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Effect of pistachio consumption on the modulation of urinary gut microbiota-related metabolites in pre-diabetic subjects☆

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Short running title: Pistachio nut modulates gut urinary metabolites

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Abbreviations:

1D NOESY-presat, one dimension nuclear overhauser effect spectroscopy pulse with a water presaturation; BMRB, Biological Magnetic Resonance Bank; CD, control diet; CI, confidence intervals; CVD, cardiovascular disease; D2O, deuterium oxide; DMA, dimethylamine; E%, energy percentage; EPIRDEM, Effect of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus; HMDB, Human Metabolome Database; HPLC, high-performance liquid chromatograph; JRES, J-resolved; MHP, N-methyl-trans-4-hydroxy-L-proline; ML-PLS-DA, multilevel partial least squares discriminant analysis; NAFLD, non-alcoholic fatty liver disease; NMR, nuclear magnetic resonance; PCA, principal component analysis; PD, pistachio diet; PQN, Probabilistic Quotient Normalization; QqQ/MS, triple quadrupole mass spectrometer; T2D, type 2 diabetes; TCA, tricarboxylic acid; TCI, inverse triple resonance; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TSP, sodium trimethylsilyl (2,2,3,3–2H4) propionate.

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Abstract

The specific nutritional composition of nuts could affect different metabolic pathways involved in a broad range of metabolic diseases. We therefore investigated whether chronic consumption of pistachio nuts modifies the urine metabolome in pre-diabetic subjects. We designed a randomized crossover clinical trial in 39 pre-diabetic subjects. Subjects consumed a pistachio-supplemented diet (PD, 50% carbohydrates, 33% fat, including 57g/d of pistachios daily) and a control diet (CD, 55% carbohydrates, 30% fat) for 4 months each, separated by a 2-week wash-out. Nuclear magnetic resonance (NRM) was performed to determine changes in 24-h urine metabolites. Significant changes in urine metabolites according to the different intervention periods were found in uni- and multivariate analysis. Score plot of the first two components of the multilevel partial least squares discriminant analysis (ML-PLS-DA) showed a clear separation of the intervention periods. Three metabolites related with gut microbiota metabolism (i.e. hippurate, p-cresol sulfate and dimethylamine) were found decreased in PD compared with CD ($P<0.05$). Moreover, cis-aconitate (intermediate of the tricarboxylic acid (TCA)) was also found decreased following PD compared with CD. Intra-group analysis showed that creatinine levels were significantly increased in PD ($P=0.023$), whereas trimethylamine N-oxide (TMAO) was found significantly reduced following PD ($P= 0.034$). Our results suggest that chronic pistachio consumption may modulate some urinary metabolites related to gut microbiota metabolism and the TCA cycle; all associated with metabolic derangements associated with insulin resistance and type 2 diabetes.

Keywords: metabolomics; pistachios; pre-diabetes.

1. Introduction

Nuts are dense foods with a complex matrix of nutrients and other bioactive components that synergically contribute to its beneficial role on different chronic metabolic diseases such as type 2 diabetes (T2D) and cardiovascular disease (CVD) [1,2]. The health benefits of nuts are mainly attributed to its richness in polyunsaturated fatty acids and its high content of fiber, phytosterols and phenolic components [1]. However, there is growing evidence that the beneficial role of nuts on health could be mediated by its capacity to modulate gut microbiota as a prebiotic food [3].

Gut microbes play an important role in several metabolic processes in the human body. They contribute to the energy harvesting capacity from the diet, the modulation of systemic inflammation and insulin resistance thereby contributing to the metabolic risk [4]. Changes in gut microbiota composition have been associated with obesity, T2D and CVDs [5,6] as well as longevity [7]. Changes in the composition of fecal bacteria with increased butyrate producers was demonstrated after almond and pistachio consumption, although changes in neither *Lactobacillus* nor *Bifidobacteria* were observed [8]. In contrast, another feeding trial administering almonds or almond skins for 6 weeks showed significant increases in *Bifidobacterium spp.* and *Lactobacillus spp.* and a repressed growth of *Clostridium perfringens* [9]. Changes in gut microbiota composition must undoubtedly modify gut-derived metabolites that are, in fact, mediator or effector molecules. However, to date no study has evaluated the effect of nut consumption on changes in gut-derived metabolites. Moreover, in the absence of fecal samples, an alternative for determining the nutritional modulation of the gut microbiota is by evaluating the different metabolites generated or catabolized by them in fluids such as urine, due to gut microbiota composition and different urine or plasma metabolite signatures are highly correlated [10].

We have previously demonstrated that pistachio consumption modulates insulin resistance (IR) status and CVD markers at systemic, cellular and molecular levels [11,12].

However, there are no previous studies evaluating changes in urinary metabolome after chronic pistachio consumption that could help to further understanding molecular consequences of their health benefit properties. We hypothesize that chronic pistachio consumption could modulate urinary metabolites towards a healthier profile.

Therefore, the aim of this study is to assess the effects of a pistachio-rich diet on different urine metabolites mainly those related with gut microbiota.

2. Material and Methods

2.1 Study characteristics

The EPIRDEM (Effect of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus) study is a randomized, controlled, crossover trial with two 4-month dietary interventions arms separated by 2-week wash-out period that was conducted in pre-diabetic subjects. The trial was registered in Clinical Trials (identifier NCT01441921) and the institutional review board of the University Hospital of Sant Joan de Reus approved the study protocol in September 2011. Executed informed written consent was obtained from all study participants.

2.2 Study population

Eligible participants were community-living men and women aged 25-65 years, with a body mass index $\leq$ 35 kg/m^2 and fasting plasma glucose levels between 100 and 125 mg/dL. Subjects were excluded if they had T2D (or use anti-diabetic drugs) or met one of the defined exclusion criteria listed in the protocol [11]. Alcohol, tobacco or drug abuse; frequent consumption of nuts or known history of allergy to them; being pregnant or wishing to become pregnant 9 months before or during the study, lactating 6 weeks before or during the study; and/or significant liver, kidney, thyroid or other endocrine diseases were excluding criteria.

2.3 Study design

At baseline, data on medical history, physical examination and fasting blood for biochemical analysis were collected. Subjects who met the inclusion criteria were randomly assigned to one of the two different intervention sequences using a computer-generated random-number table. They were instructed to follow a normocaloric diet

(calculated using WHO equations adjusted by physical activity) that provided 50% of energy (E%) as carbohydrates, 15 E% as protein, and 35 E% as total fat during the 15 days preceding each study period. After the run-in period, subjects were randomized to one of the two diet sequences: starting with a control diet (CD) followed by the pistachio supplemented diet (PD), or starting with the PD followed by the CD. The main characteristics of both intervention diets have already been published [11]. During the pistachio diet (PD) intervention, participants were supplemented with 2 ounces of pistachio (57 grams/day, half roasted and half roasted and salted).

Adherence to the intervention period was assessed by counting the empty sachets of pistachio administered and measuring plasma lutein-zeaxanthin and γ-tocopherol levels with a high-performance liquid chromatograph (HPLC) coupled to a 6490 triple quadrupole mass spectrometer (QqQ/MS) (Agilent Technologies, Palo Alto, U.S.A.) as previously described [11].

2.4 ^{1}H-NMR based targeted metabolomic profiling

2.4.1 24-h urine collection and storage

Twenty-four hour pooled urine samples were collected both at baseline and at the end of each dietary intervention period. Each subject was provided with two opaque, disinfected and airtight 2.5-L polyethylene containers. The subjects were instructed not to collect the first urine void in the morning on the first day, but after this first void, the urine collection continued until and including the morning urine void the following day. During the 24-h, the subjects were instructed to store the containers in the fridge or in another cool place, if possible. After sample arrival, instructed technicians measured diuresis, aliquoted urine and stored them at -80ºC until further use.

2.4.2 Preparation of samples for NMR

Before the NMR analysis, each sample was thawed and a volume of 400 μL was diluted with 200 of buffer phosphate solution containing 1.5 mM K_2HPO_4/KH_2PO_4, 5.8 mM of sodium trimethylsilyl (2,2,3,3–2H4) propionate (TSP) and sodium azide diluted in deuterium oxide (D2O), finally adjusted to pH 7.2.

2.4.3 Acquisition and processing of spectra

The final solution was transferred to a 5 mm NMR tube for subsequent ^{1}H NMR acquisition. The one dimension nuclear overhauser effect spectroscopy pulse with a water presaturation (1D NOESY-presat) was used to record 1D ^{1}H NMR spectra using a 600.2 MHz frequency Avance III-600 Bruker spectrometer (Bruker, Germany) equipped with an inverse triple resonance (TCI) 5 mm cryoprobe. A total of 256 transients with acquisition time equal to 2.73 s were collected across 12 kHz spectral width at 300 K into 64 k data points. A recycling delay time of 5s was applied between scans to ensure correct quantification. The frequency spectra were overfilled and an exponential line broadening of 1 Hz was applied to the FIDs before Fourier transformation, using TopSpin software (version 3.2, Bruker, Germany). The same program was used for their phasing and baseline correction. Calibration (TSP, $\delta = 0.0$ ppm), bucketing (0.001 ppm) and quantification of signals was performed through Dolphin and its on-going optimization Whale [13,14]. Statistical total correlation spectroscopy (STOCSY) and additional 2-D J-resolved (JRES) NMR experiments were performed for the purpose of confirming chemical shift assignments, using standard Bruker pulse programs.

2.4.4 Processing of the dataset

Possible wrong or inaccurate data, considered as excessive fitting error (> 8%) or low signal to noise ratio (to estimate the fitting accuracy, all the signals overlapping the one of interest are considered as 'noise'), were automatically removed. In addition, those signals with more than 25% of missing values were discarded for statistical analysis.

Accordingly, quantified metabolites were finally normalized through Probabilistic Quotient Normalization (PQN), a normalization method that improves creatinine normalization as it avoids the issues related to creatinine changes that can occur during nutritional interventions.

Metabolite identification was verified using Chenomx NMR Suite (Chenomx version 8.1 NMR Suite software package; Chenomx Inc., Edmonton, Alta., Canada) and the expected urinary metabolite concentration ranges and proton chemical shifts listed in the Human Metabolome Database (HMDB), Biological Magnetic Resonance Bank (BMRB) [15,16] and previous bibliography [17,18].

2.5 Statistical analysis

Descriptive data of participants at baseline and differences during the intervention periods are shown as means and 95% confidence intervals (95% CI) for continuous variables, and number (%) for categorical variables. Differences in all variables were evaluated by analysis of variance (ANOVA), with intervention diet as the independent and repeated measures factor. Principal component analysis (PCA), a classical unsupervised multivariate pattern recognition method, was employed to detect the presence of outliers and to examine possible carryover effect in the baseline data considering each intervention period and sequence of treatments. Multilevel partial least squares discriminant analysis (ML-PLS-DA) was used to paired comparisons of the effects of PD versus CD exploiting the crossover design with the mixOmics R package

v. 6.0.0 [19]. ML-PLS-DA is an extension of ordinary PLS-DA described by Van Velzen et al. [20] which allows separation of the intra-subject variation from the inter-subjects variation that could mask nutrition-related metabolic effects. All statistical analyses were conducted using per protocol (PP) approach and thus excluding participants who did not attend the last visit. All analyses were done using SPSS 22.0 (SPSS Inc, Chicago, IL) and R 3.2.5. All tests were 2-sided, and significance was defined as $P<0.05$.

3. Results

Out of a total of 108 participants were assessed for eligibility, 30 declined to participate and 24 did not meet the inclusion criteria. Randomization was performed for 54 participants that were randomly assigned to one of the two intervention sequences. 24h-urine data was finally available in 39 participants (5 participants dropped out of the study for personal reasons, 7 did not collect urine samples in at least one visit and 3 exhibited highly reduced levels of plasma biomarkers of pistachio consumption during PD) (Supplementary Figure 1). However, the aforementioned three subjects with reduced levels of plasma biomarkers were included in the principal component analysis (PCA) in order to explore their overall metabolomic data and thereafter decide their inclusion/exclusion in further analysis. The baseline characteristics of the study participants are shown in Table 1. No significant differences were observed between dietary interventions at baseline in any of the analyzed parameters.

After processing and filtering NMR data, we successfully identified and quantified 32 metabolites (Supplementary Table 1). N-methyl-trans-4-hydroxy-L-proline (MHP), previously found in NMR data from water soluble fractions of pistachios [21], was detected solely after PD. This intake marker was removed from further analyses.

3.1 Principal component analysis

We initially performed a PCA to explore the distribution of baseline samples according to the period (I and II) and the sequence (PD→CD; or CD→PD) of the crossover study (Supplementary Figure 2). A point cloud would indicate homogeneity at baseline. There was a high distance between first and second baseline periods in those subjects whom showed reduced levels in plasma pistachio intake biomarkers during PD, thus this reinforce their exclusion from further analysis.

3.2 Uni- and multi-variate analysis

Metabolites, and their chemical shift, with significant changes between intervention periods are reported in Table 2. Moreover, centered and scaled changes of these metabolites following each intervention period are showed in Figure 1 as box plots. Hippurate, p-cresol sulfate, cis-aconitate and dimethylamine (DMA) were found decreased in PD compared with CD. Contrary, U9.365 was found increased in PD versus CD. Moreover, we also computed paired t-tests through each intervention (baseline versus end) even though we did not found significant results in changes between interventions. Creatinine was significantly increased in PD (P = 0.023) and another unknown metabolite, U2.79, was found decreased after both CD and PD periods (P = 0.007 and P = 0.040, respectively). Interestingly, trimethylamine N-oxide (TMAO) was found significantly reduced following PD (P = 0.034).

The comparison of centered and scaled changes between interventions was evaluated using ML-PLS-DA models (Figure 2). A clear separation between treatments was observed. The highest absolute values for the loadings (data not shown) were projected into the first and second component, and concur with metabolites significantly modulated in the univariate analysis.

4. Discussion

In the present study, we demonstrated that chronic consumption of pistachio, in the context of a healthy diet, modifies the 24h-urine profile of gut-microbiota related metabolites in pre-diabetic subjects. These changes in urine metabolome were in the expected direction according to a beneficial effect of pistachio consumption on insulin resistance and type 2 diabetes.

The interest in nutritional metabolomics has been increasing due its putative role in the diagnosis and prognosis of several diseases. Previous clinical trials have demonstrated a modulatory role of macronutrients and specific food on human metabolome [22]. Nevertheless, only one cross-over study conducted in 19 subjects has evaluated the effect of 2-weeks pistachio consumption on plasma metabolomic profile compared to the nut-free period. Using ultra-HPLC/MS/MS, they found an increase in the levels of raffinose, (12Z)-9,10-Dihydroxyoctadec-12-enoate, sucrose, together with some modulations of plasma amino acids and fatty acids [23]. In our study, we found decreased urinary levels of cis-aconitate after pistachio consumption. Increased cis-aconitate, a tricarboxylic acid (TCA) cycle intermediate, was found in urine from *fa/fa* rats and *db/db* mice [24], and decreased after oral antidiabetic drug treatment [25]. Increases in urinary TCA intermediates could reflect the β-oxidation of fatty acids during the onset and development of T2D [26]. Therefore, our results support either an improvement of systemic stress caused by decreasing hyperglycemia or by the improvement of kidney tubular transport and a better overall mitochondrial function.

Beyond inner host metabolism, the complex gut microbial ecosystem produces a variety of metabolites that could play an important role in human health and disease. Recently, both pistachio and almond consumption were related with an increased butyrate-

producing phyla, supporting a prebiotic effect of nuts [8]. However, due to the volatile basis of butyrate, it cannot be analyzed with NMR.

Hippurate is one of the most studied gut-microbiota co-metabolite derived from aromatic compounds and polyphenols. Dietary protein and polyphenols ultimately lead to the degradation into quinic and benzoic acids by specific gut microbiota which are then oxidized to hippurate by hepatic mitochondrial function. Hippurate is positively associated with several metabolic diseases such as obesity, hypertension, insulin resistance and T2D [27,28]. Regarding dietary modulation, in the PREDIMED trial, a low-fat diet was associated to an increased 24h-urine hippurate and TMAO levels, compared with a Mediterranean diet supplemented with nuts [17]. Therefore, the significant lower levels of hippurate observed after PD suggest an improvement of metabolic derangements associated with IR and T2D through the modulation of gut microbiota.

Similarly, p-cresol sulfate, an abundant polyphenol and protein-derived compound was decreased after PD. Gut bacteria, such as the pathogen *Clostridium difficile*, are able to convert tyrosine into p-cresol [29]. This metabolite is largely and rapidly absorbed by the colonic mucosa cells and is excreted in urine after sulfate or glucuronide conjugation in the mucosa or the liver [30]. In humans, p-cresol is almost completely sulfonated into p-cresol sulfate by *SULT1A1* (human cytosolic sulfotransferase) [31]. This metabolite might participate in endothelial dysfunction and toxic processes; leukocyte free radical production and blocking the conversion of dopamine into noradrenaline, thus altering systemic metabolism [32,33]. Even though few studies have evaluated uremic levels of p-cresol sulfate, its production rate have been found markedly reduced in vegetarians probably because their higher fiber intake [34].

Increasing fiber intake provides increased substrate for microbial fermentation so that amino acids are consumed in microbial growth rather than broken down to waste solutes. In addition, a reduced colon transit time may limit conversion of amino acids to waste solutes when fiber intake is high [35]. These findings could partly explain our results as fiber intake was significantly higher after pistachio consumption. Overall, decreased p-cresol after pistachio consumption support its modulatory role of gut microbiota that could explain some of the healthy roles of pistachios [11].

Apart from fiber, pistachios are also a rich source of dietary choline which is required for a wide range of biological activities. Choline-derived metabolites have been associated with obesity, T2D, CVD and liver steatosis [36]. Gut microbial metabolism of choline results in the production of trimethylamine (TMA) that once absorbed is further metabolized to generate DMA and/or TMAO [37]. Conversion of choline into methylamines reduce the bioavailability of choline and results in the inability to synthesize phosphatidylcholine with subsequent accumulation of triglycerides in the liver. Importantly, high plasma concentrations of TMA and TMAO have been related to CVD risk [38], whereas high DMA levels has been previously linked to high-fat diet-induced IR, fatty liver, and T2D in experimental mice [39]. In fact, mice susceptible to non-alcoholic fatty liver disease (NAFLD) and fed with a high-fat diet displayed a high urinary excretion of DMA and TMA, and correspondingly low levels of serum phosphatidylcholine [40]. Even though pistachios are rich in choline, we observed a reduction in DMA and TMAO after its consumption that can be explained by a differential gut microbiota modulation.

Together with the modulation of some known metabolites, we found a significant modulation of U9.365 and U2.79. The lack of information regarding unknown

metabolites prevents the association of changes in them with other physiological changes. We have additionally identified and quantified MHP solely after PD. Even though the presence of this metabolite in urine has not been studied, we have shown that it could be considered a urinary biomarker of pistachio consumption as it was identified in the water-soluble fraction of pistachio nut [21].

Different strengths and limitations of our study deserve comment. Among the main strengths are its crossover design and the use of PQN to solve the problems produced by creatinine normalization in nutritional interventions. Moreover, urine composition is more influenced by diet than serum, non-invasively acquired and more low weight compounds can be profiled, although its analysis is more challenging from the analytical point of view. Finally, we used NMR, a high-throughput and nondestructive technique with high reproducibility and minimal sample preparation. Among the limitations, our study was conducted in pre-diabetic subjects, thus limiting the extrapolation of our findings to other individuals.

Chronic consumption of pistachios, in the context of a healthy diet, has a different urine metabolic fingerprinting compared with a nut-free diet. The most prominent hallmarks of these changes are related with a modulation of some microbiota-derived compounds and other metabolites related with TCA cycle. Whether these changes are related to metabolic derangements associated with IR and T2D deserves further research.

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Appendix A. Supplementary data:

Supplementary Table 1. List of 32 identified and quantified metabolites by Nuclear Magnetic Resonance.

Supplementary Figure 1. Flow chart of the study subjects considering the 24-h urine metabolomic analysis.

Supplemental Figure 2. Principal component analysis (PCA) of baseline data according to each sequence.

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LEGENDS TO FIGURES

Figure 1. Box plots of significant metabolites in each intervention period.

Box plots were constructed using values of the changes between the end and the baseline in each intervention period.

Figure 2. Score plot of the subjects projected onto components 1 and 2.

N=39. 2D ML-PLS-DA score plots of the subjects projected onto the first and the second components. Each dot represents one subject in each intervention. Circles represent control diet and triangles represent pistachio diet. Asterisk shows the centroid of each ellipse. Variance accounted by each component is showed in each axis. 2D ML-PLS-DA, 2-dimensions multi-level-partial least square-discriminant analysis.

Table 1. Baseline characteristics of the study population

Variable	Subjects (n=39)
Male/Female	20/19
Age (years)	55.3 (53.2, 57.4)
Weight (kg)	77.4 (74.0, 80.7)
BMI (kg/m^2)	28.9 (28.1, 29.8)
Waist circumference (cm)	94.7, 92.5, 96.9)
Systolic blood pressure (mmHg)	134 (130, 138)
Diastolic blood pressure (mmHg)	81 (79, 84)
Total cholesterol (mg/dL)	213.56 (203.96, 223.17)
LDL-C (mg/dL)	135.05 (125.98, 144.13)
HDL-C (mg/dL)	54.15 (49.70, 58.61)
Triglycerides (mg/dL)	122.18 (106.71, 137.65)
Fasting plasma glucose (mg/dL)	115.77 (110.61, 120.93)
Fasting plasma insulin (mU/mL)	12.95 (10.94, 14.97)
HOMA-IR	3.78 (3.12, 4.44)
HbA_{1c} (%)	5.93 (5.79, 6.07)
Dyslipidemia, n (%)	21 (53.8)
Hypertension, n (%)	18 (46.2)

Data are given as means (95% confidence intervals (CI)) or numbers (%). BMI, body mass index; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; -C, cholesterol; -P, particle; HOMA-IR, homeostatic model assessment of insulin resistance; HbA_{1c}, Glycated hemoglobin.

Table 2. Changes in 24-h urinary metabolites according to intervention period

Metabolite	δ (multiplicity)	PD vs CD	*P* value	Metabolic pathways
Cis-aconitate	5.72 (s) 3.13 (d)	↓	0.040	TCA cycle
Dimethylamine	2.72 (s)	↓	0.044	Gut microbiota-derived metabolism
Hippurate	7.84 (dd) 7.65 (tt) 7.56 (m) 3.98 (d)	↓	0.012	Gut microbiota-derived metabolism
p-cresol sulfate	7.28 (d) 7.21 (d) 2.35 (s)	↓	0.043	Gut microbiota-derived metabolism
U9.365	9.37 (m) 6.39 (d)	↑	<0.001	Unknown
MHP	4.65 (hep) 4.21 (dd) 3.96 (dd) 3.2 (dt) 3.06 (s) 2.49 (m) 2.26 (m)	↑	<0.001	Water soluble pistachio-related metabolite

↑, increase; ↓, decrease; CD, control diet; PD, pistachio diet; s, singlet; d, doublet; t, triplet; hep, heptuplet; dd, double doublet; tt, triple triplet; m, multiplet. MHP, N-methyl-trans-4-hydroxy-L-proline; TCA, tricarboxylic acid.

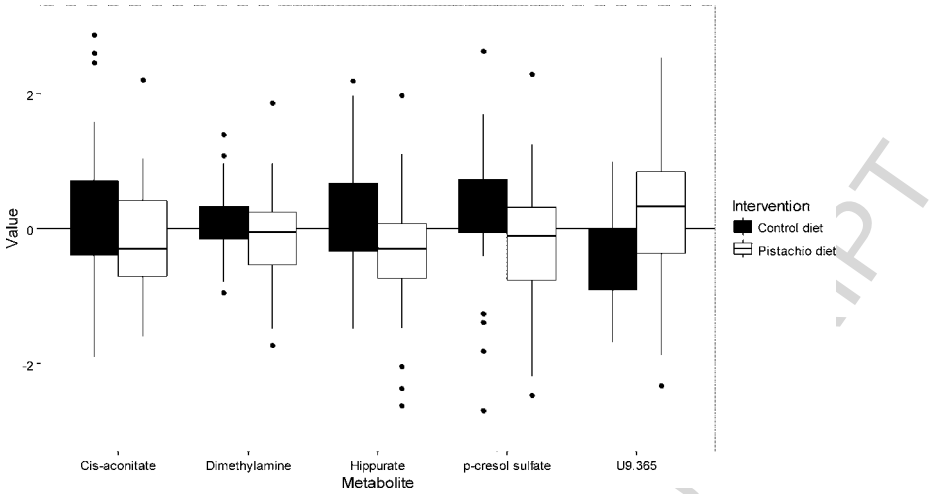

Figure 1

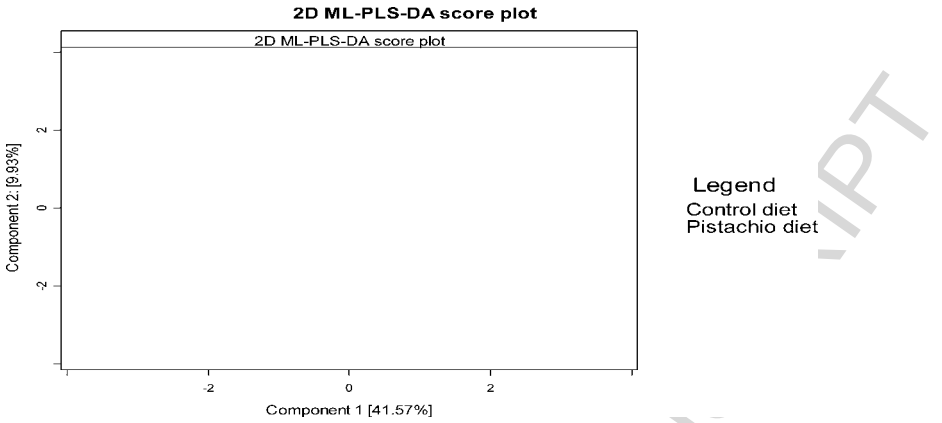

Figure 2

Highlights (maximum of 85 characters, including spaces, per highlight)

- We examined the effect of pistachio intake in urinary metabolites
- Pistachio intake modifies the metabolomic profile in pre-diabetic subjects
- Disease-related metabolites were improved following pistachio intake
- Pistachios seem to alter gut-related metabolites associated to insulin resistance