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Effects of Mediterranean Diet on plasma metabolites and their relationship with insulin resistance and gut microbiota composition in a crossover randomized clinical trial

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Abstract:	Background & Aims The Mediterranean Diet (MedDiet) may decrease the cardiometabolic risk through modulation of metabolic pathways. Furthermore, the interplay between MedDiet, metabolites and microbial metabolism may improve our understanding on the metabolic effects of this diet. We aimed to evaluate the effect of the MedDiet compared to nuts supplementation on circulating metabolites and their relationship with cardiometabolic health. We further examined whether changes in the metabolomic profiles were associated with changes in gut microbiota composition in a multi-omics integrative approach.Methods Forty-four adults with Metabolic Syndrome (MetS), (aged 37-65) participated in a randomized, controlled, crossover 2-months dietary-intervention trial with a 1-month wash-out period, consuming a MedDiet or a non MedDiet plus nuts (50 g/day). Nutritional data were collected at the beginning and the end of each intervention period using 3-day dietary records, as well as fasting blood and fecal samples. Plasma metabolites (m=378) were profiled using targeted metabolomics. Associations of these metabolites with the interventions were assessed with elastic net regression analyses. Gut microbiota composition was assessed by 16S rRNA sequencing. A sparse least regression analysis combined with a canonical correlation analysis was conducted between the plasma selected metabolites and genera in order to identify the relevant dual-omics signatures discriminating the dietary interventions.Results Changes in 65 circulating metabolites were significantly associated with the MedDiet (mainly lipids, acylcarnitines, amino acids, steroids and TCA intermediates). Importantly, these changes were associated with decreases in glucose, insulin and HOMA-IR. The network analysis identified two main clusters of genera with an opposite behaviour towards selected metabolites, mainly PC species, ChoE(20:5), TGs and medium/long-chain acylcarnitines. Conclusion Following a MedDiet, rather than consuming nuts in the context of a non-MedDiet was associated with a specific plasma metabolomic profile, which was also related to

	metabolic improvements in adults with MetS. The identified correlated network between specific bacteria and metabolites suggests an interplay between diet, circulating metabolites and gut microbiota.

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Abstract

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Methods Forty-four adults with Metabolic Syndrome (MetS), (aged 37-65) participated in a randomized, controlled, crossover 2-months dietary-intervention trial with a 1-month wash-out period, consuming a MedDiet or a non MedDiet plus nuts (50 g/day). Nutritional data were collected at the beginning and the end of each intervention period using 3-day dietary records, as well as fasting blood and fecal samples. Plasma metabolites (m=378) were profiled using targeted metabolomics. Associations of these metabolites with the interventions were assessed with elastic net regression analyses. Gut microbiota composition was assessed by 16S rRNA sequencing. A sparse least regression analysis combined with a canonical correlation analysis was conducted between the plasma selected metabolites and genera in order to identify the relevant dual-omics signatures discriminating the dietary interventions.

Results Changes in 65 circulating metabolites were significantly associated with the MedDiet (mainly lipids, acylcarnitines, amino acids, steroids and TCA intermediates). Importantly, these changes were associated with decreases in glucose, insulin and HOMA-IR. The network analysis identified two main clusters of genera with an opposite behaviour towards selected metabolites, mainly PC species, ChoE(20:5), TGs and medium/long-chain acylcarnitines.

Conclusion Following a MedDiet, rather than consuming nuts in the context of a non-MedDiet was associated with a specific plasma metabolomic profile, which was also related to metabolic

improvements in adults with MetS. The identified correlated network between specific bacteria and metabolites suggests an interplay between diet, circulating metabolites and gut microbiota.

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Keywords: Mediterranean diet, nuts, insulin resistance, plasma metabolome, gut microbiota

Abbreviations: CVD; cardiovascular disease, GC-QTOF-MS; gas chromatography time-of-flight mass spectrometry, HOMA-IR; homeostasis model assessment of insulin resistance, LC-Q-TOF-MS; liquid chromatography quadrupole time-of-flight mass spectrometry, LC-QqQ-MS; liquid chromatography with triple-quadrupole mass spectrometry, LPC; lysophosphocholine, LPE; lysophosphoethanolamine, MedDiet; Mediterranean diet, MetS; metabolic syndrome, PC; phosphocholine, T2D; type-2 diabetes, TG; triglyceride, TMA; trimethylamine, TMAO; trimethylamine N-oxide, TCA; tricarboxylic acid

1. Introduction

Diet may contribute to the risk of chronic conditions through the modulation of several biological pathways [1]. Therefore, in the era of precision nutrition, a better understanding of the biological consequences following a dietary intervention is required to build a functional readout of the major dietary factors related to health and disease. Circulating metabolites reflect individual dietary intakes, endogenous metabolic processes and other sources of variability in metabolism as the interplay with gut microbiota, which objectively allows assessing the complex metabolic responses to dietary patterns. Previous dietary interventions integrating nutrition with metabolomics have focused on a single nutrient or specific food [2], without accounting for all interactions and failing to consider important aspects such as the substitution effects of other nutrients and the synergistic or antagonistic interactions between nutrients within a dietary pattern.

Accumulating data from epidemiological and clinical trials have demonstrated that consuming a Mediterranean Diet (MedDiet) is a useful dietary strategy for the prevention of cardiovascular diseases (CVD), obesity and type 2 diabetes (T2D) [3]. The cardiometabolic benefits of this dietary pattern could be partially explained by its effects on insulin resistance [4], oxidative stress and inflammation improvements. However, the metabolic pathways through which the MedDiet exerts its beneficial effects has been little investigated. Previous randomised trials have identified changes in specific metabolites after the consumption of foods typically included in the MedDiet such as nuts [5]. On the contrary, few intervention studies exist on the effects of the MedDiet, which is also rich in other types of foods such as vegetables, fruits, legumes, whole grains, red wine and extra-virgin olive oil, on circulating metabolites [6], while the relationship between diet-induced changes in metabolomic profiles and cardiometabolic risk factors remains largely unknown. Furthermore, the role of gut microbiota in influencing host circulating metabolites in the context of dietary interventions has not been explored yet.

In the present study, we examined changes in plasma metabolites after following a MedDiet compared to the consumption of a single healthy food such as nuts in the context of a non-MedDiet among participants with Metabolic Syndrome (MetS) and whether these changes can be mediated by differences in gut microbiota composition in a multi-omics integrative approach.

2. Material & Methods

2.1 Study design and population

METADIET is a randomized, controlled, crossover, non-blinded dietary-intervention trial (**Supplemental Figure 1**) in which eligible participants were community-dwelling adults aged 25-60 years, with overweight/obesity (body mass index (BMI) 25-35 kg/m^2) and MetS according to harmonized ATPIII diagnosis criteria [7], and who regularly consumed a non-MedDiet (scoring <7 in the MedDiet score used in PREDIMED-Plus trial and volunteers with ≥7 were excluded from the trial [8]). The primary outcome of METADIET was evaluating changes in gut microbiota composition after following either a MedDiet or a regular diet supplemented with nuts (unpublished results), while changes in plasma metabolites and cardiometabolic risk factors were secondary outcomes. The exclusion criteria were: following a MedDiet considered as a punctuation ≥7 in the PREDIMED-Plus MedDiet score tool [8], presence of T2D; several chronic diseases (inflammatory, infectious, chronic obstructive pulmonary, neoplasia, endocrine, or hematological diseases), regular consumption of nuts (≥90 g/week); changes in body weight (>5 kg in the last 3 months); non-controlled hypertension; LDL cholesterol >160 mg/dL; triglycerides >400 mg/dL; use of anti-inflammatory, corticoids, hormones or antibiotics; alcohol, smoking or drug abuse; consumption of prebiotics, probiotics or laxatives. Participants were recruited from primary care centers affiliated with the University Hospital of Sant Joan and through advertisements in different media. Written informed consent

was obtained from all study participants. The Institutional Review Board approved the study protocol which accomplishes the ethical standards of the Declaration of Helsinki.

Participants who met the inclusion criteria were randomized, using a computer-generated random-number table, either to follow a MedDiet or to consume 50 g/day of mixed nuts (almonds, walnuts, hazelnuts provided free) in the context of their regular non-MedDiet for 2 months. After a 1-month washout period with no treatment, participants crossed over to the other treatment condition for the last 2 months of the study. During the MedDiet period, trained dietitians instructed participants in personalized face-to-face interviews to follow a Mediterranean dietary pattern. Participants were encouraged to adhere to the 17-point scale MedDiet [8] used in PREDIMED Plus study, emphasizing daily consumption of at least 2 servings of vegetables and 3 fruits, and weekly consumption of ≥3 servings of legumes, ≥5 servings of whole-grain cereals and pasta≥3 servings of fish and seafood and the use of extra virgin olive oil as the main culinary fat. Also, a decreased consumption of red meat and processed foods to less than 1 serving/week, and reduced use of butter and margarine, white bread and sweetened beverages was recommended. Participants were provided with biweekly menus and seasonal recipes to facilitate adherence to the MedDiet intervention. During the nuts intervention period, dietitians did not provide any other dietary advice rather than the consumption of 50 g/day of mixed nuts that were provided by free, and also written culinary advice to include nuts in regular meals with soups, creams or as side food. Adherence to the interventions was assessed either by the validated 17-item MedDiet score [8] or counting the empty nuts-packaging returned at the beginning and at the end of each intervention period, and during two intermediate control visits scheduled after 15 and 30 days from the beginning of each intervention period. Nutritional data was collected in each sampling visit using 3-day dietary records and nutrient and energy intakes were calculated using Spanish food composition tables [9,10]. Biological samples were collected at the beginning and at the end of each

intervention period (**Supplemental Figure 1**). Anthropometric data, blood pressure and biochemical measurements were conducted following regular protocols in the clinical practice and are detailed in the **Supplemental methods.**

2.2 Plasma metabolomics profiling

A total of 378 metabolites were quantified using a multiplatform approach including gas and liquid chromatography coupled to high-resolution mass spectrometry (LC-qTOF-MS, LC-QqQ-MS and GC-QTOF-MS) (**Supplemental Table 2**). The analytical procedures are specified in the **Supplemental methods.** Information about the mean and SD of the metabolites used in the present is shown in **Supplemental Table 2.**

2.3 16S rRNA sequencing and data processing

A detailed description of fecal samples collection, microbial DNA extraction and metagenomic analysis can be found in the **Supplemental methods**. Briefly, microbial DNA from fecal samples collected at the beginning and at the end of each intervention, was performed with QIAmpPowerFecal DNA kit (Qiagen, Germany) with an additional lysing step (FastPrep-24 5G Homogenizer, MPBiomedicals). Hypervariable region V4 from 16S rRNA was amplified using the Ion Metagenomics kit^{TM} (Life Technology, Carlsbad, California).

2.4 Statistical analyses

Descriptive data of participants are presented as means and 95%CI for quantitative variables, and as percentages for categorical variables. Changes in anthropometric, biochemical and nutritional data were analyzed by using linear mixed-models analysis of variance with intervention groups and periods modelled as fixed factors, baseline values as covariates and subjects as a random effect. To account for multiple testing, we adjusted P for treatment and P for treatment * period of the crude and multivariable-adjusted associations with the use of the Benjamini-Hochberg false discovery rate (FDR) procedure [11]. An FDR-adjusted P-value < 0.05 was considered to be statistically significant. The effect size was estimated via the

calculation of Cohen's, which shows the magnitude of the difference in changes in biochemical and anthropometrical parameters between MedDiet and nuts interventions (i.e. Cohen's *d* value of 0.2, 0.5 and 0.8 indicate small, medium and large effect size respectively) [12]. From a total of 378 plasma metabolites profiled, 5 metabolites with missing values >20% were removed and for those with less than 20% we used the random forest imputation approach [13] ("missForest" function of "randomForest" R package version 4.6-14). Metabolites were approximated to a normal distribution with the rank-based inverse normal transformation. Due to the high dimensionality and collinear nature of the data, logistic regression with elastic net penalty was implemented in the "glmnet" (R package, version 3.0-2) (alpha=0.5) to select the metabolites associated with the dietary interventions. We performed 10-fold cross-validation (CV) to find the optimal value of the tuning parameter that results in a mean squared error within 1-SD of the minimum [14]. The performance of the model was examined based on parameters of lambda.min. For reproducibility purposes, regression coefficients were reported using 9-10 iterations of the 10-fold CV elastic regression approach in the whole dataset. We also performed a sensitivity analysis adjusting for energy intake. A multi-metabolite score was calculated as the weighted sum of the selected metabolites with weights equal to regression coefficients from the elastic net regression model. Linear regression models were fitted to examine the association between the derived score and changes in glucose, insulin and HOMA-IR adjusting for age, sex, changes in body weight, dietary interventions and value for the respective outcome traits at the baseline examination. All analyses were performed using R statistical package 3.6.1 (www.r-project.org) (R Development Core Team, 2012). Metabolites selected from the elastic net models were mapped into a metabolic network to identify key pathways and enrichment analysis by using MetaboAnalyst 4.0.

Integration of metabolomics and 16S rRNA sequencing data was performed using DIABLO analysis in mixOmics package of R (version 6.10.9, for which we applied a full design matrix

with a value of 1 to seek for linear combinations of variables from each omic dataset that are maximally correlated between the two dietary interventions. Changes in relative abundances of 153 genera (normalized using clr function from the R package "compositions" package, version 1.40-4) and changes in the 65 selected metabolites were used. The block.splsda function with full weighted design and 10-fold CV was used to identify the optimal number of components for each omic dataset. Model performance was also evaluated using a 10-fold CV approach in order to choose the best number of latent components to be included for each omic dataset. We then evaluated the highly correlated omic variables which were able to discriminate the MedDiet from the nuts supplementation diet. Each model was built on 80% of participants (training) and the remaining 20% was used as the test set. A global overview of the correlation structure at the component level was represented with the plotDiablo function. A circosplot representing the correlations between variables of different types represented on the side quadrants was obtained with the circosplot function. Also, a network analysis was implemented by network function, in order to display the association network for regularized canonical correlation analysis and sparse partial least square regression.

3. Results

Of the 50 participants initially included in the study, 6 dropped out for personal reasons resulting in 44 participants with available metabolomic data (**Figure 1**). No significant differences in participants' baseline characteristics were observed between the two interventions (**Table 1**). Medium effect sizes (Cohen's $d = 0.5$) were observed for glucose, insulin and HOMA-IR, whereas large effect size (Cohen's $d > 1$) was observed for the MedDiet score. The mean±SD for the 17-items MedDiet score was 11.59±2.41 [increase; 4.61 (3.74, 5.48)] after the MedDiet and 9.30±3.54 [increase; 0.36 (-0.82, 1.55)] after the nuts supplemented diet. Significant decreases in the levels of glucose, insulin and the values of HOMA-IR were observed in the MedDiet compared to the nuts supplementation diet (**Table**

1). Significant differences in total lipids, saturated fatty acids (SFA), and protein intake were observed between interventions. (**Supplemental Table 3**). A higher increase of fruits, legumes, fish and a decrease of alcohol consumption was found after the MedDiet compared to the regular diet supplemented with nuts, whereas nuts consumption was significantly increased in the nuts intervention group (**Supplemental Table 3**).

Table 2 shows 65 selected metabolites ranked from the highest to the lowest elastic net positive and negative regression coefficients for the MedDiet. Thirty-five metabolites were positively associated with the MedDiet; hydroxyyperoxide-eicosapentanoic acid (HpEPE), testosterone, phosphatidylcholines (PC) (40:6, 35:1), trimethylamine (TMA), succinic acid, cholesterol esters (ChoE) (17:0, 20:5), taurolithocholic acid, amino acids (threonine, cystathione, histidine, phenylalanine), lysophosphatidylcholines (LPC) (19:0-sn1, 22:6, 16:1), carnitine species (C5-OH, C5:1, C18:2, C2:0, C16:0-OH), triglycerides (TG) (56:6, 56:7, 56:5) and lysophosphoethanolamine (LPE) 20:5-sn1. Among the 29 metabolites negatively associated with the MedDiet, the highest regression coefficients were found for LPE18:3-sn1 followed by sphingomyelin (SM) (36:0, 41:1, 32:2), 9-OxoODE, carnitines (C12:0, C5-M-DC, C12:0-OH-a), taurine and its bile acid, taurocholic acid, linolenic acid-iso2 and -iso1, dehydroepiandrosterone sulfate, androsterone sulfate-iso4, androsterone sulfate-iso2, PCs (33:2, 34:1e, 32:0, 34:2, 32:2), TG 47:0, ChoE 22:5, dihomo-y-linolenic acid-iso2 and hydroxyoctadecadienoic acid (HODE)-iso1, hydroperoxyoctadecadienoic acid (HpODE).

In order to take into account the significant difference in energy intake, we have conducted a sensitivity analysis which shown that 29 metabolites of the 65 previously selected from the unadjusted model were associated to the dietary interventions (**Supplemental Table 4**). The enrichment analysis of the selected metabolites revealed that most of them were involved in amino acids metabolism. The metabolic pathways with the highest impact were related to taurine, glycine, serine and threonine metabolism, phenylalanine and tryptophan biosynthesis,

together with tricarboxylic acid (TCA). A moderate impact was also found for the metabolism of alpha-linolenic acid, glycerophospholipid and unsaturated fatty acid (**Figure 2**). The linear regression models revealed significant inverse associations of the multi-metabolite score with changes in glucose, insulin and HOMA-IR (**Table 3**).

In **Figure 3** we plotted the multi-omic analysis including the selected metabolites from the elastic net regression and the 153 genera depicting gut microbial composition. This analysis includes a total of 38 subjects with available data after filtering procedures in gut microbiota (**Supplemental Figure 1**). No significant differences in the general characteristics of participants included in both analyses were observed (**Supplemental Table 1)**. Considering a correlation cut-off value of 0.2 we were able to find two main clusters of genera differentially correlated with a set of metabolites. The first cluster consisted of an uncultured genus of Lachnospiraceae, *Ruminococcaceae UCG002*, *Lachnoclostridium* and some genera from Prevotellaceae family which were positively correlated with changes of C16-OH, C12:0, C12-OH, PC35:1, PC40:6, and TGs 56:6, 46:7, 56:5 and also with ChoE 20:5. On the contrary, negative correlations with changes in phosphoethanolamine and taurine were found for this cluster. The second cluster, constituted by an uncultured genus from Christensenellaceae family, *Oxalobacter*, Clostridiales family XII, *Ruminococacceae UCG009, Terrisporobacter*, and a genus from Clostridiales family, presented the same associations of the first cluster but with an opposite sign.

4. Discussion

In the present study, we identified changes in several plasma metabolites associated with following either a MedDiet or a non-MedDiet supplemented with nuts in participants with overweight/obesity and MetS. These metabolites mainly included lipid species and acylcarnitines but also amino acids, steroids and TCA intermediates. Importantly, metabolites changes induced by the MedDiet, compared to a single healthy food like nuts in the context of

a regular diet, were associated with improvements in participants' metabolic risk profile, independent of changes in body weight and dietary interventions indicating their promising use in understanding the biological mechanisms behind the effects of the MedDiet.

The effect of the MedDiet on circulating metabolites has been poorly investigated. In a case-cohort study nested within the Prevention with Mediterranean Diet (PREDIMED) trial, participants following a MedDiet had a 1-year significant reduction in several lipid species, mainly PCs, PEs, ChoE and TGs, compared to participants in the low-fat diet control group, although only changes in ChoE remained statistically significant after correcting for multiple comparisons [15]. Similarly, in the Metabolic Syndrome Reduction in Navarra (RESMENA) trial conducted among 72 subjects with obesity and at least two features of MetS, who received an energy-restricted MedDiet or a low-fat diet, displayed significant changes in several lipid species, mainly PCs, LPCs and SMs after 2 months [16]. A more recent cross-sectional study that identified a metabolic profile of adherence to the MedDiet revealed that out of 67 metabolites, 45 were lipids [17]. Accordingly, in our study, following the MedDiet compared with non-MedDiet supplemented with nuts was associated with decreases in PCs and SMs. Previous observational studies have suggested a relationship between elevated levels of PC and increased risk of coronary artery disease and mortality [18], but in the PREDIMED study polyunsaturated PCs with at least 5 double bonds conferred CVD protection [19]. PCs are direct substrates for the formation of SMs by SM-synthase [20], which are hydrolyzed by sphingomyelinases activated by inflammatory cytokines and oxidative stress, to produce ceramides [21]. Whether increases in PC40:6 induced by the MedDiet are related to metabolic benefits requires further investigation. ChoE and TGs with >56 carbon atoms and >3 double bonds have been inversely associated with T2D [22] and in our study were increased after the MedDiet. These lipids could represent a pathway through which the MedDiet may have favorably affected insulin resistance.

The increase in plasma α-tocopherol after the end of the MedDiet suggests a good adherence to this dietary pattern [23]. Furthermore, given that the MedDiet and nuts are high in unsaturated fats, it is not surprising that a large proportion of lipid species consist of PUFAs [17]. Therefore, the increases in plasma LPE20:5 and LPC22:6 after the MedDiet may reflect the trend to a higher intake of fish, since plasma EPA and DHA has been identified as the most suitable biomarker of acute changes in 20:5(n-3) and 22:6(n-3) fatty acids intake [24]. Furthermore, an EPA-derived lipoxygenase metabolite, HpEPE, was highly associated with the MedDiet. Our results are in line with a previous study in which higher fish intake was associated with higher levels of highly unsaturated lipid metabolites containing 22:6 and 20:5 (n-3) [17]. An involvement of the metabolism of lysophospholipids in cardiometabolic health has been suggested, although, findings from recent lipidomic studies of LPC have been controversial [25,26]. In a previous *in vitro* study, HpEPE was found to inhibit platelet aggregation [27]. On the contrary, a positive association with the nuts supplemented diet was found for α-linolenic acid (ALA), which could also explain the increases in LPE18:3 and LPC18:3 after following this intervention. ALA is an essential n-3 fatty acid, mainly deriving from the diet, especially from nuts that has been related to cardiovascular health benefits [28]. Metabolites produced by the linoleic acid metabolism such as 9-OxoODE, 13-HpODE and its catabolic product 13-HODE increased after the nuts intervention, whereas 15-HETE produced by arachidonic acid increased after the MedDiet. Further studies are needed to understand the role of these metabolites on inflammation regulation.

Changes in different acylcarnitines were found to be associated with the dietary interventions indicating a different role of these dietary patterns on the fatty-acids β-oxidation and energy metabolism [29]. Two long-chain and three short-chain acylcarnitines were associated with the MedDiet, while one short- and two medium-chain acylcarnitines were associated with the nuts intervention. Previous studies have suggested that pathways related to acylcarnitine metabolism

can be influenced by diet. In a previous cross-sectional study, a metabolite profile composed of medium- and long-chain acylcarnitines was inversely associated with the Western dietary pattern [30]. On the other hand, long-chain acylcarnitines were associated with veganism in the EPIC-Oxford cohort [31]. In the PREDIMED study, high short- and medium-chain acylcarnitines levels were associated with a higher risk of CVD [32], while short- and long-chain acylcarnitines with higher T2D risk [33].

Besides the impact of the study dietary interventions on lipid metabolism and fatty acid oxidation, changes in amino acid metabolism were also observed in our study. Following the MedDiet was associated with increases in plasma amino acids such as phenylalanine, histidine, cystathionine and threonine. Previous studies have suggested that a Western dietary pattern or an omnivorous diet are associated with increased levels of amino acids like phenylalanine [30,34]. This could be due to the higher content of phenylalanine in animal proteins. In our study, participants assigned to the MedDiet intervention may have increased circulating amino acids concentrations possibly due to the trend for a higher fish consumption as compared to the nuts group. On the other hand, the sulfur-containing amino acid, cystine, derivative of cysteine mainly found in almonds [35] was associated with the nuts intervention group.

Increased plasma concentrations of TCA-related metabolites (i.e., succinate) after the MedDiet compared to nuts group is a novel finding. Recent evidence suggests that succinate is not only a TCA intermediary metabolite but also a by-product of gut microbial carbohydrate and amino acid fermentation [36] with pleiotropic functions, such as resolution of inflammation associated with obesity [37]. Regarding other gut microbiota-derived metabolites, increases in TMA concentrations were found after the MedDiet possibly due to the higher fish consumption observed following this dietary pattern than the non-MedDiet supplemented with nuts. TMA can be generated in the colon from choline or L-carnitine or metabolic retro-conversion of trimethylamine N-oxide (TMAO) to TMA by the gut microbiota [38]. In a study with 40 healthy

young men, consumption of meals producing TMAO provided directly from fish led to a significant increase in postprandial plasma TMA levels. However, eggs or beef consumption, providing choline and L-carnitine, respectively, resulted only in negligible TMA increases [39]. At the same time, the MedDiet was associated with increases in taurolithocholic acid, while the regular diet plus nuts with increases in taurine and its conjugated bile acid (taurocholic acid). Recent animal studies showed an increase in taurocholic acid in mice fed with a high-saturated fat diet [40], which may result in microbiota dysbiosis that can perturb immune homeostasis. Similarly, in our study, the higher intake of saturated fatty acids in the nuts group may explain these findings. In addition, previous in vitro studies have demonstrated that treatment with taurolithocholic acid significantly increased nitric oxide production [41], while taurocholic acid exerted an arrhythmogenic effect [42].

Another novel finding of our study was increased in testosterone after MedDiet and increases in three androsterone sulfate species following the nuts supplemented diet. Whether both diets provide sufficient nutrients for precursors of steroidogenesis [43] is a hypothesis that needs to be examined. Interestingly, obese men with low testosterone concentrations are more prone to metabolic disturbances such as insulin resistance [44]. A recent *in vivo* study also found a relationship between lower plasma concentrations of sulfated steroids, such as dehydroepiandrosterone sulfate and impaired glucose tolerance [45].

The dual-omic analysis demonstrated a correlation between a cluster enriched by medium-and long-chain acylcarnitines, PCs, ChoE and TGs with >56 carbon atoms and >3 double bonds and a cluster of bacteria belonging to Firmicutes (Lachnospiraceae, *Lachnoclostridium* and Ruminococcaceae) and genera from Prevotellaceae family. Genera from the Lachnospiraceae family were associated to a higher MedDiet adherence [46] whereas higher abundances of *Lachnoclostridium* were reported after a 3 months MedDiet intervention [47]. Similarly, plant-based foods diets were associated with a Prevotella enterotype [48]. On the other hand, the

second cluster, constituted of *Oxalobacter* and genera from Christensenellaceae family together with *Ruminococcaceae UCG009* and genera from Clostridiales family may reflect the effect of the nuts supplemented diet on these microbes. Nuts are rich in oxalate, the main substrate for *Oxalobacter formigens*, and their consumption has been related with a higher concentration of gastricoxalate [49]. Christensenellaceae is reported higher in relative abundance in humans with an omnivorous diet, relative to vegetarians [46]. Potential mechanisms through which the identified microbiota may affect circulating acylcarnitines, PCs, TGs and ChoE may involve short-chain fatty acids (SCFAs). Some of the genera (uncultured Lachnospiraceae, *Lachnoclostridium*, *Ruminococcaceae UCG002*, uncultured Prevotellaceae and Christensenellaceae) identified in our study are involved in production of SCFAs, which can be absorbed into the bloodstream and affect lipid metabolism [50].

Our study has several strengths such the crossover, randomized, controlled design, thus balancing the potential intra-individual variability in metabolites and gut microbiota with a longer washout period to avoid a potential carryover effect, even if we cannot completely discard due to the crossover design of the study. With regards to limitations, the relatively small sample size and the high intra-variability of gut microbiota composition could limit to reach statistically significance for some genera. Also, the lack of blinding, despite its unfeasibility, represents a potential bias that should be taken into account. Furthermore, the 16S rRNA analysis does not allow to identify bacterial species, leaving the taxonomy at genus level. Finally, diablo analysis is one of the computational methods for inferring correlation networks which is computationally challenging since sensitivity, specificity and precision have not been evaluated in reference to either real or theoretical data sets and these limitations should be accounted when interpreting our findings.

5. Conclusion

In conclusion, our study demonstrated that following a MedDiet, rather than the consumption of a non-MedDiet supplemented with nuts, was associated with changes in the plasma metabolome that were associated with insulin resistance improvements among participants with MetS. Medium- and long-chain acylcarnitines, two PCs, and TGs and ChoE with >56 carbon atoms and >3 double bonds appeared to be related with a specific gut microbiota composition supporting an interplay between diet, circulating metabolites and gut microbiota.

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Metabolomics analyses and 16S rRNA gene sequencing were conducted in the facilities of the Centre for Omics Sciences (COS) Joint Unit of Universitat Rovira i Virgili-Eurecat. Data generated and analysed in the framework of the METADIET are not publicly available due to national data regulations and for ethical reasons, because study participants only gave their consent for the use of their data by the METADIET investigators. However, collaboration for data analyses can be requested by sending a letter to the corresponding author (Mònica Bulló).

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Author Contribution

MB designed the research and was the coordinator of subject's recruitment; MB, SG, JG-G, LC-B, PA, AP-G, AR recruited participants and conducted the research, SG, JG-G and LC-B obtained data and conducted the bioinformatics analysis; SG, JG-G and CP conducted the statistical analysis; SG, CP, MB, drafted the paper; all the authors revised the manuscript for important intellectual content, and read and approved the final version.

Conflict of interest statement

The authors declare that they have no competing interests.

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Table 1. Baseline and changes in biochemical and anthropometrical parameters (n=44)

Characteristics	Mediterranean Diet		Nuts		MedDiet vs Nuts (changes)		
	Baseline	Change	Baseline	Change	p treat [1]	p treat*period [1]	Effect size Cohen's *d*
Age	50.73 (48.65, 52.80)	0 (0, 0)	50.73 (48.65, 52.80)	0 (0, 0)	N/A	N/A	
Weight (Kg)	84.81 (83.01; 86.61)	-0.7 (-1.21, -0.19)	84.43 (82.64; 86.22)	-0.02 (-0.47, 0.42)	0.156	0.580	
Waist Circumference (cm)	102.79 (101.49; 104.10)	-1.1 (-2.22, 0.02)	101.77 (100.35; 103.19)	0.74 (-1.93, 3.41)	0.617	0.726	
SBP (mmHg)	134.85 (133.14; 136.56)	-1.4 (-5.14, 2.35)	132.48 (130.74; 134.21)	-1.73 (-6.61, 3.16)	0.894	0.580	
DBP (mmHg)	85.26 (84.13; 86.39)	-0.99 (-2.99, 1.01)	84.35 (82.96; 85.75)	-2.17 (-5.53, 1.19)	0.617	0.639	
Total Cholesterol (mg/dL)	215.09 (210.09; 220.09)	-7.82 (-14.99, -0.64)	212.41 (207.10; 217.72)	-0.16 (-9.04, 8.73)	0.189	0.639	
LDLc (mg/dl)	136.93 (132.86; 141.00)	-6.66 (-13.33, 0.02)	133.27 (128.95; 137.60)	-2.65 (-8.31, 3.01)	0.474	0.381	
HDLc (mg/dL)	50.48 (48.71; 52.24)	-1.23 (-3.13, 0.68)	50.61 (48.84; 52.39)	0.02 (-1.56, 1.61)	0.565	0.793	
VLDLc (mg/dL)	27.68 (25.77; 29.59)	0.07 (-2.47, 2.61)	28.52 (26.91; 30.14)	-1.44 (-4.55, 1.67)	0.617	0.681	
Triglycerides (mg/dL)	138.66 (129.12; 148.20)	0.07 (-12.44, 12.58)	142.84 (134.75; 150.94)	13.25 (-29.51, 56.01)	0.617	0.681	
Glucose (mg/dL)	100.55 (98.61; 102.48)	-3.18 (-6.53, 0.17)	97.45 (95.77; 99.14)	2.25 (-0.47, 4.97)	0.046	0.580	0.53 (0.10, 0.96)
Insulin (mcUI/mL)	14.46 (13.33; 15.58)	-1.44 (-3.1, 0.21)	12.44 (11.33; 13.54)	2.35 (0.03, 4.66)	0.046	0.210	0.56 (0.12, 0.99)
HOMA-IR	3.65 (3.32; 3.98)	-0.42 (-0.96, 0.12)	1.01 (2.73; 3.29)	0.8 (0.03, 1.56)	0.046	0.210	0.55 (0.11, 0.97)
Zonulin (ng/mL)	40.08 (38.93; 41.23)	0.52 (-1.67, 2.7)	40.45 (39.51; 41.40)	1.04 (-1.04, 3.11)	0.770	0.830	
17-items MedDiet score	6.98 (6.61; 7.35)	4.61 (3.74, 5.48)	8.93 (8.46; 9.40)	0.36 (-0.82, 1.55)	0.001	0.381	-1.22 (-1.53, -0.89)

All values are given as means (95% CI). Changes in anthropometric and biochemical parameters between dietary interventions were analyzed analyzed by using linear mixed-models analysis of variance with intervention groups and periods modelled as fixed factors, baseline values as covariates and subjects as random effect. Cohen's *d* indicates the effect size of MedDiet vs. Nuts group. Abbreviations: SBP; systolic blood Pressure, DBP; diastolic blood pressure, LDLc; low density lipoprotein cholesterol, HDLc; high density lipoprotein cholesterol, VLDLc; very low-density lipoprotein cholesterol, HOMA-IR; homeostatic model assessment of insulin resistance, MedDiet; Mediterranean Diet, N/A; not available.

[1] Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Table 2. Plasma metabolites ranked from the highest to the lowest elastic net positive or negative regression coefficients for the MedDiet intervention.

Metabolites	Coefficient	Metabolites	Coefficient
HpEPE	0.958	LPE18:3-sn1	-0.649
Testosterone	0.579	SM36:0	-0.563
PC40:6	0.537	9-OxoODE	-0.517
TMA	0.518	C12:0	-0.334
Succinic acid	0.407	C5-M-DC	-0.320
ChoE(17:0)	0.388	Taurine	-0.284
Taurolithocholic acid	0.380	Linolenic acid-iso2	-0.283
Threonine	0.356	SM41:1	-0.282
LPC19:0-sn1	0.306	Dehydroepiandrosterone sulfate	-0.281
C5-OH	0.304	Hydroxyproline trans	-0.236
3-Phosphoglyceric acid	0.291	PC33:2	-0.235
LPC 22:6	0.278	Linolenic acid-iso1	-0.229
Cystathione	0.273	androsterone sulfate-iso4	-0.222
Histidine	0.231	PC34:1 e	-0.218
C5:1	0.219	Taurocholic acid	-0.215
C18:2	0.215	PC32:0	-0.213
Glycoursodeoxycholic acid	0.205	TG47:0	-0.198
Phenylalanine	0.199	ChoE(22:5)	-0.191
C2:0	0.197	PC34:2	-0.188
Glycerol-1-phosphate	0.172	dihomo-y-linolenic acid-iso2	-0.188
TG56:6	0.166	androsterone sulfate-iso2	-0.178
PC38:2	0.161	C12_0-OH-a	-0.172
alpha-tocopherol	0.145	HODE-iso1	-0.140
TG56:7	0.139	HpODE	-0.134
15-HETE	0.131	Cystine	-0.112
ChoE(20:5)	0.128	9.12.13-TriHOME	-0.109
LPE20:5-sn1	0.095	SM32:2	-0.106
Fumaric acid	0.089	Phosphoethanolamine	-0.096
3-Hydroxybutyric acid	0.088	LPC18:3-sn1	-0.078
PC35:1	0.088	PC32:2	-0.059
Nervonic acid	0.075		
C16:0-OH	0.058		
cis-10 heptadecenoic acid	0.041		
LPC16:1	0.026		
TG56:5	0.025		

Abbreviations: ChoE: cholesterol ester; HETE: hydroxyeicosatetranoic acid; HpEPE: hydroxyperoxy-eicosapentaenoic acid; HpODE: hydroperoxyoctadecadienoic acid; LPC: lysophosphatidylcholine; LPE: lysophosphoethanolamine; OxoODE: oxo-octadecadienoic acid; PC: phosphatidylcholine; SM: sphingomyelin; TG: triglycerides; TMA: trimethylamine-N-oxide; TriHOME: trihydroxyoctadecenoic acid

Table 3. Linear regression analysis examining the associations of 1-SD increase of multi-metabolite score with changes of main cardio-metabolic parameters.

Changes	(Mean±SE)	p value
Glucose (mg/dL)	-0.72± 0.34	0.020
Insulin (mcUI/mL)	-0.57± 0.24	0.010
HOMA-IR	-0.56 ± 0.23	0.010

Values are given as means ± standard error. Model was adjusted for sex, age, weight changes, dietary interventions and value for the respective outcome traits at the baseline examination.

Figure legend

Figure 1: Flow Diagram of the study design METADIET

Figure 2. Metabolite set enrichment analysis

Over representation analysis (ORA) was implemented in the enrichment analysis of MetaboAnalyst 4.0 using the hypergeometric test to evaluate whether a particular metabolite set is represented more than expected by chance within the metabolites previously selected. On the left there is a bar-plot representation of this test and on the right the same information is plotted in a network with a clustering of the identified metabolic pathways

Figure 3. DIABLO graphical and numerical outputs on dual-omics analysis

a) Sample scatterplot from plotDiablo displaying the first component in each data set (upper diagonal plot) and Pearson correlation between each component (lower diagonal plot); b) Loading plot of each feature selected on the first component of both data sets, with color indicating the class with a maximal mean expression value for each feature; c) Circos plot of the final dual-omics signature; d) Relevance network visualization of the selected features at a correlation cut off value of 0.2.

Figure 1

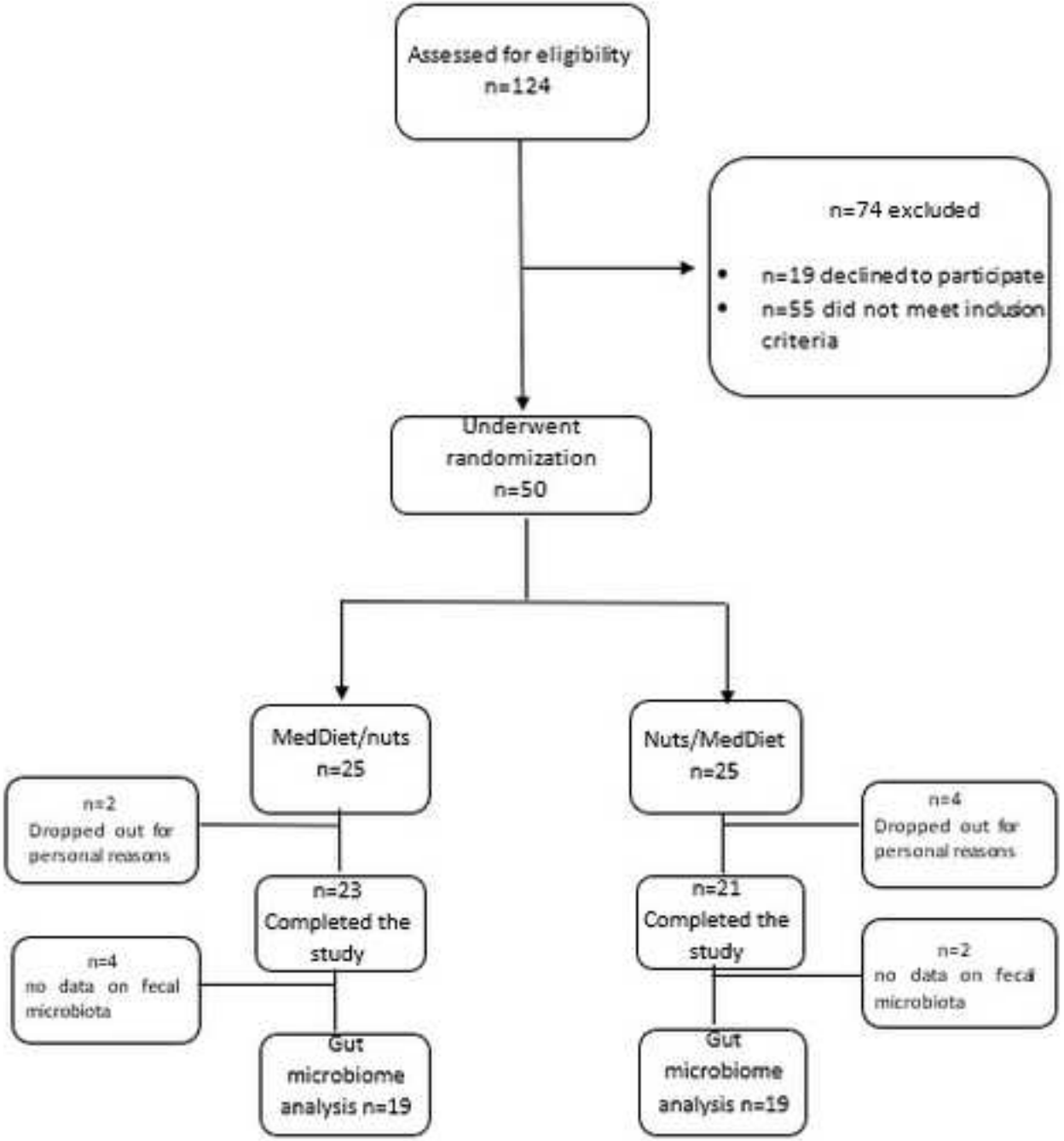

Figure 2

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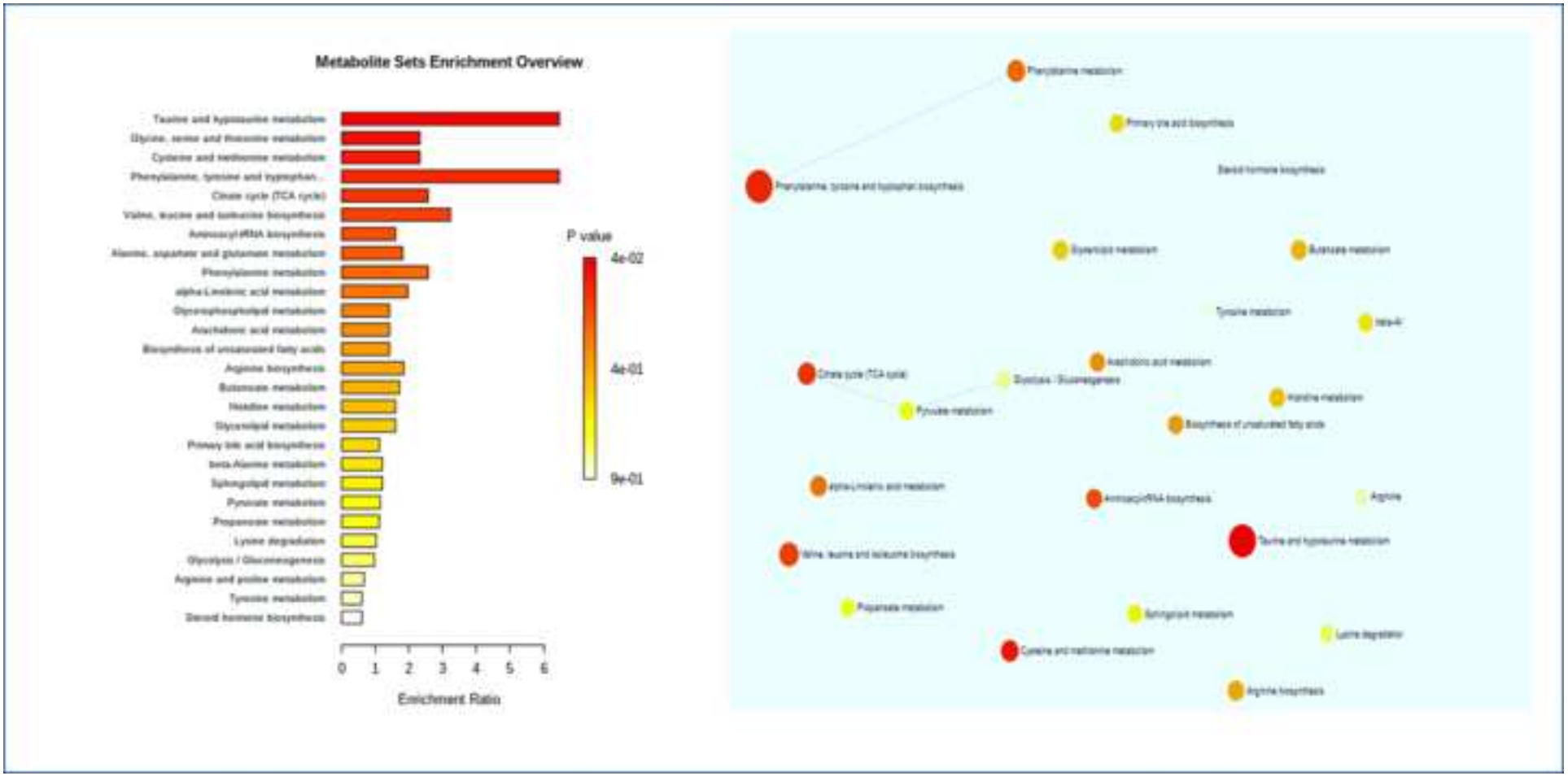

Figure 3

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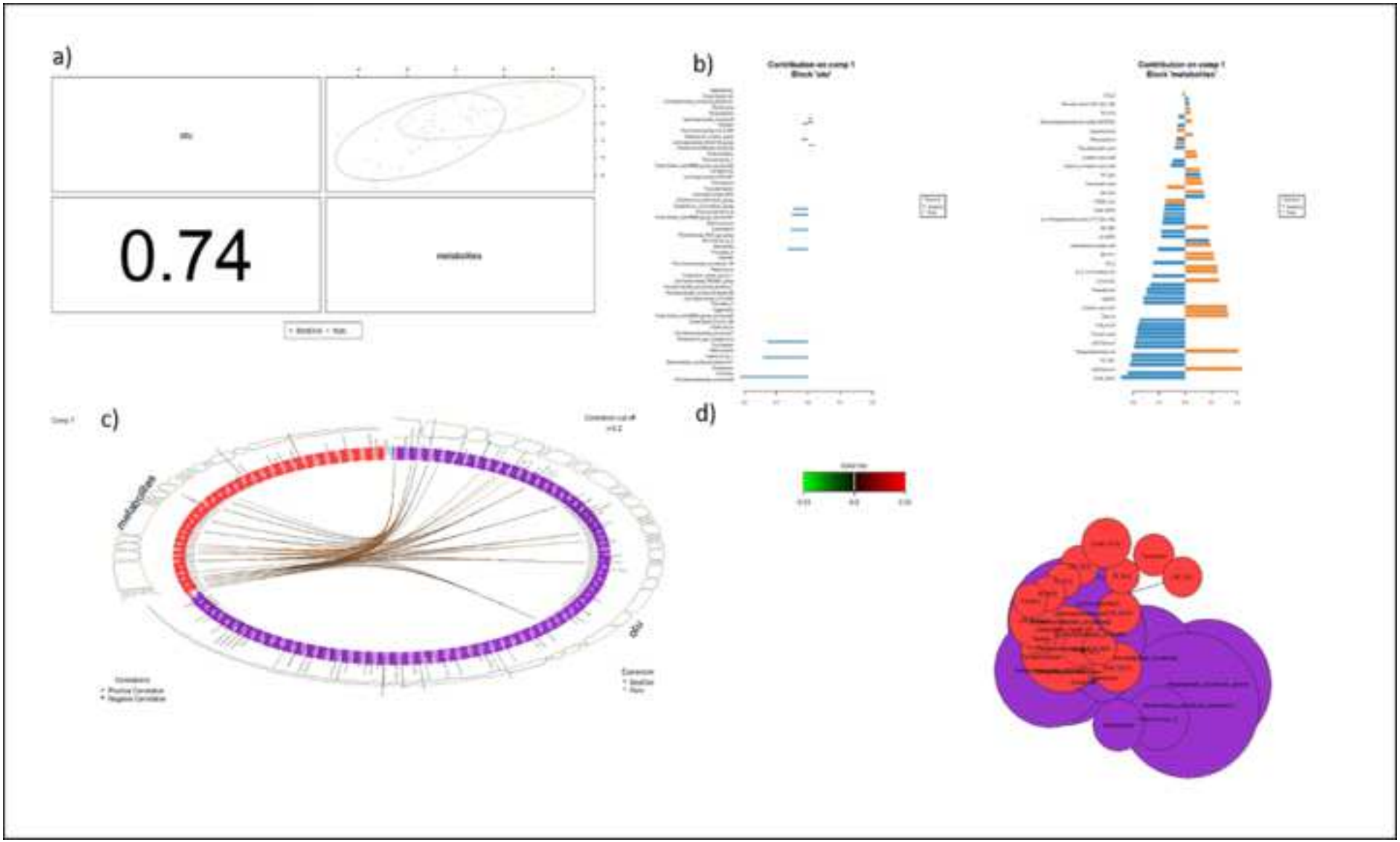

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	3
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	3-4
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	5
	2b	Specific objectives or hypotheses	6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	6
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	6
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	9
Sample size	7a	How sample size was determined	Not available
	7b	When applicable, explanation of any interim analyses and stopping guidelines	Not available
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	6
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Not available
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	Not available
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	Not available
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	Not available

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	Not available
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	7
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	7
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	9
	13b	For each group, losses and exclusions after randomisation, together with reasons	9
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Not available
	14b	Why the trial ended or was stopped	Not available
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	9
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	9
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)	10
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	10
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Not available
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Not available
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	16
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	11-12
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	13-14-15
Other information			
Registration	23	Registration number and name of trial registry	2
Protocol	24	Where the full trial protocol can be accessed, if available	2
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	1-2

*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.