory cytokines and the activation of antioxidant genes [2]. Furthermore, polyphenols undergo
97 extensive modifications by the gut microbiota and, consequently, affect the intestinal microbial
98 ecosystem. For such reasons, polyphenols could represent elective bioactives to develop dietary
99 intervention strategies to counteract detrimental effects of IP.
100 To the best of our knowledge, human intervention studies aimed at investigating the role of polyphenols
101 in the modulation of IP are still lacking. Within this context, the MaPLE (Microbiome mAnipulation
102 through Polyphenols for managing Leakiness in the Elderly) randomised, controlled, crossover trial
103 was designed to assess whether a high intake of polyphenol-rich foods in older subjects would reduce
104 IP and improve markers of inflammation and vascular function.
105
106 **2. Materials and Methods**
107 *2.1 Setting and subjects' recruitment*
108 The MaPLE trial was carried out at Civitas Vitae (OIC Foundation, Padua, Italy), an institution
109 including residential care and independent residences for older subjects. The setting was selected in
110 order to enable a significant control of most of the experimental variables affecting dietary intervention
111 studies as previously described [15]. 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 [15-18]. 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 [19]. 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 [15].

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 [15]. 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 were collected for the analysis of metabolic and functional markers.

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) [15].

2.5 Anthropometrical and physical evaluations

Height and weight were measured according to Lohman et al international guidelines [20]; body mass index (BMI) was calculated according to the formula – weight (kg)/height (m^2). Reference scores were

163 defined according to international guidelines [20]. Blood pressure was obtained in resting, seated
164 position following the JNC 7 guidelines [21].
165
166 *2.6 Blood sampling and analysis*
167 After an overnight fast, blood samples were drawn in Vacutainer tubes containing silicon gel for serum
168 and maintained at room temperature for at least 30 min. Serum was then obtained by tube centrifugation
169 (1400 g x 15 min, 4°C), splitted in small aliquots into specific vials and stored at -80°C until analysis.
170 Samples were used for the evaluation of several metabolic and functional parameters [15].
171 In particular, glucose, insulin, lipid profile (total cholesterol, triglycerides), liver and renal function
172 (i.e. aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, creatinine)
173 were analysed using a standardized routine-use automatic biochemical analyser (ILAB 650,
174 Instrumentation Laboratory, Lexington, MA). Serum concentration of low-density lipoprotein
175 cholesterol (LDL-C) and non-high-density lipoprotein cholesterol (HDL-C) were estimated by using
176 the Friedewald formula [22] and by subtracting HDL-C from total cholesterol (TC), respectively. In
177 addition, the homeostasis model assessment of insulin resistance (HOMA-IR) was performed, and
178 values > 3 were considered as a criterion for insulin resistance [23]. The Cockroft-Gault (C-G) index
179 based on creatinine clearance was calculated according to the formula previously defined in literature
180 [24,25].
181
182 *2.7 Evaluation of IP*
183 Serum samples for IP evaluation (at recruitment and at each time point of intervention) were defrosted
184 at room temperature and serum zonulin level was assessed by using the Immunodiagnostik® ELISA
185 kit (Bensheim, Germany). The assay, based on a competitive Elisa method, consisted in the addition
186 to each sample (including standard and control samples) of a biotinylate zonulin tracer (at first step)
187 and the use of a pre-coated 96-well plate with polyclonal anti-zonulin antibody. The peroxidase-
188 labelled streptavidin addition was used to bind the biotinylate zonulin tracer. After the reaction, the

189 plate reader TECAN Infinite F200 (Tecan Group Ltd. Mannedorf, Switzerland) was used to read the
190 fluorescence at 450 nm. Serum zonulin concentrations were quantified by using a standard curve
191 calculated by a 4-parameter algorithm as reported by the manufacturer.
192
193 *2.8 Evaluation of inflammatory markers*
194 C-reactive protein (CRP), Tumour Necrosis Factor α (TNF-α) and interleukin-6 (IL-6) levels were
195 quantified using specific ELISA kits (R&D Systems, Biotechne, Abingdon, UK). Specifically, CRP
196 (DCRP00), IL-6 (HS600B), and TNF-α (HSTA00E) were quantified in serum at the beginning and
197 the end of each intervention period.
198
199 *2.9 Evaluation of vascular markers*
200 Serum samples at each time point were used to quantify vascular cell adhesion molecule-1 (VCAM-1)
201 and intercellular adhesion molecule-1 (ICAM-1) by using an ELISA kit (Booster® from Vinci
202 Biochem S.r.l., Vinci, Italy). After competitive treatment with antibodies and fluorophore, fluorescence
203 was read by a TECAN Infinite F200 plate reader. A 4-parameter algorithm was used to create the
204 standard curve and to calculate serum concentrations.
205
206 *2.10 Statistical analysis*
207 Sample size was calculated based on previous published data [18,26]. It was estimated that 50 subjects
208 were needed to detect a 30% decrease in plasma zonulin with 80% power and alpha=0.05 with an
209 estimated drop-out rate of 15%.
210 Differences between treatments were computed by ANOVA for repeated measures design (using the
211 Least Significant Difference - LSD test - as post hoc analysis to evaluate differences among means).
212 In addition, although a relatively high zonulin level was used as an inclusion criterion [15], we found
213 interesting to verify whether the response to dietary treatments could differ in subjects stratified with
214 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. 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**. Age ranged between 60 and 98 years old with a median value of 77 years old. Age

239 distribution was comparable in men and women. A high inter-individual variability was observed for
240 several markers and in particular BMI (IQR: 22.5;30.7), glucose (IQR: 86;113) and total cholesterol
241 levels (IQR 167;242).
242
243 *3.3 Correlation analysis of subjects' characteristics based on HSZ or LSZ at baseline*
244 Serum zonulin levels were positively correlated with creatinine ($p = 0.033$) and triglycerides ($p =$
245 0.004) considering all participants (**Figure 2A**). However, the correlation of zonulin with creatinine
246 clearance, as C-G index, was not significant. A positive correlation was evidenced among
247 inflammatory markers (i.e. IL-6, TNF-α, CRP); in addition, a positive correlation emerged between
248 CRP levels and BMI ($p = 0.021$), and TNF-α and TG ($p = 0.0009$) (**Figure 2A**).
249 When subjects were stratified according to high *versus* low serum zonulin levels at baseline, HSZ
250 group showed a positive correlation between zonulin and HOMA index ($p = 0.037$), and creatinine (p
251 $= 0.025$) (**Figure 2B**). This last correlation was not confirmed when C-G index was used. Regarding
252 LSZ subjects, no significant correlation was observed between serum zonulin levels and the other
253 markers under study (**Figure 2C**).
254
255 *3.4 Compliance to the dietary intervention*
256 The nutrient composition of the diet consumed by participants during both treatment periods is reported
257 in **Table 2**. A comparable pattern of food consumption was evidenced, except for the PR-products
258 provided in the PR-diet. Energy and overall composition of the diet did not differ in the two periods of
259 intervention (PR-diet *vs* C-diet). Following the PR-diet a small decrease in animal proteins and lipids
260 and an increase in carbohydrates and fibre intake (less than 1 g as a mean) was observed with respect
261 to the C-diet. Overall, a high adherence to the dietary protocol was registered: the subjects accepted
262 and easily consumed all the PR-products provided daily and no adverse effects were reported. On the
263 whole, during the PR-diet treatment subjects increased their total polyphenol intake by approximately
264 70% (**Table 2**).

3.5 Effect of dietary interventions on markers under study

Table 3 shows the results concerning anthropometrical and physical characteristics, biochemical, inflammatory and vascular 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 evidenced a significant reduction of diastolic blood pressure following the PR-diet intervention, while uric acid decreased following the C-diet.

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 lower during C-diet intervention. Finally, a time effect ($p = 0.039$) was observed for total cholesterol with a trend towards reduction following both interventions. No significant effect was found for the remaining variables.

Considering gender (**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 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 systolic ($p = 0.042$) and diastolic blood pressure ($p = 0.043$) showing a reduction after the PR-diet, 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 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.

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 and a significant decrease of diastolic blood pressure ($p = 0.01$), glucose levels ($p = 0.049$) and a trend towards a reduction of IL-6 ($p = 0.097$); conversely a significant increase in uric acid levels ($p = 0.03$) was found after C-diet (data not shown). After stratifying subjects by BMI (**Table 1**), 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 uric acid and IL-6 serum levels ($p = 0.027$ and $p = 0.049$ respectively) 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 an increase ($p = 0.027$) in uric acid after C-diet was observed (data not shown).

4. Discussion

In this study, we have shown that modifying the diet of older subjects by including small portions of PR-products can positively affect IP, evaluated as serum zonulin concentrations. 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 here evaluated as zonulin level. Zonulin, also known as prehaptoglobin-2, is a 47-kDa protein produced mainly by epithelial cells (e.g. in the gut) which is able to reversibly modulate paracellular permeability [27]. 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 as it happens in different physiological and pathological conditions [18]. Moreover, several studies have reported correlations between the results obtained through the most common and validated IP test (based on lactulose/mannitol urine excretion evaluation following standardised sugar intake) and those with serum zonulin levels [17,28-31].

Increased serum zonulin levels and impaired IP condition have been previously found in individuals with metabolic disorders, such as diabetes and obesity [28]. 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 [28].

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 [32] 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 [33].

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 [34]. 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 [35].

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 [36]. 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 [37-42]. Other 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 [43-48].

With regard to the vascular function markers, it is well known that vascular oxidative stress increases with age and different studies found elevated levels of both VCAM-1 and ICAM-1 in older compared to younger individuals [49-50]. High polyphenol intake has been inversely associated with a reduced risk of cardiovascular events and mortality [51], possibly by decreasing the levels of reactive oxygen species and adhesion molecules or by inducing the production of vasodilators [52]. In the present study, we could not demonstrate an effect of the PR dietary pattern in terms of modulation of ICAM-1 and VCAM-1 as it has been previously documented following an intervention with freeze-dried wild blueberry drink, freeze-dried polyphenol-rich whole grape powder, green tea extract or beverage [37-39]. However, a protective effect was found by other research groups following the administration of different berries and grape products [53-54]. Nevertheless, no specific information on direct association between IP and vascular function has been previously reported.

The aging process is not only associated to 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 [54]. Data from the literature suggest a potential role of polyphenols and polyphenol-rich foods in the modulation of blood pressure [55]. In the present study, most of the subjects showed normal blood pressure levels or a mild hypertension treated with drugs [56]. The PR-diet intervention significantly reduced diastolic blood pressure in both men and women. Our results are partially in line with that of other studies reporting partial or no effects of these foods on blood pressure [57-64]. In addition, it is noteworthy to highlight that we found also a significant reduction of systolic blood pressure in women, but not in men. In this regard, the impact of gender in the response to treatments of hypertension has been recently reviewed underlying the kidneys, renin-angiotensin system, relaxin, and developmental programming as potential contributors to the differences observed [65].

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 [51,66,67]. 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 [68-70], orange juice/hesperidin [64], pomegranate [66,67] and different fruit juices [71]. On the contrary, beneficial effects were documented following the consumption of cocoa products, dark chocolate, and flavan-3-ols [58,72,73], berries [74,75] and black cumin [76,77]. 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

392 a limitation not enabling a strong emphasis on a potential gender specific response to the dietary
393 treatment.
394 Overall, the main outcome of the MaPLE RCT is evidence that support the notion that IP reduction
395 can be obtained through a sustainable inclusion in the diet of polyphenol-rich food sources and this
396 may support also the reliability of non-invasive dietary intervention as potential strategies to improve
397 IP in the older subjects. It is noteworthy that only limited research has been carried out to provide
398 evidence for the efficacy of dietary treatments in the management of IP [2], and just one observation
399 was recently published considering both healthy adults and older subjects as target population;
400 although that, the study did not find an effect of dietary fibre (i.e. sugar beet derived pectins) on
401 multiple IP parameters [78]. In the present trial, with respect to that by Wilms et al. [78], the inclusion
402 of PR products, the type, duration and strict control of dietary intervention (i.e. compliance to
403 polyphenol-rich products intake and overall dietary plan during the whole intervention study) and
404 subjects' characteristics could have been reasons to explain the difference in the results obtained.
405 Finally, since older subjects are generally low consumers of dietary fibre, the possibility to introduce
406 other beneficial molecules could be of utmost importance for the exploitation directed to the
407 maintenance of host functional and metabolic homeostasis.
408 The MaPLE study has several strengths represented by the well-controlled protocol of intervention
409 including the setting, the daily preparation of products and the continuous interaction with the
410 participants. On the other hand, it has also some limitations related mainly to the relatively small
411 sample size. Furthermore, the evaluation of IP using also the gold standard method (i.e. multi-sugar
412 test, difficult to apply in the population under study) or multiple IP markers could have provided more
413 insight on the impact of the diet on this condition.
414

415 **5. Conclusions**

416 In conclusion, the MaPLE RCT has demonstrated the feasibility and efficacy of a PR dietary pattern,
417 providing approximately 700 mg of total polyphenols daily for 8 weeks, in the modulation of IP

evaluated by means of serum zonulin levels and on limited associated markers. These results are novel and have potentially important clinical implications. Further intervention studies should be performed aimed at investigating the role of non-pharmacological treatment 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. 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.

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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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Figure (flow diagram)

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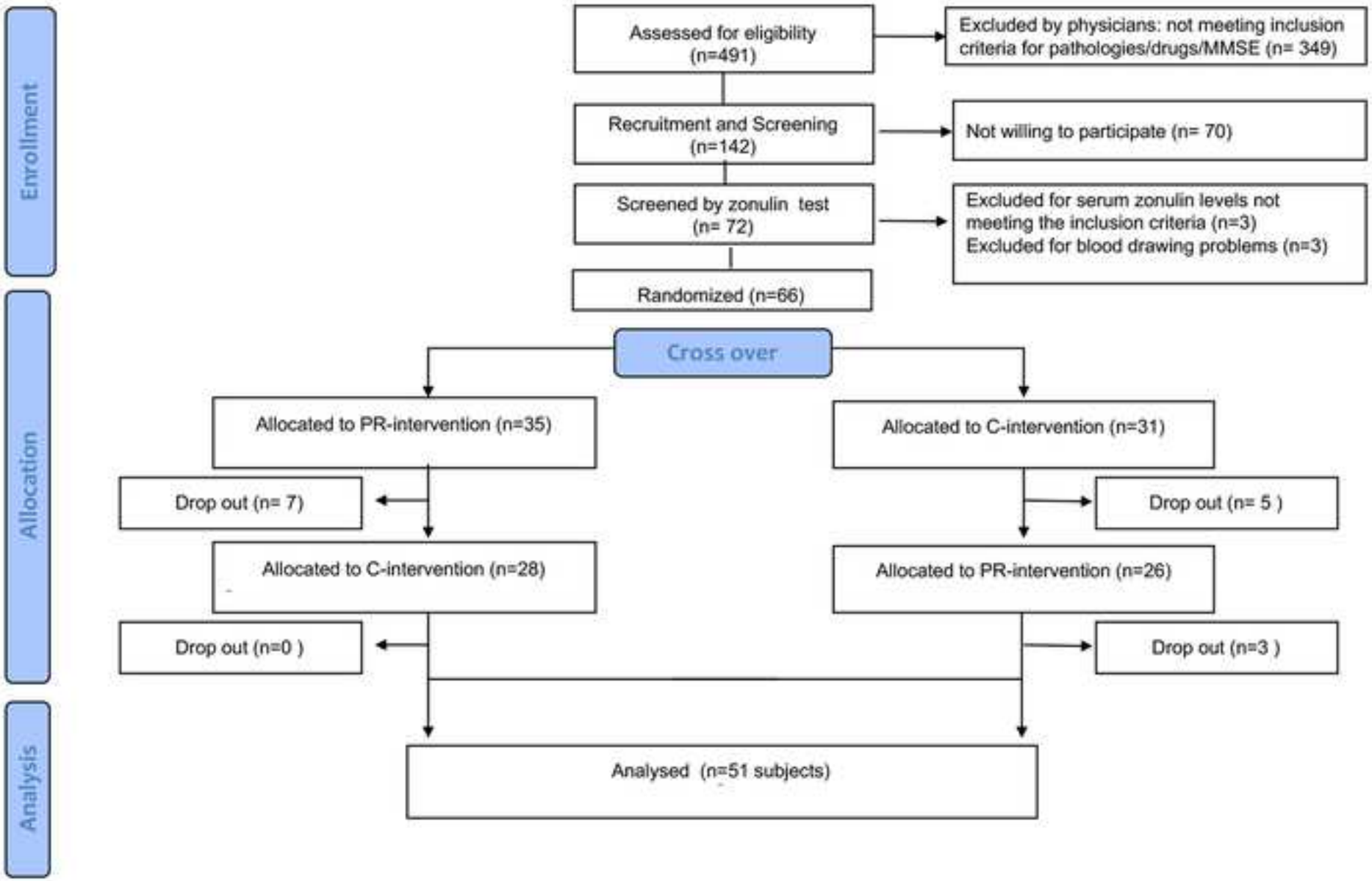

Figure 2A

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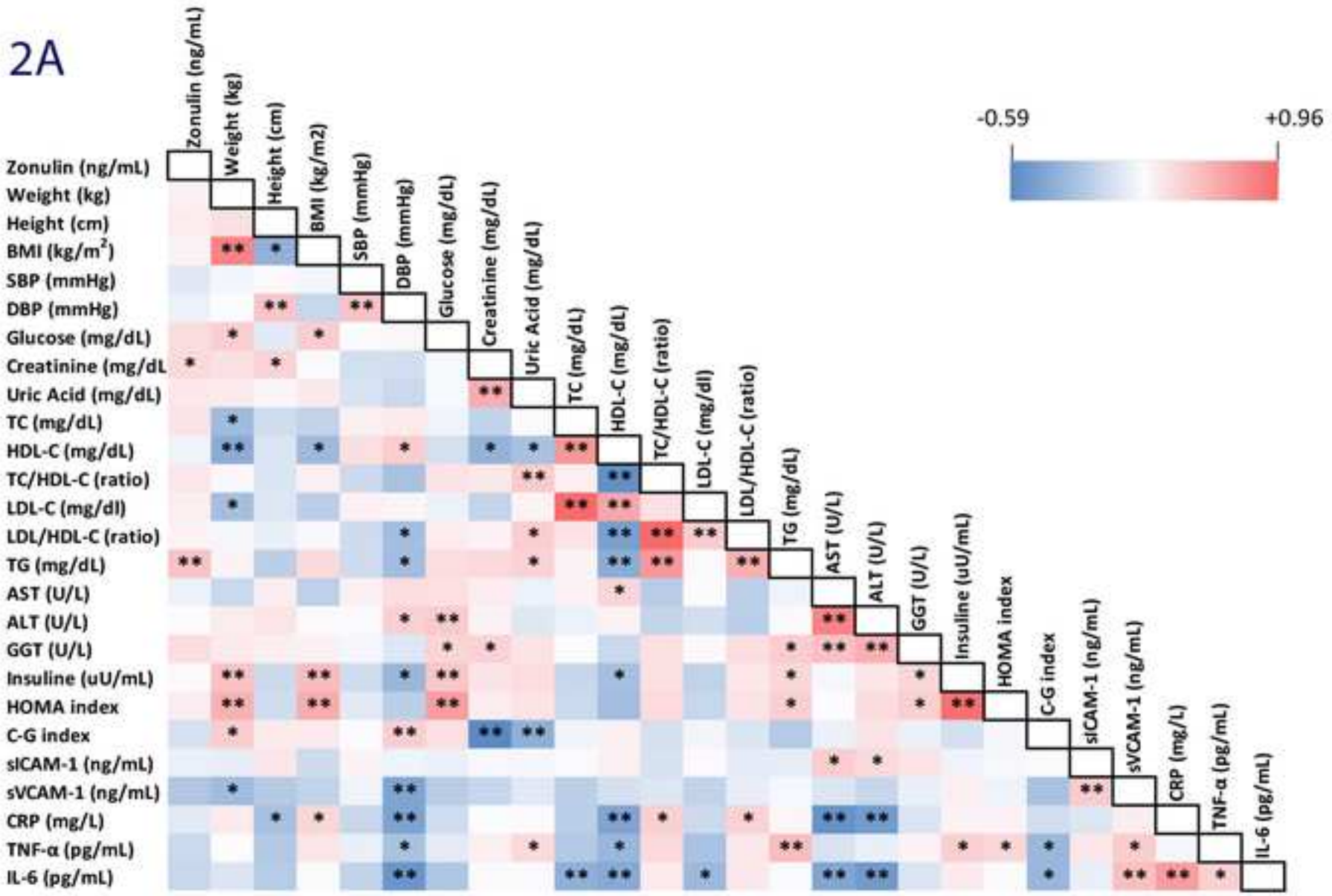

Figure 2B

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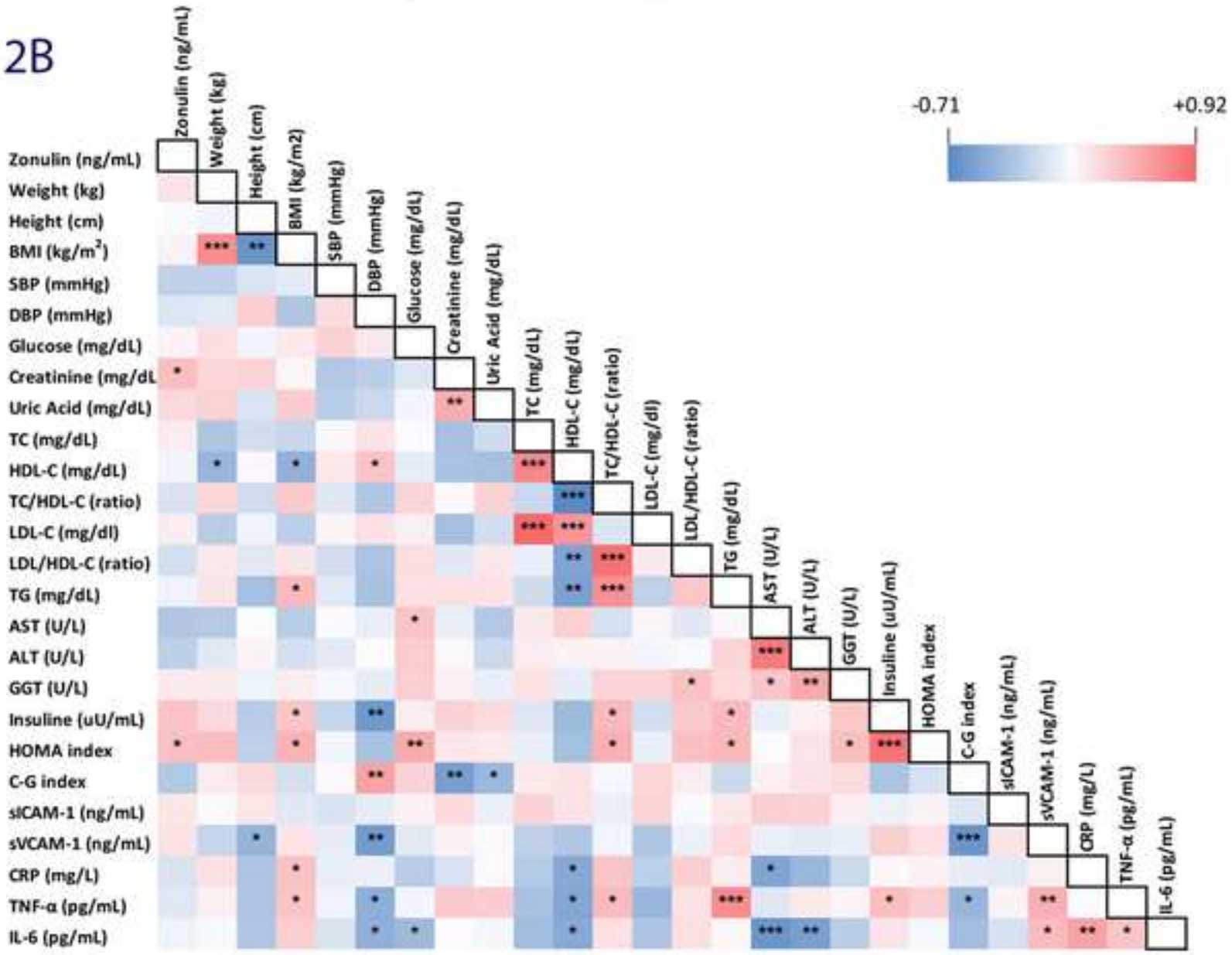

Figure 2C

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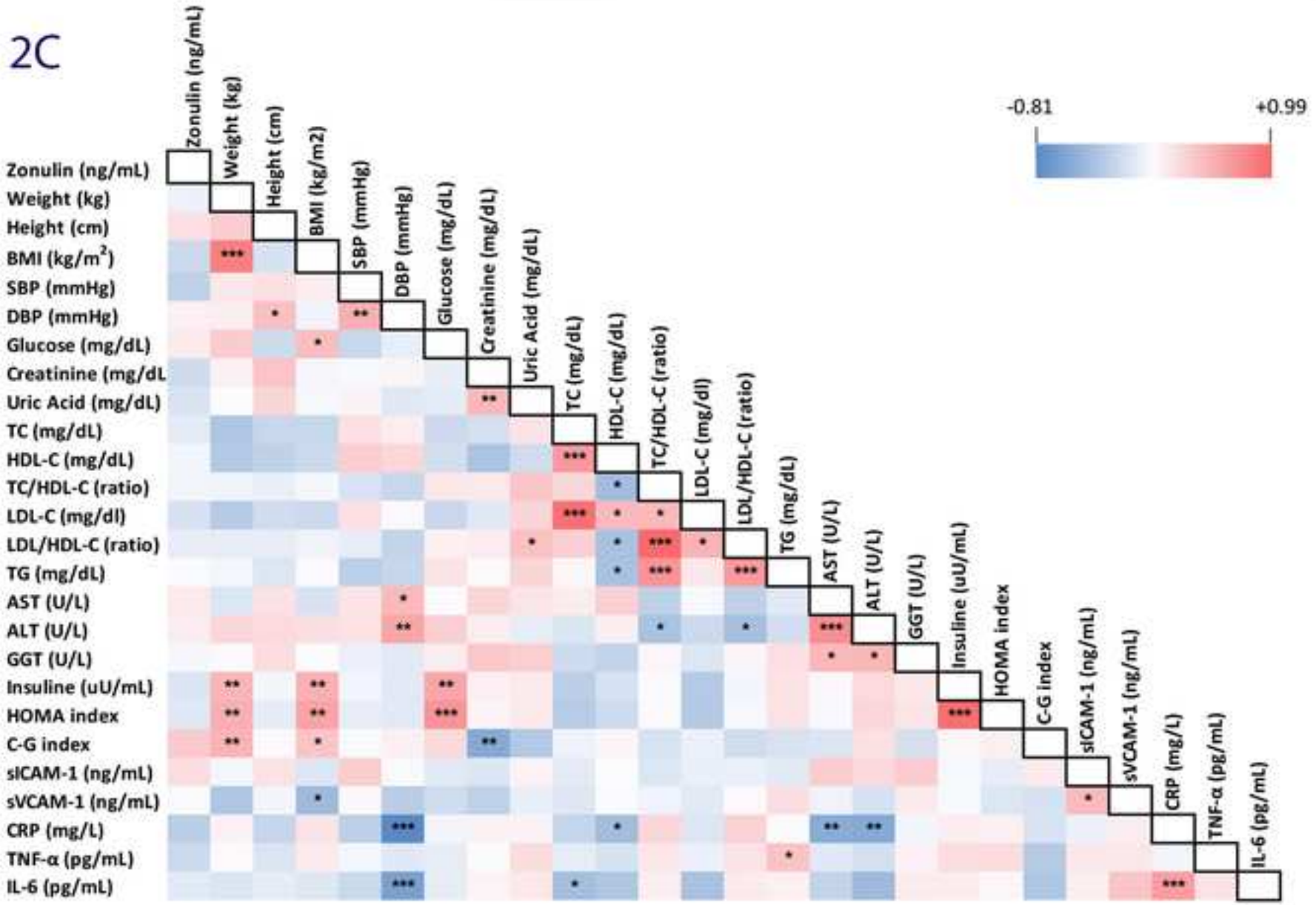

Table 1 – Baseline characteristics of subjects selected for the study

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

Table 2 – Effect of intervention on nutrient and polyphenol intake

Variables	PR- diet	C diet	P value
Energy (Kcal)	1537 ± 183	1559 ± 153	0.365
Total carbohydrates (% of energy)	47.2 ± 5.4	45.2 ± 5.2	0.016
Protein (% of energy)	17.7 ± 1.8	18.0 ± 1.9	0.185
Animal proteins (% of energy)	66.5 ± 8.2	68.9 ± 7.3	0.013
Vegetable proteins (% of energy)	27.3 ± 6.5	28.7 ± 6.8	0.100
Total lipids (% of energy)	34.9 ± 4.7	36.9 ± 4.7	0.012
SFA (% of energy)	11.3 ± 2.3	11.8 ± 2.5	0.179
MUFA (% of energy)	15.2 ± 2.8	16.4 ± 2.7	0.012
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	0.291
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	0.005
Cholesterol (mg)	216.3 ± 62.2	210.8 ± 67.0	0.587
Total carbohydrates (g)	188.6 ± 24.2	184.2 ± 27.0	0.286
Proteins (g)	66.7 ± 10.5	68.9 ± 8.7	0.063
Animal proteins (g)	45.0 ± 9.8	48.0 ± 8.7	0.003
Vegetable proteins (g)	17.7 ± 3.8	19.3 ± 3.7	0.001
Total lipids (g)	59.1 ± 13.3	63.1 ± 11.3	0.040
SFA (g)	19.2 ± 5.5	20.3 ± 5.3	0.209
MUFA (g)	26.0 ± 5.5	28.6 ± 6.0	0.004
PUFA (g)	5.6 ± 2.0	6.9 ± 2.6	<0.001
Total ω-3 (g)	1.0 ± 0.4	1.1 ± 0.4	0.315
Total ω-6 (g)	4.5 ± 1.7	5.7 ± 2.3	<0.01

Fibre (g/day)	17.4 ± 3.3	16.4 ± 3.2	0.006
Calcium (mg)	736.9 ± 207.7	875.0 ± 233.2	<0.001
Iron (mg)	8.5 ± 1.7	9.2 ± 1.6	0.003
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	0.012
Vitamin E (mg)	8.5 ± 2.2	8.9 ± 2.3	0.366
Vitamin B_1 (mg)	0.9 ± 0.2	0.9 ± 0.2	0.123
Folates (µg)	233.3 ± 66.0	250.8 ± 72.7	0.126
Vitamin B_6 (mg)	1.4 ± 0.3	1.5 ± 0.3	0.079
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

Table 3- Effect of 8-week intervention with PR-diet and C-diet on anthropometrical, physical, biochemical, functional characteristics 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
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

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2,3
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4,5
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	
Participants	4a	Eligibility criteria for participants	5
	4b	Settings and locations where the data were collected	5,6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	6,7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7,8,9
	6b	Any changes to trial outcomes after the trial commenced, with reasons	/
Sample size	7a	How sample size was determined	9,10
	7b	When applicable, explanation of any interim analyses and stopping guidelines	
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	7

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	/
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	9,10
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	9,10
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	10-13
	13b	For each group, losses and exclusions after randomisation, together with reasons	10,11
Recruitment	14a	Dates defining the periods of recruitment and follow-up	/
	14b	Why the trial ended or was stopped	/
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	11
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	/
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	/
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	/
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	10-13
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	/
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	17,18
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	18
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	14-18
Other information			
Registration	23	Registration number and name of trial registry	2
Protocol	24	Where the full trial protocol can be accessed, if available	/
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	19

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Table 3A- Effect of 8-week intervention with PR-diet and C-diet on anthropometrical, physical, biochemical, functional markers and serum zonulin levels in women

Women (n = 29)	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)	68.8 ± 13.8	69.0 ± 13.8	68.3 ± 12.8	68.2 ± 13.4	0.062	0.878	0.416
BMI (kg/m^2)	27.0 ± 5.7	27.1 ± 5.7	26.9 ± 5.4	26.8 ± 5.7	0.071	0.785	0.416
SBP (mmHg)	125.4 ± 8.8	120.6 ± 12.3	124.8 ± 9.5	125.7 ± 8.3	0.089	0.165	0.042
DBP (mmHg)	74.6 ± 7.1	71.7 ± 9.2	73.5 ± 6.8	74.7 ± 7.0	0.362	0.299	0.043
Glucose (mg/dL)	117.1 ± 80.9	109.3 ± 50.2	113.2 ± 60.2	107.9 ± 47.8	0.375	0.167	0.698
Creatinine (mg/dL)	0.81 ± 0.31	0.83 ± 0.37	0.82 ± 0.40	0.78 ± 0.33	0.211	0.481	0.134
Uric Acid (mg/dL)	5.5 ± 2.0	5.5 ± 1.9	5.6 ± 2.2	5.2 ± 1.8	0.390	0.184	0.079
TC (mg/dL)	200.7 ± 54.7	201.7 ± 53.8	197.1 ± 51.5	194.5 ± 57.3	0.183	0.787	0.645
HDL-C (mg/dL)	49.2 ± 16.9	48.9 ± 16.2	48.9 ± 17.0	48.8 ± 18.1	0.764	0.813	0.945
LDL-C (mg/dL)	121.3 ± 39.5	121.3 ± 36.8	119.6 ± 36.5	117.4 ± 41.2	0.341	0.608	0.691
TC/HDL (ratio)	4.4 ± 1.2	4.4 ± 1.1	4.3 ± 1.2	4.3 ± 1.2	0.261	0.957	0.712
HDL/LDL-C (ratio)	2.6 ± 0.7	2.6 ± 0.8	2.6 ± 0.8	2.6 ± 0.8	0.614	0.999	0.234
TG (mg/dL)	149.3 ± 99.3	152.8 ± 86.5	142.8 ± 97.6	139.7 ± 92.0	0.030	0.973	0.593

AST (U/L)	17.4 ± 5.7	17.4 ± 5.9	16.8 ± 5.5	16.5 ± 4.4	0.180	0.731	0.732
ALT (U/L)	13.3 ± 8.2	12.7 ± 7.2	12.3 ± 6.1	12.2 ± 5.2	0.304	0.596	0.709
GGT (U/L)	32.7 ± 32.5	33.4 ± 30.6	36.7 ± 45.8	31.4 ± 26.7	0.621	0.449	0.257
Insuline (uU/mL)	8.8 ± 8.2	7.4 ± 4.3	9.3 ± 8.1	7.4 ± 4.8	0.711	0.092	0.785
HOMA index	3.5 ± 7.2	2.2 ± 2.3	3.3 ± 6.0	2.3 ± 2.7	0.790	0.181	0.593
C-G index	68.8 ± 32.4	69.0 ± 32.9	69.8 ± 33.1	71.1 ± 32.9	0.179	0.524	0.660
sVCAM-1 (ng/mL)	1025.1 ± 499.2	1097.8 ± 562.6	1609.0 ± 2172.6	1250.3 ± 773.9	0.066	0.467	0.208
sICAM-1 (ng/mL)	56.6 ± 18.7	59.9 ± 25.0	55.9 ± 20.8	54.1 ± 20.1	0.336	0.200	0.121
CRP (mg/L)	6.4 ± 7.8	6.0 ± 8.1	5.6 ± 6.4	8.3 ± 10.6	0.448	0.424	0.140
TNF-α (pg/mL)	1.7 ± 1.3	1.5 ± 0.6	1.5 ± 0.8	1.4 ± 0.6	0.303	0.266	0.583
IL-6 (pg/mL)	4.6 ± 3.3	5.0 ± 6.3	4.8 ± 4.4	6.7 ± 11.9	0.174	0.487	0.328
Zonulin (ng/mL)	41.0 ± 9.0	38.5 ± 9.5	42.3 ± 10.1	45.8 ± 10.0	0.004	0.694	0.010

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

Table 3B - Effect of 8-week intervention with PR-diet and C-diet on anthropometrical, physical, biochemical, functional markers and serum zonulin levels in men

Men (n = 22)	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)	79.4 ± 13.6	79.8 ± 13.5	78.7 ± 12.7	78.4 ± 12.7	0.142	0.815	0.199
BMI (kg/m^2)	26.6 ± 5.7	26.6 ± 5.8	26.5 ± 5.6	26.4 ± 5.5	0.228	0.436	0.125
SBP (mmHg)	129.6 ±16.5	129.5 ± 16.1	128.6 ± 10.1	126.9 ± 12.8	0.536	0.432	0.600
DBP (mmHg)	79.3 ± 9.8	76.7 ± 9.0	78.0 ± 6.0	79.8 ± 7.2	0.558	0.683	0.073
Glucose (mg/dL)	110.8 ± 48.2	105 ± 31.6	102.5 ± 25.3	102.8 ± 20.4	0.295	0.358	0.317
Creatinine (mg/dL)	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	0.733	0.308	0.460
Uric Acid (mg/dL)	5.7 ± 1.4	5.9 ± 1.4	6.0 ± 1.5	6.0 ± 1.5	0.203	0.521	0.259
TC (mg/dL)	187.4 ± 45.9	173.4 ± 39.4	184.3 ± 46.2	179.7 ± 40.9	0.677	0.003	0.106
HDL-C (mg/dL)	44.4 ± 10.6	43.5 ± 10.0	44.5 ± 11.5	44.5 ± 11.5	0.597	0.616	0.522
LDL-C (mg/dL)	116.6 ± 33.1	107.7 ± 28.5	112.3 ± 34.0	109.8 ± 30.6	0.714	0.020	0.954
TC/HDL-C (ratio)	4.33 ± 1.07	4.07 ± 0.80	4.26 ± 0.95	4.19 ± 1.05	0.829	0.039	0.284
LDL/HDL-C (ratio)	2.7 ± 0.7	2.5 ± 0.6	2.6 ± 0.6	2.5 ± 0.7	0.549	0.110	0.129
TG (mg/dL)	128.2 ± 67.7	115.9 ± 55.4	140.2 ± 85.5	130.2 ± 96	0.282	0.082	0.893

AST (U/L)	18.2 ± 5.0	17.5 ± 4.3	18.8 ± 5.0	19.6 ± 5.9	0.042	0.933	0.220
ALT (U/L)	14.3 ± 5.6	13.8 ± 5.9	15.2 ± 7.3	16.1 ± 7.4	0.074	0.729	0.387
GGT (U/L)	46.7 ± 30.0	42 ± 30.8	41.7 ± 30.4	43.8 ± 31.1	0.590	0.610	0.131
Insuline (uU/mL)	7.5 ± 3.3	6.9 ± 2.5	7.2 ± 4.0	7.2 ± 4.0	0.872	0.509	0.498
HOMA index	2.1 ± 1.1	1.8 ± 1.0	1.9 ± 1.2	1.8 ± 1.0	0.453	0.274	0.588
C-G index	78.0 ± 40.7	82.4 ± 48.4	80.2 ± 49.4	79.1 ± 45.7	0.694	0.156	0.125
sVCAM-1 (ng/mL)	921.6 ± 569.6	957.8 ± 824.6	939.0 ± 653.2	888.8 ± 547.8	0.737	0.920	0.724
sICAM-1 (ng/mL)	56.6 ± 23.0	60.0 ± 33.7	60.4 ± 27.7	57.9 ± 26.2	0.695	0.886	0.305
CRP (mg/L)	7.3 ± 9.9	5.7 ± 7.1	4.1 ± 4.5	3.7 ± 4.0	0.032	0.384	0.644
TNF-α (pg/mL)	1.3 ± 0.9	1.4 ± 0.5	1.2 ± 0.4	1.3 ± 0.5	0.218	0.721	0.859
IL-6 (pg/mL)	4.3 ± 4.1	3.4 ± 2.5	3.3 ± 2.6	3.3 ± 3.0	0.255	0.347	0.300
Zonulin (ng/mL)	43.1 ± 12.1	39.7 ± 8.1	43.5 ± 12.1	42.4 ± 15.2	0.409	0.141	0.497

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