

Pharmacological therapy determines the gut microbiota modulation by a pomegranate extract nutraceutical in metabolic syndrome: a randomized clinical trial

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List of Abbreviations: **AD**, anti-diabetic; **ALP**, alkaline phosphatase; **ALT**, alanine aminotransferase; **ANOVA**, analysis of variance; **AST**, aspartate-aminotransferase; **BDNF**, brain-derived neurotrophic factor; **BP**, blood pressure; **CVD**, cardiovascular disease; **GGT**, γ -glutamyl transferase; **GLP-1**, glucagon-like peptide-1; **GM**, gut microbiota; **GRS**, genetic risk score; **GWAS**, genome-wide associations studies; **HGF**, hepatocyte growth factor; **HOMA-IR**, homeostatic model assessment for insulin resistance; **HP**, anti-hypertensive; **hs-CRP**, high-sensitivity C reactive protein; **HWE**, Hardy-Weinberg equilibrium; **LBP**, lipopolysaccharide-binding protein; **LD**, linkage disequilibrium; **LDH**, lactate dehydrogenase; **LEfSe**, linear discriminant analysis effect size; **LL**, lipid-lowering; **MAF**, minor allele frequency; **MCP-1**, monocyte chemoattractant protein-1; **MetS**, metabolic syndrome; **PAI-1**, plasminogen activator inhibitor type-1; **PE**, pomegranate extract; **PICRUSt**, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; **PYY**, peptide YY; **RBP4**, retinol-binding protein-4; **sICAM**, soluble intercellular adhesion molecule-1; **SNP**, single-nucleotide polymorphism; **sVCAM**, soluble vascular adhesion molecule-1; **TBARs**, thiobarbituric acid reactive substances; **Tchol**, total cholesterol; **TG**, triglycerides; **TNF- α** , tumour necrosis alpha; **UM**, urolithin metabotype; **UPLC-ESI-QTOF-MS**, ultra-performance liquid chromatography with electrospray ionisation coupled to quadrupole time-of-flight mass spectrometry.

Keywords: Gut microbiota, inter-individual variability, metabolic syndrome, polyphenol, single-nucleotide polymorphisms.

Abstract

Scope: Poly-pharmacological therapy shapes the gut microbiota (GM) in metabolic syndrome (MetS) patients. The effects of polyphenol-rich sources in poly-medicated MetS patients are unknown.

Methods and Results: A randomized, placebo-controlled, double-blinded, and crossover trial in poly-medicated MetS patients (n=50) explored whether the effects of a pomegranate extract nutraceutical (PE, 320 mg phenolics/day for one month) were affected by the drug therapy. We evaluated, considering the lipid-lowering (LL-), anti-hypertensive (HP-) and(or) anti-diabetic (AD-) treatments: GM (16S rRNA sequencing), short-chain fatty acids, 40 inflammatory-metabolic and endotoxemia-related biomarkers, associations between biomarkers and GM with 53 cardiometabolic dysfunctions-related single-nucleotide polymorphisms (SNPs), and the influence of urolithin metabotypes (UMs). Representative SNPs-GM associations after PE included *Lactococcus* and *ClostridiumXIVa* with rs5443-*GNB3* and *ClostridiumXIVa* with rs7903146-*TCF7L2* and rs1137101-*LEPR*. PE decreased sICAM-1 in LL-patients and the lipopolysaccharide-binding protein in all the patients. PE did not affect the other patients' markers as a group or stratifying by UMs. After PE, *Lactococcus* increased in AD-, LL- and HP-patients, *Bifidobacterium* increased in LL- and AD-, while *Clostridium XIVa* decreased in non-LL- and non-HP-patients.

Conclusion: The prebiotic effect of PE depended on the medication, mainly on HP-treatments.

Targeting GM could complement MetS therapy, but the patients' drug therapy should be considered individually.

1. Introduction

Metabolic syndrome (MetS) is a cluster of metabolic traits that increase the risk of cardiovascular disease (CVD).^[1,2] The worldwide prevalence of MetS is not homogenous. MetS shows an

age-associated prevalence of 24.3% in Europe,^[3] which increases in the United States up to 35% or even 50% in individuals older than 60 years.^[4] The harmonized criteria for diagnosing MetS, according to different societies such as the International Diabetes Federation, American Heart Association, International Atherosclerosis Society, etc.^[2] include any three of the following five diagnostic criteria: i) waist circumference (abdominal adiposity) >94/80 cm (Caucasian males/females), or body mass index (BMI) > 30 kg m⁻², ii) raised triglycerides (TG) (≥ 150 mg dL⁻¹) or under specific treatment for this abnormality, iii) reduced HDL-cholesterol (HDLc) (< 40 mg dL⁻¹ in males and < 50 mg dL⁻¹ in females) or under specific treatment for reduced HDLc, iv) raised blood pressure (BP) (systolic BP ≥ 130 or diastolic BP ≥ 85 mmHg) or under treatment for hypertension, and v) raised fasting plasma glucose (≥ 100 mg dL⁻¹) or previously diagnosed type 2 diabetes. MetS is a silent disease with multi-etiological components dealing with lifestyle, including sedentary habits, smoking, alcohol abuse, high-sugar, and high-fat dietary patterns, but also with the genetic makeup of individuals.^[5] Lifestyle interventions are the first approach to prevent the worsening of CVD risk factors. However, if a lifestyle change is not sufficient, then drug therapies for treating the individual risk factors must be indicated.^[1]

Many mechanisms can contribute to the etiology of MetS.^[6] Obesity can lead to impaired intestinal wall permeability and promote metabolic endotoxemia, chronic low-grade inflammation, oxidative stress, and metabolic and pro-thrombotic abnormalities, which dramatically increase CVD risk.^[7] Furthermore, the causality of the gut microbiota in the pathogenesis of MetS has also been demonstrated through fecal transplant experiments.^[8]

Preclinical research and randomized human intervention studies suggest the multi-target potential of dietary phenolics against chronic-degenerative diseases, including MetS.^[9] Functional foods and nutraceuticals, including prebiotics, have been proposed as complementary strategies to improve MetS traits.^[10,11] In this regard, dietary (poly)phenolic-rich sources such as red wine and derived extracts,^[12,13] pomegranate,^[14,15] and other Mediterranean products^[16] have shown some benefits against MetS. However, the clinical evidence for (poly)phenol effects is limited and often controversial. As recently reviewed,^[17] the basis of this controversy lies in a large number of variables that lead to a sizeable

inter-individual variability: the type of phenolic compound and its dietary food matrix, the two-way interaction between the gut microbiota and phenolics, the individuals' genetic makeup and possible associations of genetic polymorphisms with gut microbial groups.^[18] Besides, some polyphenols are metabolized by particular gut microbial ecologies that produce specific microbial metabolites (postbiotics) with distinctive activity. This unique metabolism leads to the so-called polyphenol-related metabotypes, which contribute to explain the different individuals' responses to polyphenols consumption. This is the case of ellagic acid that yields the urolithin metabotypes (UMs), and isoflavones that gives rise to the equol and non-equol producer metabotypes.^[17,19,20] Finally, the possible effect of (poly)phenols on human health will much depend on whether the approach is preventive in healthy or at-risk individuals or adjuvant in medicated patients.

Targeting gut dysbiosis has been proposed to improve MetS traits.^[21] However, there is no full consensus regarding the term "gut dysbiosis" ("imbalanced relative abundances" of microbial groups) and "healthy microbiome".^[22,23] In this regard, we recently reported how the gut microbiota in MetS patients was shaped by their poly-pharmacological treatments associated with their main traits, being hypertension and(or) its associated medication, the primary trait involved in the shaping of the gut microbiota. Besides, an overabundance of lipopolysaccharide-producing microbial groups from the Proteobacteria phylum was observed.^[24] Therefore, there is no distinctive gut microbiota signature in MetS patients under drug therapy to serve as a standard target for prebiotics.^[24]

Consequently, in the present study, our primary aim was to evaluate whether the modulation of the gut microbiota after consumption of a polyphenol-rich pomegranate extract (PE) nutraceutical with known prebiotic effects could be affected by the poly-pharmacological treatments in MetS patients.^[25,26] Besides, as secondary outcomes, and always considering pharmacological therapy as a possible critical variable, we also aimed to explore: i) the effect of PE consumption on metabolism, inflammation, and endotoxemia-related markers, ii) the involvement of UMs in the impact of PE, and iii) the possible associations of 53 SNPs from 43 genes mainly related to obesity and cardiometabolic diseases with the above markers and also with the gut microbiota of the patients at baseline and after PE consumption.

2. Materials and Methods

2.1. Patients and Study Design

The trial protocol was approved by the Reina Sofía University Hospital Clinical Ethics Committee (Murcia, Spain) and the Spanish National Research Council's Bioethics Committee (Madrid, Spain) (reference AGL2015-64124-R). The clinical trial was conducted following the guidelines established in the Declaration of Helsinki (1975) and its amendments. Eligible participants were adult metabolic syndrome (MetS) patients under secondary pharmacological prevention and without previous cardiovascular disease events. The diagnosis of MetS was based on the joint interim statement of the following societies: International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity.^[2] The study was fully explained to the patients who gave their written informed consent before participating. These patients recently participated in a baseline study that explored the associations between their gut microbiota and their primary CVD risk factor and (or) their associated medication.^[24] The protocol was registered at clinicaltrials.gov as NCT04075032. The patients were randomly divided into two arms using a computerized random number list. The intervention design was double-blind, crossover, randomized, and placebo-controlled, one month each, with a total of 3-months of follow-up (**Figure 1**). The primary outcome in this pilot trial was to evaluate the change in the composition of the patients' fecal microbiota after the consumption of a PE nutraceutical. Unfortunately, we were forced to stop recruiting patients, and unable to increase the sample size due to the current pandemic situation. However, the current sample size was similar to that of a previous study with 49 overweight-obese individuals where significant prebiotic and blood lipid-lowering effects were observed after consuming the same PE nutraceutical.^[19,26]

Exclusion criteria were: pregnancy or lactation, established cardiovascular disease (coronary or peripheral artery disease, stroke, etc.), previous gastrointestinal surgery, consumption of ellagitannin-rich sources (e.g., pomegranates, strawberries, walnuts, raspberries, blackberries) or dietary supplements such as nutraceuticals or pre/probiotics 1 week before the inclusion and during the trial, as well as the use of antibiotics within 1 month prior and during the study. Patients were asked not to change their dietary

habits and lifestyle, which had previously been specified in questionnaires provided by the physicians (adherence to the Mediterranean diet, moderate physical activity, smoking cessation, etc.). A representative (and summarized) daily dietary pattern (approximately 1,750-2,000 kcal/day) consisted of i) a cup of skimmed milk and 40 g bread for breakfast; ii) a serving of fruit before lunch; iii) a serving of vegetables + rice or legumes (chickpeas, lentils, etc.) + 150 g fish (or 100 g breast chicken) + a serving of fruit for lunch; and iv) a serving of vegetables + 200 g boiled potatoes + 1 boiled egg (or 150 g fish or 100 g breast chicken) + a serving of fruit. The specific food items consumed for three days before collecting biological samples at each time-point were also recorded. The consumption of ellagitannin-rich foods during the study was prohibited from a list provided for this purpose.

CVD risk factors (dyslipidemia, type-2 diabetes, and hypertension) and the medication to treat them are unavoidable linked variables in these patients (i.e., we cannot dissociate the specific effect due to the patients' pathological condition vs. the isolated effect of the medication). Therefore, hereafter we refer to AD- vs. non-AD, LL- vs. non-LL, and HP- vs. non-HP consumers as those patients treated vs. not treated with oral anti-diabetic, lipid-lowering, and anti-hypertensive drugs, respectively.

2.2. Study Products

The PE nutraceutical and placebo (microcrystalline cellulose) (Laboratorios Admira S.L., Alcantarilla, Murcia, Spain) were provided in identical hard gelatine capsules containing 450 mg of the corresponding product and bottled with a specific blind code for patients and researchers. The phenolic composition of PE was analyzed by HPLC-DAD-ESI-MS/MS as previously described,^[27] and can be found in Table S1, Supporting Information. The total content of ellagitannins per capsule of PE was estimated by acid hydrolysis, resulting: 72.9±1.1 mg free ellagic acid, 69.3±0.9 mg gallagic acid dilactone, 10.3±0.1 mg sanguisorbic acid, 3.1±0.04 mg gallic acid, 2.9±0.2 mg punicalin, and 1.9±0.2 mg valoneic acid dilactone. The total content of phenolic compounds per capsule was ~160 mg.

2.3. Dosage Information

Each patient consumed daily two capsules of PE (900 mg extract day⁻¹, containing 320 mg phenolics) or placebo (900 mg microcrystalline cellulose day⁻¹) for 1 month in a crossover fashion with 1 month of wash-out between treatments (Figure 1). The same PE was also assayed in previous trials with colorectal cancer patients,^[28-30] and overweight-obese subjects.^[19,26] However, physicians were reluctant to administer more than 2 capsules day⁻¹ to prevent potential interactions with the poly-medication since it had not been previously evaluated. The patients were asked to return the remaining capsules at each visit for evaluation of compliance. The daily phenolic dose was approximately equivalent to 234 mL of pomegranate juice concerning the phenolic content.^[27]

2.4. Sampling Procedures

Fasting peripheral blood samples were collected between 8 and 9 AM to minimize circadian variations, in the supine position and after 20 min of rest. Patients also provided urine and feces, collected in the morning of each visit. Four time-points were analyzed in the present trial (urine, plasma, and feces), i.e., baseline, 4 weeks (T1), 8 weeks (T2), and 12 weeks (T3) (Figure 1). Besides, the patients also provided a urine sample 3 days after starting the consumption of PE to determine their urolithin metabotype (UM) (Figure 1). Blood samples were collected in vacutainers, with or without EDTA, to obtain the plasma or serum, respectively, after centrifuging at 2,000 x g at 4 °C for 10 min. All samples were stored at -80 °C until further analysis.

2.5. Serobiochemical Variables, Metabolic, Inflammatory and Endotoxemia Markers

Ghrelin, peptide YY (PYY), tumor necrosis alpha (TNF- α), leptin, adiponectin, soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), retinol-binding protein-4 (RBP4), glucagon-like peptide-1 (GLP-1), interleukin-6 (IL-6), plasminogen activator inhibitor type-1 (PAI-1), resistin, brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), monocyte chemoattractant protein-1 (MCP-1), P-selectin, and C-peptide were determined in plasma samples by immunoassay in a customized panel (ThermoFisher, USA) with Luminex xMAP technology,

following manufacturer's instructions. The acute phase liver reactant lipopolysaccharide-binding protein (LBP), a surrogate marker of metabolic endotoxemia since plasma LPS determination shows a number of limitations, mainly due to the presence of endogenous inhibitors^[31,32] LBP was quantified using a commercial ELISA kit (HycultBiotech, Uden, The Netherlands) as described elsewhere.^[26] All samples were analyzed in triplicate, and the intra- and inter-assay coefficients of variation (CVs) were below 10% for all markers. Red and white cell series were determined from whole blood by an automated hematology analyzer (LH 780; Beckman Coulter, Fullerton, CA, USA). Serum samples were used to measure serobiochemical variables: total cholesterol (Tchol), LDL-cholesterol (LDLc), HDLc, TG, glucose, protein, alkaline phosphatase (ALP), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), γ -glutamyl transferase (GGT), urea, creatinine, albumin, bilirubin, sodium, chlorine, calcium, potassium and phosphorus using automated biochemical auto-analyzers (Advia Systems, Siemens Healthcare Diagnostic Inc., Deerfield, IL). Insulin was quantified with the IMMULITE 2000 analyzer (DPC, LA, USA), and insulin resistance was calculated with the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR).

2.6. Analysis of Urolithins in Urine and Patients Metabotypes

Urine samples (baseline, 3 days, T1, T2, and T3) (Figure 1) were processed and analyzed by UPLC-ESI-QTOF-MS to determine the metabolic profiling of urolithins and the corresponding patients' UMs as previously reported.^[28]

2.7. Single Nucleotide Polymorphisms (SNPs)

2.7.1. SNPs Selection, DNA Extraction, and Genotyping

Sixty candidate genes and single-nucleotide polymorphisms (SNPs) related to MetS (i.e., involved in cardiovascular diseases, obesity, diabetes, inflammation, etc.) were selected after reviewing the published literature and using the Single Nucleotide Polymorphism Database (dbSNP).^[5,33-35] Phenotypes associated with risk alleles, according to the literature, were used to establish the allele risk and whether a variant was favorable or unfavorable for each SNP. The potential biological and regulatory functions were analyzed

with the HaploReg v4.1 and Regulome DB databases (Table S2, Supporting Information).^[36,37] Genomic DNA was extracted from blood samples using the NucleoSpin[®] Blood Kit (Macherey-Nagel, Germany) and frozen at -80 °C until further analysis. Genotyping was performed at the GENYAL Platform (IMDEA-Food, Madrid, Spain) using the OpenArray[™] AccuFill[™] System (Life Technologies Inc. Carlsbad, CA, USA) and the TaqMan Genotyper Software v1.3 (autocaller confidence level > 90%) for data analysis, as previously described.^[38]

2.7.2. *Quality Control Analysis of SNPs*

SNPs were screened for eligibility using quality control criteria of call rate > 90%, Hardy-Weinberg equilibrium (HWE) ($P > 0.05$), minor allele frequency (MAF) > 5%, and no linkage disequilibrium (LD), which were analyzed with the web tool SNPStats.^[39] Six SNPs were removed after all quality control checks: rs1800896-*IL10* and rs5082-*APOA2* for not accomplishing HWE, rs16139-*NPY* because MAF was < 5%, and rs9930333-*FTO*, rs9935401-*FTO*, rs9928094-*FTO* were in LD. Finally, 54 SNPs were selected to be analyzed in this group of patients (Table S2, Supporting Information).

2.7.3. *SNPs Analysis*

Associations between SNPs and markers related to metabolism, inflammation, and endotoxemia, as well as some relevant bacterial groups, were analyzed using a linear regression model using SNPstats.^[39] Age, sex, and drug therapy were considered as confounding factors. The inheritance model chosen for each SNP was selected according to the variants' effect (favorable and unfavorable) found in the literature: i) dominant model (AA vs. Aa + aa) when homozygous minor allele and heterozygous had the same effect, ii) recessive model (AA + Aa vs. aa) when homozygous wild-type allele and heterozygous had the same effect, and iii) codominant model (AA vs. Aa vs. aa) when each genotype had a different effect. Akaike information criteria (AIC) was used to choose the inheritance model that best fits the data when no consensus was available for a given SNP.

An unweighted genetic risk score (GRS) was also elaborated. The GRS in MetS patients was compared with that of a healthy children-adolescence cohort (5 to 17 years old) that were genotyped for the same SNPs and using the same genotyping platform (Table S3, Supporting Information).^[40] GRS was calculated by adding the number of risk alleles of each volunteer for each SNP (i.e., 0 for no risk allele, 1 for one risk allele, 2 for two risk alleles) as previously reported.^[40-43] Variants with a doubtful or unknown effect (variants in black color in Tables S2, S3) and participants with no complete genotyped data were excluded from the GRS calculation. The SNP rs1801253-*ADRB1* was also eliminated because of the low call rate in the children-adolescence cohort. Finally, the GRS score was calculated for 46 SNPs in 50 MetS patients and 359 children-adolescents.

2.8. Short Chain Fatty Acids (SCFAs) Analysis

SCFAs were measured in fecal samples using a gas chromatography-mass spectrometer (GC-MS) (Agilent 7890A coupled with an Agilent 5975C mass selective detector) (Agilent Technologies, Santa Clara, CA, USA), as described elsewhere.^[24] Identification and quantification of SCFAs were performed with the available standards butyric acid, isobutyric acid, valeric acid, isovaleric acid, propionic acid, and acetic acid.

2.9. Gut Microbiota Analysis

Bacterial DNA was isolated from stool samples following the protocol of the NucleoSpin[®] Tissue DNA Purification Kit (Macherey-Nagel, Germany). The V3-V4 variable region of 16S rRNA gene was sequenced to analyze the gut microbiota composition using a read length of 2 x 300 bp paired-end run (MiSeq Reagent Kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Spain), following Illumina protocols (Illumina Inc., San Diego, CA, USA). Chimeric sequences and sequences that could not be aligned were removed from the data set. Final reads per sample were $99,929 \pm 55,321$. Data processing to obtain taxonomic classification, alpha-diversity and richness (Shannon and Chao1 indexes), and potential bacterial functions (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)) were carried out as previously described.^[24] Shannon and Chao1 indexes

were estimated based on a randomly selected 30,992 reads per sample. Rarefaction curves were calculated with RDPipeline (<http://pyro.cme.msu.edu/>), and beta-diversity was performed based on the Bray-Curtis dissimilarity index. The quantitative analysis of *Gordonibacter* by qPCR was determined as previously described.^[44]

2.10. Statistical Analysis

SPSS Software v.26.0 (SPSS Inc., Chicago, IL, USA) and Sigma Plot v.13.0 (Systat Software, San Jose, CA, USA) were used to perform the statistical analysis and the data graphs, respectively. The Shapiro-Wilk test was used to assess data normality. All possible combinations between variables and groups of patients (as one group or according to their medication) were carried out. However, only results in which a significant association was found are shown later. Intra-group comparisons were carried out using repeated measured analysis of variance (ANOVA) or a Friedman's test, when data were normally or non-normally distributed, respectively. Inter-group multiple comparisons were analyzed by *t*-test Bonferroni or Tukey's test, according to normal or non-normal data distribution, respectively. All the analyses were adjusted by age and sex. Comparisons between two independent groups (for example, LL-medication vs. no LL-medication, UM-A vs. UM-B, etc.) were tested using the independent *t*-test or the Mann-Whitney *U* test when data distribution was normal or non-normal, respectively. Differences between two dependent groups (before vs. after PE or placebo, etc.) were explored using the paired Student's *t*-test, or Wilcoxon Signed Rank Test when data were normally or non-normally distributed, respectively. Chi-square (X^2) test was used to study relationships between categorical variables (metabotype vs. medication, etc.). Spearman's rank correlation was used to analyze possible associations with some variables (LBP vs. bacterial groups, SCFAs vs. metabolic or inflammatory markers, etc.). Permutation multivariate analysis of variance (PERMANOVA) was used with 9,999 permutations to test significance in beta diversity between groups. Differentiation of specific bacterial taxa in relative abundance between two groups (before vs. after PE or placebo consumption, medication vs. no medication, etc.) was carried out with the Linear Discriminant Analysis (LDA) Effect Size (LEfSe)

algorithm using the online interface Galaxy (<http://huttenhower.sph.harvard.edu/galaxy/root>). Statistical significance was set at $P < 0.05$. Marginal significance was considered when $0.1 > P > 0.05$.

3. Results

3.1. Characterization of the Study Population

Sixty-nine eligible MetS patients were contacted, and 62 finally agreed to participate. All these patients were under poly-pharmacological treatment against hypertension, and(or) diabetes, and(or) dyslipidemia to prevent cardiovascular events as previously reported.^[24] The poly-medication consisted of lipid-lowering drugs (LL) (statins, fibrates, and ezetimibe), anti-hypertensive drugs (HP) (angiotensin II receptor blockers, angiotensin-converting enzyme inhibitors, diuretics, β -1 adrenergic blockers, calcium channel blockers) and oral anti-diabetics (AD) (metformin, glucosurics, incretins, and others) (Table S4, Supporting Information). **Table 1** shows the demographic characteristics and baseline laboratory values of the patients. Some of these baseline values have been recently reported.^[24]

A total of 50 patients (24 women and 26 men) completed the study with nearly 100% protocol compliance. Significantly sex-specific differences were found for HDLc, leptin, and adiponectin values, higher in women than men. In contrast, insulin, HOMA-IR, LBP, creatinine, and GGT values were higher in men (Table 1). Besides, Table S5 (Supporting Information) shows the baseline characteristics of the patients who completed the trial, according to their allocation in the two arms of the trial design (Figure 1). Significant differences (although small and clinically irrelevant) were observed between both arms in GGT, $\text{TNF}\alpha$, and phosphorus (Table S5).

3.2. Laboratory Values at Baseline and After PE Consumption according to Drug Therapy

Serobiochemical markers related to hepatic, renal, and muscle damage did not show any clinically relevant change throughout the trial (results not shown). No side effects were reported by any patient upon consumption of PE or placebo (intolerance, dyspepsia, nausea, etc.).

We explored possible associations between the baseline levels of the analytes shown in Table 1 with the pharmacological therapy followed by the MetS patients. From all the analyses performed, **Table 2** only shows those inflammatory and metabolic markers with significantly different values at baseline, depending on the patients' drug therapy. The concentration of sICAM-1 was lower in LL-treated vs. non-LL treated patients, and PYY levels were higher in AD- vs. non-AD consumers. Remarkably, HP-consumers (i.e., hypertensive patients) showed higher levels of ghrelin, resistin, leptin, and HGF (Table 2). Therefore, hypertension and (or) the intake of anti-hypertensive drugs was associated with markers related to higher food intake, insulin resistance, and metabolic abnormalities. Besides, significant differences in baseline LBP values were only found between HP- and non-HP consumers.

After placebo or PE consumption, all possible associations between the analytes and the patients as a whole group or after clustering according to the type of drugs consumed (LL vs non-LL, AD vs non-AD, HP vs non-HP) were explored, but only those results with statistical significance are shown. Placebo consumption and wash-out did not modify any analyte from Table 1. After PE consumption, no significant differences were found within-subjects analyses or between time-points in most of the metabolic and inflammatory markers or serobiochemical variables. From all the analytes explored, only sICAM-1 values in LL-treated patients ($P = 0.006$) decreased significantly after PE consumption (Table 2). Besides, PE modified the baseline associations between drug therapy and sICAM-1, PYY, and HGF, but did not change those for ghrelin, resistin, and leptin (Table 2). Only after PE consumption, within-subjects analyses showed a small but significant decrease in LBP values (**Figure 2**). This decrease was quantitatively similar in all the patients, and thus, it was independent of the patients' medication.

We also explored possible interactions between SNPs (Table S2, Supporting Information) and all the analytes from Table 1, before and after PE consumption, and considering the different patient's medication, but no clear interactions were found (results not shown).

The same approach was followed for SCFAs. Their levels at baseline and after placebo or PE consumption, as well as all the possible associations with all the analytes and patients' medications,

were explored. At baseline, significantly lower levels of butyric (1.7-fold, $P = 0.004$) and acetic (1.8-fold, $P = 0.007$) acids were observed in LL vs. non-LL consumers (results not shown). We also explored potential baseline associations between SCFAs and the analytes listed in Table 1, depending on the patients' medication and the different time-points of the trial (Figure 1). However, neither consistent baseline associations were found, nor did PE consumption significantly modify fecal SCFAs (results not shown).

3.3. The Prebiotic Effect of PE Depends on the Patients' Drug Therapy

The gut microbiota of the patients as whole or grouped, according to their medication, remained unchanged after placebo consumption and wash-out (results not shown).

We first explored the possible modulation of the gut microbiota after PE consumption in the whole group. In this case, LDA (Figure 3A) and cladogram (Figure S1, Supporting Information) from LEfSe analyses showed the modulation of some bacterial groups, including the well-known probiotic genera *Lactococcus* (family Streptococcaceae) and *Bifidobacterium* (family Bifidobacteriaceae). Other groups also increased after PE consumption, such as the genera *Hespellia* (family Lachnospiraceae), *Aestuariuspira* (family Rhodospirillaceae), and *Tessaracoccus* (family Propionibacteriaceae) (Figure 3A, Figure S1). On the other hand, the abundance of the *Bacillaceae* family and the genera *ClostridiumXIVa* (family Clostridiaceae) and *Weisella* (family Leuconostocaceae) decreased after PE consumption (Figure 3A, Figure S1). The Firmicutes to Bacteroidetes ratio (F/B), alpha-diversity (Shannon and Chao 1 indexes), and metabolic functions after PICRUST analysis remained unchanged after PE consumption (results not shown). No significant differences in beta-diversity before and after PE treatment were observed at the genus, family, and phylum level ($P > 0.05$). Rarefaction curves were similar before and after PE consumption showing no differences in diversity (Figure S2, Supporting Information).

When the patients were stratified according to their pharmacological treatments, the composition of the gut microbiota differed depending on the presence or not of each medication (LDA in Figure S3 and cladogram in Figure S4, Supporting Information). The corresponding LDA from LEfSe analyses

showed that PE consumption did not significantly modify the gut microbiota of non-AD consumers (Figure 3B, Figure S1). In the rest of the groups, the prebiotic effect of PE differed according to the patients' drug therapy (Figure 3C-G, Figure S1). For example, PE consumption increased *Bifidobacterium* in all the groups except in those patients treated with HP (Figure 3G, Figure S1). The genus *Lactococcus* increased after PE consumption, but only in AD- (Figure 3C, Figure S1), LL- (Figure 3E, Figure S1), and HP-treated patients (Figure 3G, Figure S1). Also, PE consumption increased the genus *Hespellia* in AD- and HP-, but not in LL-treated patients. Another contrasting effect was observed in the genus *Weissella*, from the family Leuconostocaceae, whose abundance remained unaltered in AD- (Figure 3C), decreased in LL- (Figure 3E, Figure S1) and increased in HP-treated patients (Figure 3G, Figure S1), after PE consumption.

Other modifications, specifically associated with the medication, were those related to the increase of *Buttiauxella* (family Enterobacteriaceae) in AD-, *Corynebacterium* (family Corynebacteriaceae) in non-LL (Figure 3D, Figure S1), *Campylobacter* (family Campylobacteraceae) in LL- (Figure 3E, Figure S1), and *Aestuariuspira* in HP-treated patients (Figure 3G, Figure S1). However, the latter genus, absent at baseline, was present only in 6 patients after PE consumption. The increase of *Tessaracoccus* was only observed in the non-HP group (Figure 3F, Figure S1), and *Alloscardovia* (family Bifidobacteriaceae) in HP-treated patients (Figure 3G, Figure S1), after PE consumption. Regarding the decrease of the abundance of microbial groups after PE consumption, the genus *Delftia* (family Comamonaceae) decreased in AD- (Figure 3C), the genera mentioned above *Weissella* in LL- (Figure 3E, Figure S1), *Bacillus* in HP- (Figure 3G, Figure S1) and *Clostridium XIVa* in non-LL and non-HP treated patients (Figure 3D and 3F, respectively; Figure S1). The F/B ratio, alpha-diversity (Shannon and Chao1 indexes) and rarefaction curves were evaluated for each group at each time-point, and no significant differences were observed. At baseline, beta-diversity showed significant differences between HP and non-HP at the phylum level ($P = 0.015$), and marginally significant at the genus level ($P = 0.056$). The differences were significant when comparing AD vs non-AD at the family and genus levels ($P = 0.020$ and $P = 0.008$, respectively). This dissimilarity was not found after placebo or PE consumption. The analyses of the metabolic functions associated with bacterial groups (PICRUSt

analysis) revealed that only the pathway dealing with a reduction of metabolism of cofactors and vitamins was observed after PE consumption in LL-consumers. A correlation analysis (Spearman's rank correlation) was made between this metabolic pathway (KEGG2_Metabolism_of_cofactors_and_vitamins) and the microbial groups modulated by PE consumption in LL-consumers. However, no significant correlations were found.

Figure 4 shows the relative abundance of representative bacterial groups before and after PE consumption, i.e., *Bifidobacterium* (Figure 4A-C), *ClostridiumXIVa*, (Figure 4D-F), and *Lactococcus* (Figure 4G-I), highlighting the specific change after PE, depending on the patients' medication. AD-treatments did not affect the prebiotic effect of PE on these microbial groups (Figure 4C,F,I). However, in the case of *Bifidobacterium*, LL- tended to hamper (Figure 4A), and HP- treatments significantly hampered (Figure 4B) the prebiotic effect of PE. In the case of *ClostridiumXIVa*, both LL- (Figure 4D) and HP- (Figure 4E) treatments hampered the modulatory effect of PE. Although each drug treatment seemed to promote PE effects on *Lactococcus* (Figure 4G-I), the increase did not significantly differ from that observed in the patients that did not consume each specific medication.

3.4. Associations between SNPs and Gut Microbial Groups

We next tried to unravel possible associations between the panel of SNPs (Table S2, Supporting Information) and representative bacterial taxa that changed after PE consumption (*Bifidobacterium*, *Lactococcus*, *ClostridiumXIVa*, *Weisella*, and *Corynebacterium*). Tables S5, S6, and S7, Supporting Information, show those significant associations between the relative abundance of microbial groups and SNPs (favorable, unfavorable, and neutral genotypes) at baseline and after PE consumption, and according to the drug therapy. Several significant associations were found, but only a few were consistently observed in the three groups of patients. The favorable variants of rs4343-*ACE* (A/A) and rs1801253-*ADRB1* (C/G-G/G) were associated with a higher abundance of *Corynebacterium* at baseline, but after PE consumption, the association for both SNPs only persisted in AD-consumers. At baseline, the unfavorable variant of rs662799-*APOA5* (A/G-G/G) was associated with higher *Lactococcus* levels. However, this association was not observed after PE consumption. Moreover, after

PE consumption, a higher abundance of *Lactococcus* was associated with the unfavorable variant of rs5443-*GNB3* (T/T), and a lower abundance of *ClostridiumXIVa* was associated with the unfavorable variants of both rs5443-*GNB3* (C/T-T/T) and rs7903146-*TCF7L2* (C/T-T/T). Finally, a higher abundance of *Weisella* and *ClostridiumXIVa* were associated with the favorable variants of rs8061518-*FTO* (A/G-G/G) and rs1137101-*LEPR* (A/G-G/G), respectively, after PE consumption. In the rest of the cases, a miscellaneous of sporadic associations were observed for each time-point (either baseline or PE), microbial group, and medication (Tables S5, S6, and S7, Supporting Information).

3.5. No differences in the genetic risk score (GRS) of MetS patients compared to a healthy cohort

We calculated a GRS and compared it with that of a healthy cohort in an attempt to assess the possible contribution of the selected panel of SNPs to the susceptibility to having metabolic syndrome. In other words, the GRS was calculated to assess whether having certain genetic variants associated with MetS is reflected with a greater predisposition to suffer it, compared to a healthy population (for example, a cohort of healthy children-adolescents), so that significant differences should be clearly observed between both GRS. Thus, a GRS with 46 SNPs from genes mainly related to obesity and cardiometabolic dysfunctions was calculated as described in the Materials and Methods Section. The maximum and minimum values of GRS were 0 and 92 points, respectively (higher scores indicated a theoretically higher genetic risk dealing with obesity, diabetes, inflammation, and MetS). The GRS for this group of MetS patients was compared with that of a healthy children-adolescents cohort (Tables S2, and S3, Supporting Information). The patients' GRS was 42.5 ± 3.9 points (mean \pm SD), ranging from 34 to 54. In the case of the children-adolescents, the GRS was 42.8 ± 4.1 , ranging from 33 to 54. Therefore, both GRSs were unexpectedly nearly identical.

3.6. Urolithin metabotypes (UMs) and urolithin-producing bacteria in MetS patients

In the present study, the majority of patients were UM-A (72%) compared to UM-B (26%) and UM-0 (2%, i.e., only one patient, who was excluded from further association analyses). Consequently, further comparisons were carried out between UM-A and UM-B.

No significant associations were found between UM-A or UM-B and the CVD risk factors, i.e., in this specific group of patients, belonging to a particular UM was not related to a characteristic CVD trait or associated medication. No significant differences were found between UMs and the baseline levels of serobiochemical variables, metabolic-inflammatory markers, LBP, and SCFAs (results not shown). Finally, the effects after consumption of PE on any analyte and the intestinal microbiota were not different when considering all the patients as a single group or after grouping them according to their UMs. Besides, the higher or lower urine or fecal excretion of total or individual urolithins (mainly urolithin A, isourolithin A, urolithin B, and urolithin C) was not associated either with the level of analytes after PE (results not shown).

We also explored in this group of patients the relative abundances of the specific urolithin-producing genera *Gordonibacter* (predominant in UM-A), and *Ellagibacter* (a biomarker of UM-B). Besides, the possible influence of drug therapy was also assessed. At baseline, the gut microbiota of UM-A patients was enriched in *Gordonibacter* ($P = 0.000$), and *Ellagibacter* abundance was much higher in UM-B patients ($P = 0.009$). The percentage of relative abundance of *Gordonibacter* and *Ellagibacter* in UM-A patients was 0.063 ± 0.095 and 0.061 ± 0.121 , respectively, while in UM-B patients the abundance was 0.003 ± 0.006 and 0.136 ± 0.106 (mean \pm SD). Although the relative abundance differed between UMs, *Gordonibacter* qPCR analysis revealed that the quantity of *Gordonibacter* was similar in both UMs. Regarding the medication, after analyzing all the associations, only non-AD consumers showed a higher *Ellagibacter* abundance than AD consumers, but the value was only marginally significant ($P = 0.053$).

After PE consumption, the abundance of these genera did not increase significantly. However, a marginal statistical difference was observed in the abundance of *Gordonibacter* in UM-A patients ($P = 0.061$), and *Ellagibacter* in UM-B patients ($P = 0.086$). In non-HP consumers, the increase of *Ellagibacter* abundance was higher than HP consumers ($P = 0.034$). No significant differences were found in the changes of *Gordonibacter* and *Ellagibacter* after PE consumption in the rest of the medications.

4. Discussion

There is abundant research in animal models that describe the benefits of dietary polyphenol sources, including pomegranate, against different MetS traits (adiposity, hypertension, diabetes, and dyslipidemia).^[45-47] Also, various randomized human interventions with dietary polyphenols or polyphenol-rich foods (resveratrol, tart cherry juice, berries, green tea, red wine, etc.) have shown a variety of effects against subjects with MetS features, often with limited or even controversial results.^[48-51] Although some trials with polyphenol-derived products have recruited some medicated MetS patients,^[52,53] in general, the component “under drug therapy” has not been sufficiently addressed since either it is usually present in the exclusion criteria of the protocols or the statistical analyses do not include the type of medication as a crucial variable.

Regarding pomegranate, its effect on diagnosed MetS patients has been scarcely approached. The effects have been mainly attributed to the “antioxidant” phenolic fraction (ellagitannins) of pomegranate. However, these phenolics are not bioavailable but extensively metabolized by the gut microbiota to yield urolithins, postbiotics with acknowledged biological activity.^[17,25,54,55] The evidence in humans associated with ellagitannin-rich sources through randomized clinical trials is still limited.^[56] Nevertheless, we have observed lipid-lowering, anti-inflammatory, prebiotic, and cancer chemopreventive activities for the same pomegranate extract (PE) nutraceutical assayed here in previous clinical trials with non-medicated overweight-obese subjects^[19,26] and newly-diagnosed colorectal patient.^[29,30,57]

We have recently reported the heterogeneity of the gut microbial profile in MetS patients as a function of their drug treatments and(or) primary CVD risk factors (diabetes, hypertension, and dyslipidemia).^[24] In this regard, the drug therapy as a critical variable, and especially in MetS, has not generally been addressed since preclinical research with MetS models does not include drug therapy, and human interventions usually omit pharmacological treatments or mix medicated patients with non-medicated ones, or directly exclude MetS patients under medication.^[14,15,52,58] Therefore, the challenging objective in the present trial was to evaluate possible adjuvant effects of a pomegranate extract (PE) nutraceutical in poly-medicated MetS patients, something unexplored so far. Besides, the

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possible prebiotic effects of polyphenols or derived food sources or nutraceuticals in poly-medicated MetS patients had not been approached so far.

In the context of pomegranate and diagnosed MetS, Kojadinovic et al.^[14] did not find significant effects on the main traits such as raised glucose, dyslipidemia, or blood pressure upon pomegranate juice consumption (300 mL day⁻¹) for 6 weeks in non-medicated women with MetS traits (n=23). These authors found an increase of arachidonic acid saturated fatty acids, as well as a specific modest decrease of thiobarbituric acid reactive substances (TBARs) in erythrocytes. Another study showed that pomegranate juice (500 mL day⁻¹) for one week modestly lowered high-sensitivity C reactive protein (hs-CRP) levels and blood pressure in non-medicated individuals with MetS traits (n=30). However, a significant increase in TGs and very-low LDLc (VLDLc) concentrations, as well as a borderline significant increase in HOMA-IR values, were also observed.^[15] None of these studies explored possible associations between pomegranate phenolics and the effects observed, neither the potential impact on the gut microbiota nor the effect of drug therapy since it was within the exclusion criteria.

We first attempted to identify possible clusters, through PCA analyses, which could integrate the global differential effects of PE consumption on all the quantitative variables studied as a function of patients' medication. However, the only clusters identified were related to patients' medication but not as a consequence of PE consumption (results not shown).

In the present trial, in agreement with the above studies, we did not find any significant effect of the PE nutraceutical on blood pressure, lipids, and a panel of inflammatory and metabolic-related markers (Table 1), except sICAM-1 in LL-treated patients. sICAM belongs to the intercellular adhesion molecules expressed in the healthy endothelium. It is activated by cytokines and thus during the initial atherosclerosis stages. sICAM is one of the best immunoglobulin representing the risk in primary prevention of CVD, showing a vascular inflammation status.^[59]

The same PE nutraceutical was reported to improve dose-dependently the blood lipid profile in overweight-obese subjects, but only in those belonging to the so-called urolithin metabotype-B (UM-B).^[19] Recent evidence suggests that these UMs can be biomarkers of particular microbial ecologies,^[60] which could be useful to explain the inter-individual variability upon consumption of

polyphenols.^[17] However, we did not observe significant effects either when the patients were clustered according to their UMs, although we cannot discard that the dose was not enough to exert effects in this poly-medicated group. Notably, the UMs distribution was different (lower proportion of UM-B individuals) from that previously reported in MetS patients with lower medication.^[61] Therefore, this suggests that the poly-pharmacological treatments disturb the reported UMs distribution in the population,^[62] which deserves further investigation.

A small but significant decrease of the endotoxemia-surrogate marker LBP was observed in all the patients after PE intervention, independently of their medication and UMs. These results agree with our previous study in overweight-obese subjects and colorectal cancer patients in which the same PE also decreased LBP values.^[26,30] However, while plasma LBP reduction was significantly associated with both *Faecalibacterium* and *Odoribacter* increase and *Parvimonas* decrease in non-medicated overweight-obese individuals, no clear association between LBP decrease and the modulation of the gut microbiota was found in the present study. Despite the reduction of plasma LBP levels, PE intervention did not revert the baseline overabundance of the phylum Proteobacteria and representative genera such as *Escherichia-Shigella* in HP-consumers.^[24]

The prebiotic effects of PE were somewhat heterogeneous, depending on the patients' drug therapy. F/B ratio, the Shannon and Chao1 indexes and beta-diversity seemed not to reflect the changes of the gut microbiota after PE consumption. However, *Bifidobacterium* and *Lactococcus*, genera that include well-known probiotics in functional foods and dietary supplements,^[63] were increased after PE. Some species from these genera have been reported to counteract obesity and metabolic dysfunctions in animal models^[64] and obese individuals,^[65] including non-medicated MetS patients.^[66] The increase of *Lactococcus* seemed to be favored by the medication, whereas HP treatments especially hampered the rise of *Bifidobacterium* after PE. In the case of non-LL and non-HP consumers, PE consumption decreased the abundance of *ClostridiumXIVa*, which was prevented in LL- and HP-consumers. The *ClostridiumXIVa* cluster, also known as *Clostridium coccoides-Eubacterium rectale* group,^[67] consists of 21 non-sporulating and spore-forming species. This cluster involves contrasting species, and their potential effects should be evaluated individually.^[67] Nevertheless, *C. coccoides* abundance has been

reported to be higher in (non-medicated) MetS vs. non-MetS obese individuals^[68] and correlated with various traits associated with MetS such as BMI, TGs, etc.^[68,69]

Genome-wide associations studies (GWAS) have identified SNPs associated with MetS traits. For example, the rs7903146-*TCF7L2* and rs9939609-*FTO* SNPs are major candidate genes predisposing to MetS,^[70] as well as variants of the genes *ADIPOQ*, *SREBF1* and, *GNB3*, among others, which are associated with diabetes, hypertension, and dyslipidemia in MetS.^[5] The significance of some gene-nutrient interactions (adiponectin, leptin receptor, transcription factor 7-like 2 (*TCF7L2*), among others) has been reported to influence insulin resistance in MetS patients.^[71] However, the role of gene-nutrient interaction through interventions with polyphenol-rich sources in MetS patients has been scarcely approached. Most interventional studies dealing with gene-nutrient interactions focus on only one or a few SNPs. In this regard, Rezazadeh et al.^[72] reported in 49 non-medicated women with MetS features the decrease of triglycerides vs. placebo in those MetS that carried the A allele of rs9939609-*FTO* upon consumption of an artichoke leaf extract containing chlorogenic acid derivatives, while no interaction was observed with the SNP rs7903146-*TCF7L2*. The same authors,^[58] and using the same artichoke leaf extract, reported the decrease of insulin and HOMA-IR in 68 non-medicated MetS patients (the same 49 women plus 19 men), but only in those that carried the TT genotype of rs7903146-*TCF7L2*. The apparent discrepancy with their previous study, regarding the interaction or not with rs7903146-*TCF7L2*, was not discussed. In the present trial, we explored the interaction between 53 SNPs and 40 analytes (Table 1). Unfortunately, despite this effort, no consistent interactions either at baseline or after PE consumption were observed (results not shown).

Regarding the potential genetic susceptibility to MetS development, the selection of a specific group of patients with established MetS, and under secondary pharmacological prevention should yield a higher proportion of unfavorable variants from the selected SNPs compared to that of healthy children-adolescents, at least in those SNPs from genes previously reported to be related to MetS predisposition (*TCF7L2*, *FTO*, *ADIPOQ*, *SREBF1*, *GNB3*, etc.).^[5] Unexpectedly, MetS patients and a healthy children-adolescents cohort did not differ from the frequency of unfavorable SNP variants, either individually or collectively, like a GRS, as previously used in other studies.^[43] These results

suggest that environmental factors and lifestyle (diet, physical activity, smoking, etc.) were more important than these specific genetic variants in the development of MetS. However, we acknowledge that these results should be confirmed with more trials and larger groups of poly-medicated MetS patients.

The host genotype was reported to contribute to the variability of the composition of the human gut microbiome, although this remains controversial.^[18,73] To the best of our knowledge, only Lim et al.^[74] have reported SNP-microbiota associations in MetS, specifically that Actinobacteria and *Bifidobacterium* were significantly linked to the minor allele at the rs651821-*APOA5* SNP, which is involved in MetS development. However, with hundreds or probably thousands of SNPs potentially involved in MetS development, these authors focused only on one SNP, which they acknowledged as a limitation of their study. Other studies have reported the association of genetic variants in genes such as *FUT2*, involved in mucus composition.^[75] In contrast, others failed to find any association between gut microbiota and SNPs related to inflammatory bowel disease risk.^[76]

We have made here a substantial effort to identify how the patients' genotype could affect the composition of their microbiota, where perhaps a host-microbiota effect, independent of the medication, could have been identified. However, we only found a collection of sporadic associations either at baseline or after PE consumption, with somewhat contradictory meaning in many cases, i.e., unfavorable variants linked to the increase of "beneficial groups", favorable variants associated with either the rise of "harmful groups" or the decrease of "beneficial groups", etc. Recently, Ortega-Vega et al.^[77] published an interesting study that described in 441 adults, several associations between microbial groups, cardiometabolic risk markers, and various SNPs; some of them also included in our trial. Overall, their conclusions supported the inconsistency of associations between host genetics cardiometabolic health and gut microbiota, i.e., genetic risk variants with impaired cardiometabolic markers were associated with both beneficial and detrimental microbial groups.^[77]

Our study presents some limitations. Although the same PE nutraceutical has reported exerting prebiotic effects after 3 weeks,^[26] and the gut microbiota can be even modulated in short periods such as 3 days,^[78] the dose assayed in these patients for 4 weeks might have been insufficient to observe more

relevant effects. Besides, this is a pilot study that prevented the subgrouping of patients from establishing a definite difference within specific drug treatments. Also, we acknowledge that the binomial cardiovascular risk factors and drug therapy cannot be dissociated, which prevents distinguishing strictly whether the gut microbiota from MetS patients is mainly affected by their primary traits (hypertension, diabetes, and(or) dyslipidemia) and(or) by the associated drug treatments, or by both.^[24,79] Besides, the 16S rRNA gene sequencing determines microbial profiles at a particular moment in time, and genus level, which is essential since some genera contain species with contrasting health effects, and thus they should be analyzed individually. Finally, the small sample size (n=50) has probably prevented finding more consistent associations between SNPs, markers, and gut microbiota.

Therefore, in the present trial, we have considered a combination of targets, variables and approaches to assess the potential effects of PE that include: i) markers related to metabolism, inflammation, and endotoxemia, ii) possible SNPs-markers and SNPs-microbiota interactions, iii) involvement of patients' urolithin metabotypes (UMs) in the effects observed, iv) modulation of the gut microbiota and v) the poly-medication of the patients as a potential determinant variable in the effects of PE. Overall, most PE effects were presumably prevented by patients' poly-pharmacological treatments. PE consumption was safe at the dose assayed since no adverse effects were observed. UMs did not influence PE effects in MetS patients. Besides, no consistent or clearly interpretable associations between SNPs and markers, as well as SNPs and gut microbiota were observed before and after PE consumption. Finally, the modulation of the gut microbiota after PE consumption depended on patients' drug therapy, i.e., *Lactococcus* increased in AD-, LL- and HP-patients, *Bifidobacterium* increased in LL- and AD-, while *Clostridium XIVa* decreased in non-LL- and non-HP-patients, among other prebiotic effects.

Overall, we consider that the group of poly-medicated patients who have participated responds to a "real" scenario. The choice of non-medicated MetS patients in previous studies with dietary sources could obey to an attempt to avoid the high interference of pharmacological treatments with the presumed effects sought. However, to explore potential effects within this variability is precisely the real challenge.

In the context of precision medicine, our results highlight that targeting the gut microbiota with prebiotic functional foods or nutraceuticals, such as pomegranate-derived products, could complement MetS therapy. However, this approach should consider MetS patients individually, according to their CVD risk factors and associated medication. We believe we have paved the way for considering in future clinical trials, with poly-medicated patients, the large number of interacting variables that participate in the possible adjuvant effects of (poly)phenolic-rich functional foods or nutraceuticals against chronic-degenerative diseases such as MetS.

Author Contributions

J.C.E. designed the study; A.M. recruited and followed-up the patients; A.C.M., C.E.I.A and M.V.S. performed the experiments and analyzed the data; J.C.E. wrote the manuscript; A.C.M., C.E.I.A, A.M. and M.V.S. critically reviewed the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Supporting Information is available from the Wiley Online Library or the author.

Table S1. Detailed phenolic content of the pomegranate extract (PE) consumed by the MetS patients.

Table S2. Description of the genotyped single-nucleotide polymorphisms (SNPs) in MetS patients.

Figure S1. Cladograms from LEfSe analyses of gut microbiota that show significant differences in the fecal microbiome of MetS patients, before and after PE consumption, in the whole group (A) or depending on their drug therapy (B-G). LL, lipid-lowering drugs; HP, anti-hypertensive drugs; AD, oral anti-diabetics; PE, pomegranate extract. No statistically significant difference was observed neither after placebo consumption nor in the wash-out before crossing-over (results not shown). Red bars, before PE consumption; Green bars, after PE consumption. The comparison of time-points was as follows: T1 vs. baseline in arm-1, and T3 vs. T2 in arm-2, according to Figure 1.

Table S3. Description of the genotyped single-nucleotide polymorphisms (SNPs) in a healthy childhood-adolescence cohort.

Table S4. Pharmacological treatments followed by MetS patients.

Table S5. Baseline characteristics of the metabolic syndrome (MetS) patients that completed the trial and were randomly allocated to the two arms.

Figure S2. Rarefaction curves before and after pomegranate extract (PE) consumption.

Figure S3. LDA from LEfSe analyses showing the differently abundant bacterial groups in MetS patients, depending on their therapy, before (**A-C**) and after PE consumption (**D-F**).

Figure S4. Cladograms from LEfSe analyses showing the differently abundant bacterial groups in MetS patients, depending on their therapy, before (**A-C**) and after PE consumption (**D-F**).

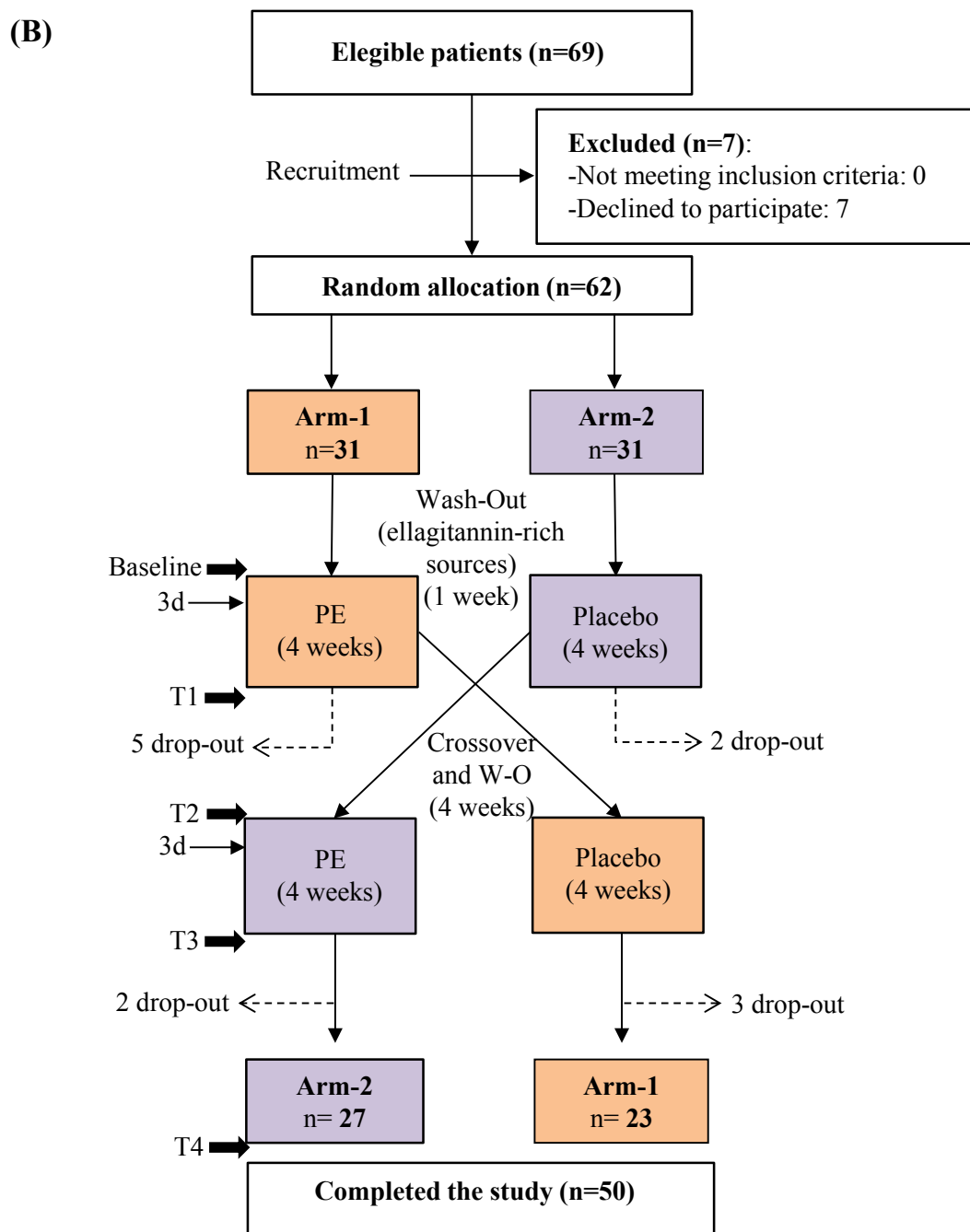
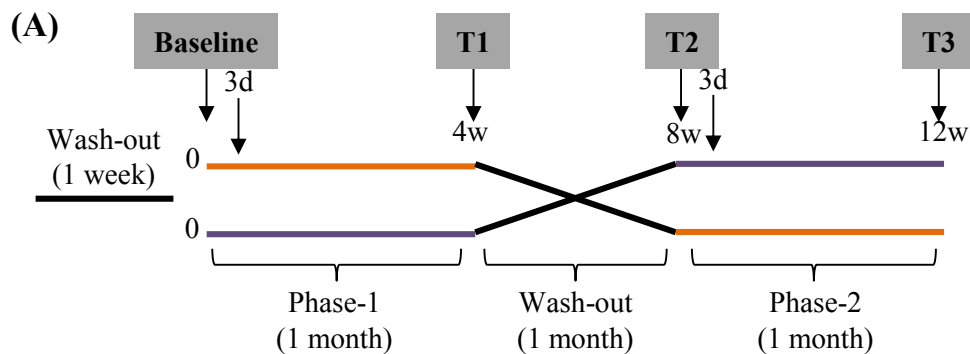
Table S6. Associations between SNPs and gut microbial groups in HP- and non-HP treated patients, before and after PE consumption.

Table S7. Associations between SNPs and gut microbial groups in AD- and non-AD treated patients, before and after PE consumption.

Table S8. Associations between SNPs and gut microbial groups in LL- and non-LL treated patients, before and after PE consumption.

Figure Captions

Figure 1. (A) Study design. (B) Flow chart of the trial.



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Figure 2. Plasma lipopolysaccharide-binding protein (LBP) concentration in MetS patients at baseline (comparison between all the patients and HP- and non-HP consumers), and after wash-out, and pomegranate extract (PE) and placebo consumption in all the patients. Groups with different letters were significantly different ($P < 0.05$). At baseline, LBP values were only different between HP- and non-HP consumers. After PE consumption, no significant differences were observed, taking into account the medication of the patients.

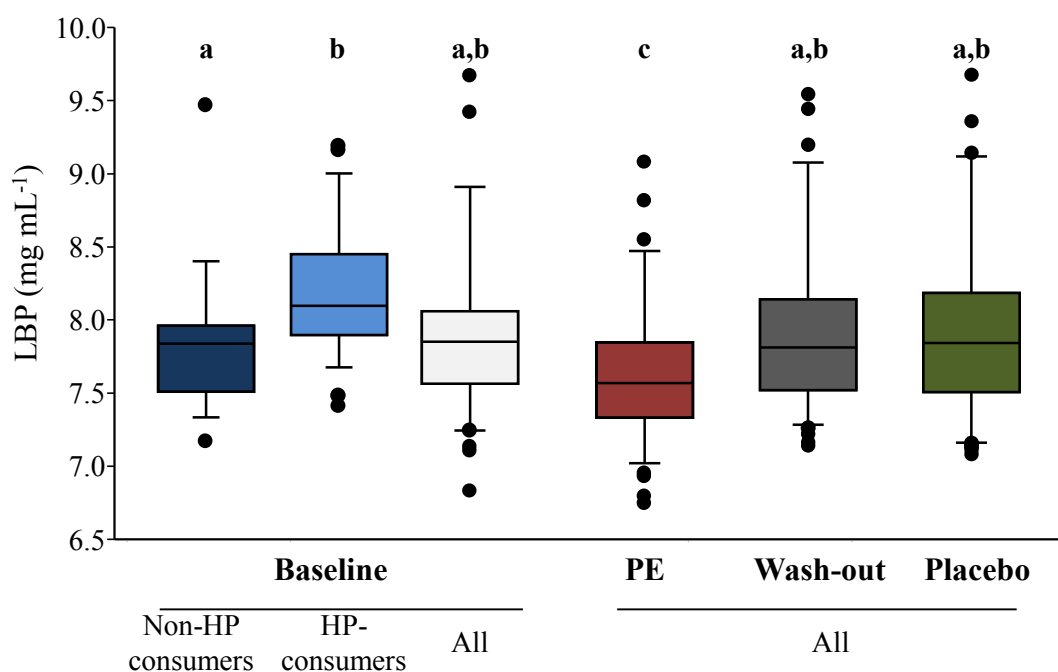


Figure 3. Linear discriminant analysis (LDA) effect size (LEfSe) of gut microbiota that shows significant differences in the fecal microbiome of MetS patients, before and after PE consumption, in the whole group (A) or depending on their drug therapy (B-G). LL, lipid-lowering drugs; HP, anti-hypertensive drugs; AD, oral anti-diabetics; PE, pomegranate extract. No statistically significant difference was observed neither after placebo consumption nor in the wash-out before crossing-over (results not shown). Red bars, before PE consumption; Green bars, after PE consumption. The comparison of time-points was as follows: T1 vs. baseline in arm-1, and T3 vs. T2 in arm-2, according to Figure 1.

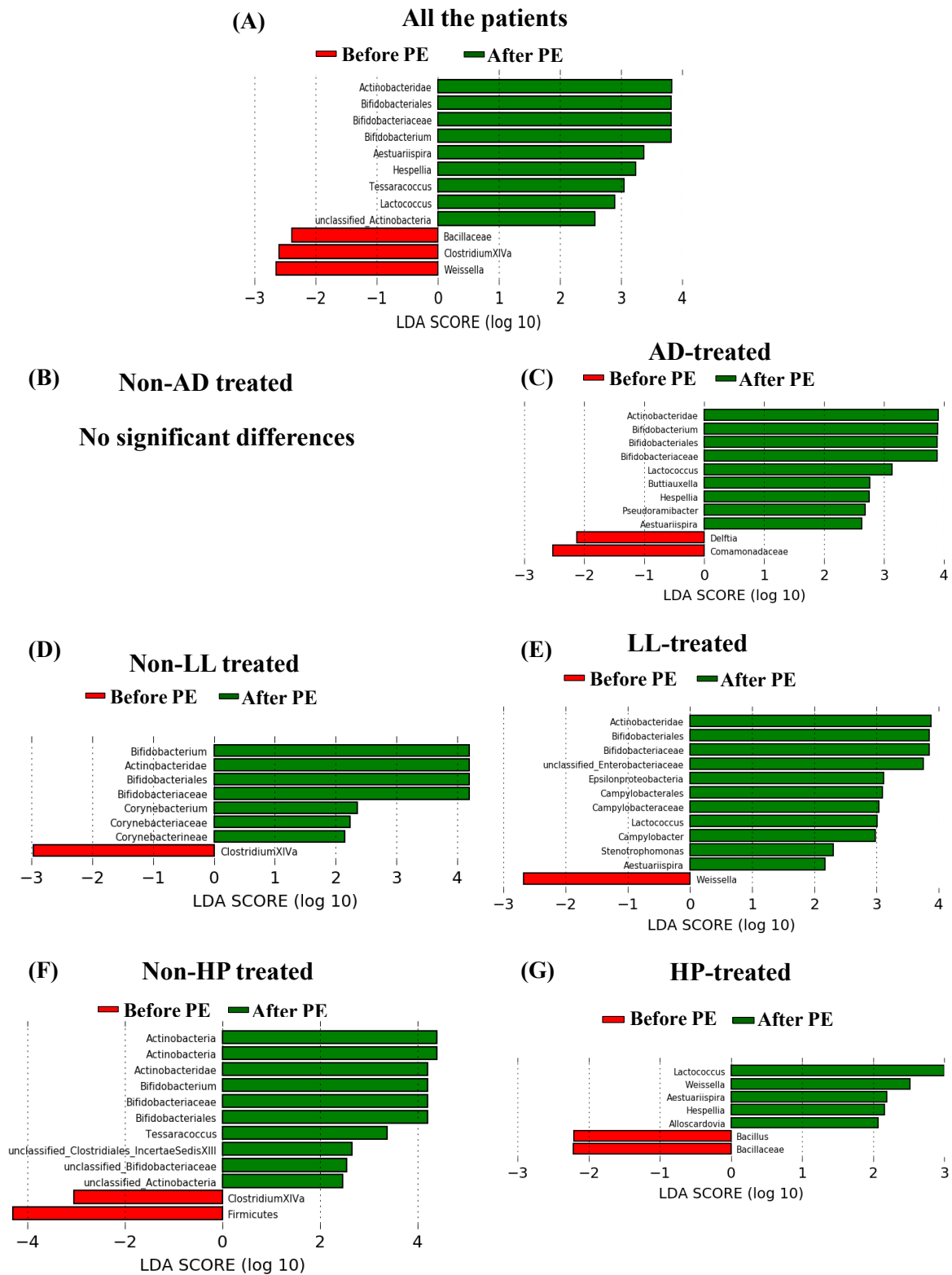


Figure 4. Relative abundances of *Bifidobacterium* (A-C), *ClostridiumXIVa* (D-F), and *Lactococcus* (G-I) before (orange bars) and after (red bars) PE consumption; and specific change after PE, depending on the drug therapy. Significant ($P < 0.05$) and marginally significant ($0.1 > P > 0.05$) differences are highlighted in bold.

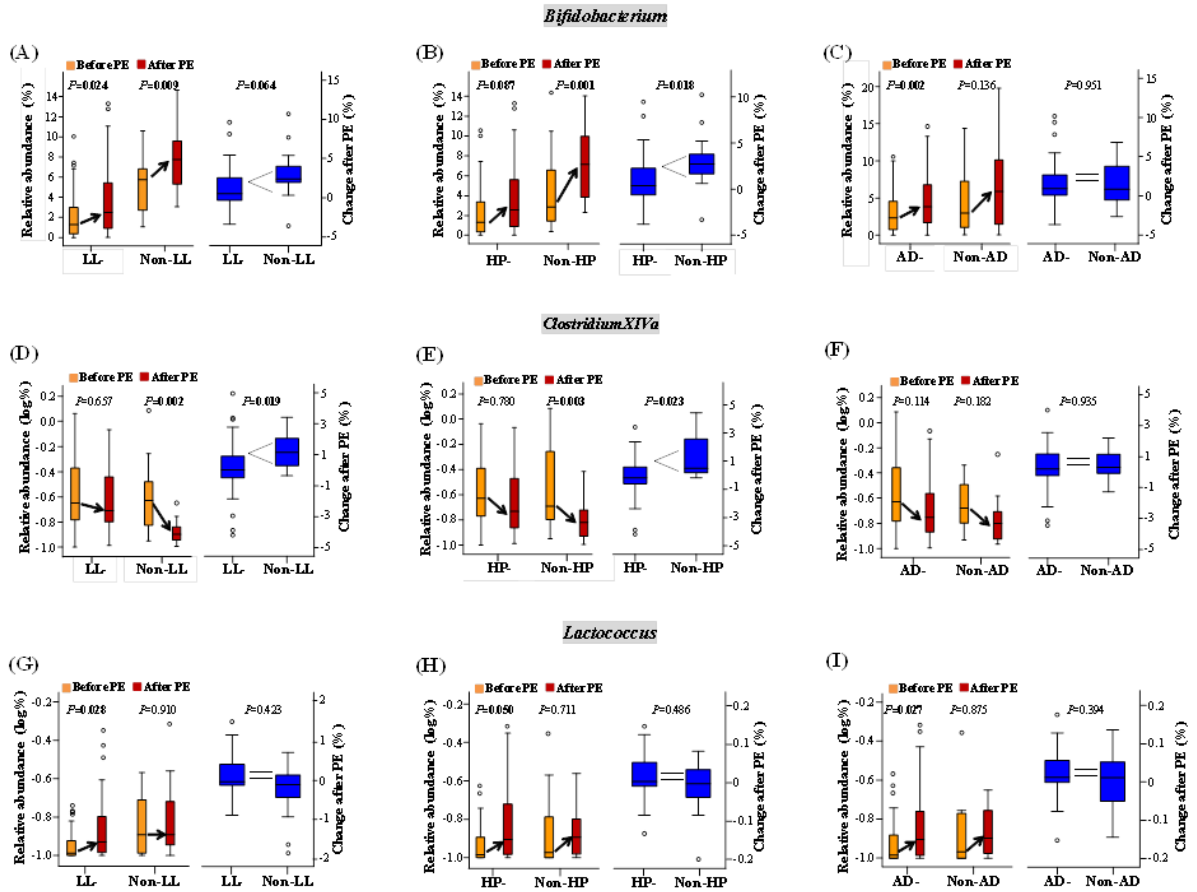


Table 1. Baseline demographic and laboratory values of the metabolic syndrome (MetS) patients that completed the trial.¹

	Women (n = 24)	Men (n = 26)	P-value
Age	55.0 (35.0–74.0)	56.0 (29.0–76.0)	0.736
BMI (kg m ⁻²)	33.6 (30–48.6)	32.9 (30.4–45.3)	0.573
Drug treatments:			
AD-consumers	20 (83.3%)	22 (84.6%)	0.902
LL-consumers	19 (79.1%)	20 (77.0%)	0.848
HP-consumers	17 (70.1%)	19 (73.1%)	0.860
Systolic BP (mm Hg)	136.0 (96.0–190.0)	134.0 (99.0–201.0)	0.165
Diastolic BP (mm Hg)	79.0 (55.0–102.0)	82.0 (60.0–96.0)	0.817
Tchol (mg dL ⁻¹)	186.0 (116.0–249.0)	158.0 (106.0–269.0)	0.077
LDLc (mg dL ⁻¹)	105.3 (49.0–163.0)	85.5 (46.0–163.0)	0.079
HDLc (mg dL ⁻¹)	51.0 (28.5–85.5)	46.0 (30.0–65.0)	0.023*
TG (mg dL ⁻¹)	118.0 (48.0–479.0)	149.0 (64.0–611.0)	0.065
Glucose (mg dL ⁻¹)	95.5 (79.0–194.0)	114.0 (73.0–232.0)	0.307
Insulin (μU l ml ⁻¹)	9.7 (2.9–34.3)	15.9 (6.4–74.8)	0.027*
HOMA-IR (U)	2.7 (0.9–9.3)	5.0 (1.4–15.1)	0.012*
Bilirubin (U L ⁻¹)	0.4 (0.2–0.7)	0.5 (0.2–1.1)	0.257
Protein (U L ⁻¹)	7.0 (6.5–8.1)	7.2 (6.4–8.5)	0.682
GGT (U L ⁻¹)	17.0 (8.0–147.0)	31.0 (12.0–283.0)	0.012*
AST (U L ⁻¹)	18.0 (12.0–43.0)	21.0 (11.0–64.0)	0.125
ALT (U L ⁻¹)	19.0 (11.0–59.0)	23.0 (9.0–68.0)	0.105
ALP (U L ⁻¹)	74.0 (44.0–279.0)	73.0 (38.0–317.0)	0.957
LDH (U L ⁻¹)	175.0 (122.0–354.0)	179.0 (139.0–524.0)	0.511
Calcium (mg dL ⁻¹)	9.5 (8.9–10.8)	9.6 (8.7–10.4)	0.920
Phosphorus (mg dL ⁻¹)	3.5 (2.3–4.6)	3.3 (1.9–4.3)	0.174
Sodium (mEq L ⁻¹)	141.0 (137.0–147.0)	141.0 (137.0–145.0)	0.444
Potassium (mEq L ⁻¹)	4.5 (4.3–5.4)	4.5 (3.9–5.5)	0.260
Chlorine (mEq L ⁻¹)	101.5 (95.0–105.0)	103.0 (99.0–105.0)	0.211
Albumin (g dL ⁻¹)	4.5 (4.0–5.0)	4.6 (3.9–5.1)	0.687
Urea (mg dL ⁻¹)	39.0 (23.0–87.0)	34.0 (22.0–88.0)	0.624
Creatinine (mg dL ⁻¹)	0.7 (0.5–2.0)	0.9 (0.6–1.9)	0.024*
LBP (μg mL ⁻¹)	7.9 (7.2–9.2)	8.1 (7.1–10.4)	0.022*
Ghrelin (pg mL ⁻¹)	750.4 (321.3–2019.0)	758.6 (474.5–1214.0)	0.727
TNF-α (pg mL ⁻¹)	155.3 (39.7–315.3)	155.7 (63.8–333.4)	0.821
GLP-1 (pg mL ⁻¹)	34.3 (7.1–137.8)	54.7 (4.7–184.0)	0.331
IL-6 (pg mL ⁻¹)	39.1 (10.4–96.2)	31.3 (9.5–123.0)	0.177
PYY (pg mL ⁻¹)	80.9 (52.8–116.9)	77.5 (42.7–105.5)	0.173
PAI-1 (ng mL ⁻¹)	1.6 (0.5–4.2)	1.5 (0.6–3.6)	0.801
Resistin (pg mL ⁻¹)	505.8 (138.0–1258.0)	409.8 (191.2–966.6)	0.341
Leptin (ng mL ⁻¹)	2.2 (1.0–6.5)	1.5 (0.8–3.4)	0.003*
HGF (pg mL ⁻¹)	133.0 (72.6–273.9)	142.9 (80.5–464.0)	0.727
MCP-1 (pg mL ⁻¹)	52.8 (29.0–116.9)	43.3 (17.7–91.6)	0.143
P-Selectin (ng mL ⁻¹)	26.2 (11.2–62.9)	32.3 (14.7–64.1)	0.351
C-peptide (pg mL ⁻¹)	254.0 (118.9–673.4)	233.0 (125.3–502.7)	0.472
BDNF (pg mL ⁻¹)	7.6 (1.1–143.3)	13.3 (3.8–121.1)	0.560

Adiponectin ($\mu\text{g mL}^{-1}$)	12.5 (3.9–51.1)	5.5 (1.5–39.0)	0.000*
sICAM-1 (ng mL^{-1})	414.8 (300.2–751.7)	393.8 (266.1–1225.6)	0.522
RBP4 ($\mu\text{g mL}^{-1}$)	43.3 (20.4–90.7)	37.8 (18.5–120.5)	0.534
sVCAM-1 (ng mL^{-1})	285.7 (205.1–896.3)	336.5 (131.5–943.7)	0.382
Acetic acid ($\mu\text{mol L}^{-1}$)	1249.6 (414.0–3432.4)	1697.1 (264.0–3373.2)	0.219
Propionic acid ($\mu\text{mol L}^{-1}$)	960.2 (292.5–3176.9)	1228.0 (167.9–2749.6)	0.135
Isobutyric acid ($\mu\text{mol L}^{-1}$)	127.5 (13.6–410.0)	167.9 (50.8–396.4)	0.180
Butyric acid ($\mu\text{mol L}^{-1}$)	1100.9 (241.5–3218.2)	1187.7 (148.1–3608.1)	0.204
Isovaleric acid ($\mu\text{mol L}^{-1}$)	180.7 (14.1–760.9)	264.1 (78.6–676.5)	0.210
Valeric acid ($\mu\text{mol L}^{-1}$)	194.7 (20.0–415.1)	284.2 (17.3–608.5)	0.166
UM-A (%)	20 (83.3%)	16 (61.5%)	0.125 (sex
UM-B (%)	4 (16.7%)	9 (34.6%)	vs.
UM-0 (%)	0 (0.0%)	1 (3.9%)	metabotype)

¹Values are expressed as median and (range). The drug therapy is detailed in Table S4. Some baseline data were previously published.^[24] *Significantly different values ($P < 0.05$). BMI, body mass index; AD, oral anti-diabetic drugs; LL, lipid-lowering drugs; HP, anti-hypertensive drugs; BP, blood pressure; Tchol, total cholesterol; LDLc, LDL-cholesterol; HDLc, HDL-cholesterol; TG, triglycerides; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; GGT, γ -glutamyl transferase; AST, aspartate aminotransferase, ALT, alanine aminotransferase; ALP, alkaline phosphatase, LDH, lactate dehydrogenase; LBP, lipopolysaccharide-binding protein; TNF- α , tumor necrosis alpha; GLP-1, glucagon-like peptide-1; IL-6, interleukin-6; PYY, peptide YY; PAI-1, plasminogen activator inhibitor type-1; HGF, hepatocyte growth factor; MCP-1, monocyte chemoattractant protein-1; BDNF, brain-derived neurotrophic factor; sICAM-1, soluble intercellular adhesion molecule-1; RBP4, retinol-binding protein-4; sVCAM-1, soluble vascular adhesion molecule-1; UM-A, urolithin metabotype A; UM-B, urolithin metabotype B; UM-0, urolithin metabotype 0.

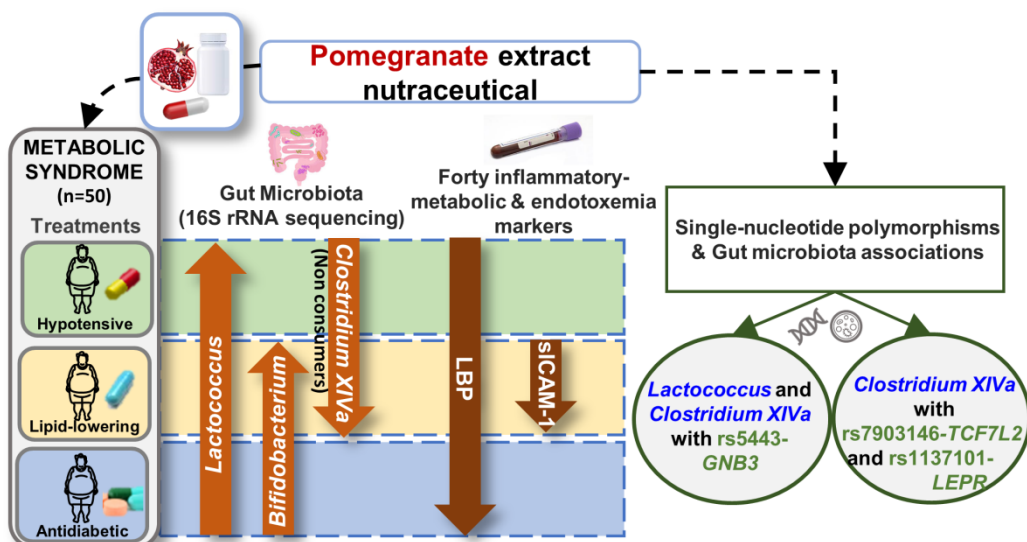
Table 2. Inflammatory and metabolic markers at baseline with significant differences as a function of the drug treatments, and the effect of PE consumption.¹

	Baseline	<i>P</i> -values	PE	<i>P</i> -values	Baseline vs PE
	Median and		Median and		<i>P</i> -values
	(range)		(range)		
sICAM-1 (ng mL⁻¹)					
LL-treated	368.8 (260-1225)	0.012	314.4 (187-3260)	0.214	0.006
Non-LL treated	458.7 (355-683)		363.3 (247-1047)		
PYY (pg mL⁻¹)					
AD-treated	77.9 (51-117)	0.040	81.8 (57-135)	0.057	0.683
Non-AD treated	62.1 (43-94)		69.5 (52-90)		
Ghrelin (pg mL⁻¹)					
HP-treated	765.7 (474-2019)	0.028	808.7 (559-1331)	0.005	0.583
Non-HP treated	684.3 (321-953)		644.2 (303-967)		
Resistin (pg mL⁻¹)					
HP-treated	496 (138-1258)	0.007	542.4 (130-1202)	0.007	0.889
Non-HP treated	347 (187-662)		369.5 (129-813)		
HGF (pg mL⁻¹)					
HP-treated	143.6 (73-464)	0.016	166.5 (80-393)	0.068	0.729
Non-HP treated	110.1 (80-170)		126.7 (88-178)		
Leptin (ng mL⁻¹)					
HP-treated	1.9 (0.9-6.5)	0.041	1.9 (0.8-6.8)	0.017	0.857
Non-HP treated	1.5 (0.8-4.0)		1.5 (0.8-2.4)		

¹Markers initially selected were those with significant differences at baseline, depending on the medication. The rest of the markers did not show significant differences either at baseline or after PE consumption (results not shown).

Text (Graphical Abstract):

Clinical trials with prebiotics, such as polyphenol-rich pomegranate products, in poly-medicated metabolic syndrome patients (MetS), is challenging. We explored the effects on i) metabolism, inflammation, endotoxemia, and gut microbiota (GM), ii) the role of urolithin metabolites and iii) associations between SNPs, biomarkers, and GM. Targeting GM with prebiotics could complement MetS therapy, but the cardiovascular risk factors and associated medication should be considered individually.



Pharmacological therapy determines the gut microbiota modulation by a pomegranate extract nutraceutical in metabolic syndrome: a randomized clinical trial