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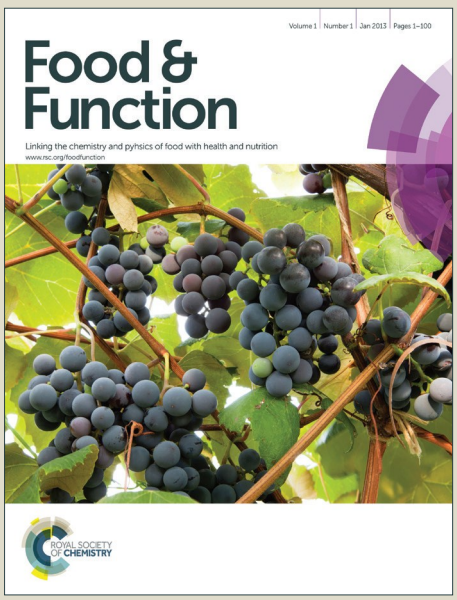

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Urinary metabolomic profile following the intake of meals supplemented with cocoa extract in middle-aged obese subjects

Idoia Ibero-Baraibar [a,b], Ana Romo-Hualde [b], Carlos J. Gonzalez-Navarro [b], M. Angeles Zulet [a,b,c,d], J. Alfredo Martinez [a,b,c,d] *

[a] Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain

[b] Centre for Nutrition Research, University of Navarra, Pamplona, Spain

[c] Instituto de Investigación Sanitaria de Navarra (IdiSNA), Navarra, Pamplona, Spain

[d] CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III, Madrid, Spain

*Corresponding author:

J. Alfredo Martinez

Department of Nutrition, Food Science and Physiology, University of Navarra,
C/Irunlarrea 1, 31008 Pamplona, Spain.
E-mail: jalfmtz@unav.es

Abstract

Metabolomics is used to assess the compliance and bioavailability of food components, as well as to evaluate metabolic changes associated with food consumption. This study aimed to analyze the effect of consuming ready-to-eat meals containing a cocoa extract, within an energy restricted diet on urinary metabolomic changes. Fifty middle-aged volunteers [30.6 (2.3) kg/m^2] participated in a 4-week randomised, parallel and double-blind study. Half consumed meals supplemented with 1.4g of cocoa extract (645mg polyphenols) while the remaining subjects received meals without cocoa supplementation. Ready-to-eat meals were included within a 15% energy restricted diet. Urine samples (24 h) were collected at baseline and after 4 weeks and were analyzed by high-performance-liquid chromatography-time-of-flight-mass-spectrometry (HPLC-TOF-MS) in negative and positive ionization modes followed by multivariate analysis. The relationship between urinary metabolites was evaluated by the Spearman correlation test. Interestingly, the principal component analysis discriminated between the baseline group, control group at the endpoint and cocoa group at the endpoint ($p<0.01$), although in the positive ionization mode the baseline and control groups were not well distinguished. Metabolites were related to theobromine metabolism (3-methylxanthine and 3-methyluric acid), food processing (L-beta-aspartyl-L-phenylalanine), flavonoids (2,5,7,3',4'-Pentahydroxyflavanone-5-*O*-glucoside and 7,4'-dimethoxy-6-C-methylflavanone), catecholamine (3-methoxy-4-hydroxyphenylglycol-sulphate) and endogenous metabolism (uridine monophosphate). These metabolites were present in higher ($p<0.001$) amounts in the cocoa group. 3-methylxanthine and L-beta-aspartyl-L-phenylalanine were confirmed with standards. Interestingly, 3-methoxy-4-hydroxyphenylglycol-sulphate was positively correlated with 3-methylxanthine (rho=0.552; $p<0.001$) and 7,4'-dimethoxy-6-C-methylflavanone (rho=447; $p=0.002$). In conclusion, the metabolomic approach supported the compliance of the volunteers with the intervention and suggested the bioavailability of cocoa compounds within the meals.

Introduction

Metabolomics is an emerging *omics* tool applied to evaluate metabolic modifications in body fluids [1]. The interest for metabolomics has recently increased in nutritional science, but it is also used in clinical, pharmaceutical and toxicological fields in order to explain biological functions and biochemical responses of the organism [2].

In nutrition science, metabolomic analysis is used to detect biomarkers of food intake, which are metabolites produced from the absorption, digestion and metabolism of dietary components [3-4]. Moreover, metabolomics is used for identifying biomarkers of nutritional exposure related to endogenous changes and alteration of metabolic pathways induced by dietary components [5]. Two are the principal types of metabolomic analyses. On the one hand targeted metabolomics is used to analyse predetermined metabolites in body fluids in order to assess the nutritional compliance or endogenous changes associated with dietary consumption [6]. On the other hand, untargeted metabolomics is focused on investigating metabolomic changes in plasma, urine or faeces to identify new biomarkers of dietary exposure after the intake of a specific food or dietary pattern [6-8].

The consumption of fruits and vegetables, which are rich sources of antioxidants, are related to the reduction of some diseases [9]. Those benefits are principally linked to the polyphenol content of plants [10]. However, in order to establish a relationship between the consumption of plants and the effects on health, polyphenols need to be bioavailable for the organism [11-12].

Cocoa is a rich source of antioxidants due to its content in flavanols [13], the consumption of which has been associated with the prevention or improvement of some health disorders such as hypertension, endothelial dysfunction, inflammation and oxidative stress, among others [14-16]. Indeed, the European Food Safety Authority (EFSA) has approved a claim concerning the benefits of cocoa consumption on endothelial function [17]. Nevertheless, it seems that the bioavailability of cocoa flavanols is poor[11]. Cocoa monomers and some dimers are directly

absorbed into the circulation and are sulphated, methylated and glucoronidated by phase-II enzymes in the enterocyte and liver [11]. In contrast, oligomers and procyanidins reach the colon, where they are degraded by colonic microflora producing phenolic acids that then are absorbed into the circulation and metabolized in the liver [11, 18], finally to be excreted in bile and urine [11]. On the other hand, manufacturing procedures such as high temperatures and the storage conditions are able to modify the polyphenolic content in cocoa [19].

The common consumption of cocoa is as cocoa bar or as a powder mixed with milk [20]. However, the inclusion of cocoa flavanols within other different food matrices has to date received little attention. In this context, the aim of the present study was to assess the effect of consuming ready-to-eat meals containing 1.4 g of cocoa extract as part of a 4 week weight loss diet, on urinary metabolome in order to investigate the compliance of overweight/obese middle-aged subjects to the intervention, as well as to evaluate the presence of metabolomic changes of interest for human health.

Material and methods

Subjects

The inclusion criteria were age between 50 and 80 years and a body mass index (BMI) between 27.0 and 35.5 kg/m^2. The exclusion criteria to participate in the study have been previously reported elsewhere [21]. Fifty subjects were recruited to participate in the study, but only 47 completed it (24 in control and 23 in cocoa groups) [21]. The study was carried out in the Metabolic Unit of the University of Navarra (Spain) from April to July 2012 and all participants signed written informed consent before starting the intervention. The trial was approved by the Research Ethics Committee of the University of Navarra (ref. no 006/2012). Moreover, Helsinki Declaration guidelines and further updates were followed. The trial was registered in www.clinicaltrials.gov with the number NCT01596309.

Study design

The study was a 4 week, randomised, placebo-controlled double-blind parallel nutritional intervention. One week before the beginning of the study volunteers were asked to exclude cocoa and cocoa containing products from their habitual diet and three days before starting the trial they were required to consume a low-polyphenol diet without energy restriction. After that, subjects were randomised to the control or cocoa groups using the "random between 1 and 2" function in Microsoft Office Excel (Microsoft Iberica, Spain). Cocoa and control groups were provided with a variety of ready-to-eat meals (dishes and desserts), supplemented with cocoa extract in the case of cocoa consumers and without cocoa extract supplementation in the case of control group. Meals were supplied by Tutti Pasta S.A. company (Navarra, Spain). Volunteers had to choose a dish and a dessert daily from the variety of dishes and desserts they had available for each week. All the volunteers were provided with the same variety of dishes and desserts, but they were not instructed to consume them in a predetermined order. On the other hand and taking into account that participants were overweight/obese, both groups were prescribed with the same hypocaloric diet (-15% energy restriction), which provided 45% of total energy value from carbohydrates, 30-33% of energy from lipids and 22-25% of energy from proteins. Moreover, volunteers were provided with a list of forbidden polyphenol-rich foods and were requested not to change their physical activity patterns during the intervention. Three days before the end of the intervention volunteers were required to consume pre-determined types of test meals from the variety of meals they had available for the last week of the intervention in order to make all the volunteers reach the endpoint of the study in similar nutritional conditions and to avoid nutritional interferences. The day before the intervention (at the beginning and at the endpoint of the study) volunteers collected a 24 h urine specimen. On the first day and the last day of the intervention clinical and biochemical measures were also taken in fasting conditions early in the morning.

The consumption of ready-to-eat meals was assessed by a notebook where volunteers self-reported the name of the test dish and dessert they consumed daily. Volunteers reported a high

adherence, as reported previously [21]. In addition, nutritional intake was analysed by a 3-day validated food-recall questionnaire, which was completed at the beginning and the end of the study. As was the case in other studies, the data were analysed using the DIAL software (Alce Ingenieria SL, Madrid, Spain)[22] and no differences between groups concerning macronutrient consumption were found [23].

Ready-to-eat meals

Each ready-to-eat dish and dessert was supplemented with 0.7 g of cocoa extract in the case of cocoa group, while the control group received the same meals without cocoa extract supplementation. Considering that the daily consumption consisted of a ready-to-eat dish and a dessert, volunteers following the cocoa treatment consumed 1.4 g of cocoa extract daily, which was composed of [mean (SD)]: 140.4 (7.1) mg of theobromine, 645.3 (32.3) mg of total polyphenols as catechin, 414.3 (20.7) mg of flavanols as catechin, 153.4 (7.7) mg of epicatechin, 14.6 (0.7) mg of catechin, 99.4 (5.0) of procyanidin B2, 13.4 (0.7) mg of procyanidin B1 and 133.5 (6.7) mg of oligomeric procyanidins. Total polyphenol content was determined by the Folin-Ciocalteu method and flavanoids and theobromine were analysed by high performance liquid chromatography (HPLC) [21]. The cocoa extract was supplied by Nutrafur S.A (Murcia, Spain). The cocoa extract was added to the ready-to-eat meals before a pasteurization process (95 ºC during 15-20 min). The temperature was then decreased to 10ºC and subsequently, the ready-to-eat meals were ultra-frozen for 90 min and stored at t -20 ºC. At the time of consumption, volunteers had to heat the meals using the microwave, where they were heated reaching the 65 ºC in the core of the meal.

Clinical and blood biochemical measurements

Clinical and blood biochemical measurements were analysed as previously reported in Ibero-Baraibar *et al* (2014) [21]. Briefly, the measurements of height and weight were taken in underwear after an overnight fast. Weight and BMI calculation were determined by a dual-

energy X-ray absorptiometry (DEXA Lunar Prodigy, GE Medical Systems, Madison, WI, USA), following standardized and validated procedures [24]. Systolic and diastolic blood pressure (SBP and DBP), were taken 3 times with an automatic monitoring device (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, Netherlands), using the average value obtained from the last two measurements as established by Wold Health Organization criteria[21].

Overnight fasting blood samples were collected from 8:00 to 9:30 a.m at baseline and at the end of the intervention using EDTA and CLOT tubes. After sample centrifugation (15 min, 1500 g, 4ºC) plasma and serum aliquots were stored at -80ºC until the analysis. Plasma glucose and total cholesterol were measured by colorimetry in a Pentra C200 autoanalyser (Horiba Medical, Montpellier, France). Plasma insulin (Mercodia,Uppsala, Sweden) was determined with specific ELISA kits in a Triturus auto-analyser (Grifols, Barcelona, Spain) [21]. HOMA-IR was calculated as follows: HOMA-IR= [glucose (mmol/L) x insulin (mU/ml)]/22.5.

Urine collection

Twenty-four hours urine specimens were collected the day prior to the beginning of the study and the day before the endpoint of the intervention. Urine samples were collected by all the volunteers in a urine container and chilled at 4 ºC. As has been done in other studies, urine samples were stored in vials of 1 mL at -80 ºC until analysis [25]. Considering the number of baseline and endpoint samples, a total of 94 samples were collected, 2 samples from each subject.

Reagents and Materials

All solvents used were of Liquid Chromatography–Mass Spectrometry (LC-MS) grade and obtained from Scharlau (Scharlab, Sentmenat, Spain). Water (18.2 MΩ) was obtained from an Ultramatic system from Wasserlab (Barbatáin, Spain). The other standards were of analytical or higher grade, and were purchased from Sigma Aldrich (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany).

Sample preparation and High Performance Liquid Chromatography-Time-Of-Flight-Mass Spectrometry (HPLC-TOF-MS) analysis

The urine samples were thawed and centrifuged for 10 min at 10.000 rpm. A 100 µL aliquot of the supernatant was diluted with 100 µL of Milli-Q water and vigorously vortexed. The solution was transferred to a vial for the subsequent analyses. Agilent Technologies 1200 liquid chromatographic system equipped with a 6220 Accurate-Mass TOF LC/MS, operated in positive electrospray ionization mode (ESI+) or negative electrospray ionization mode (ESI-), controlled by MassHunter Workstation 06.00 software (Agilent Technologies, Barcelona, Spain) was used for the analysis. The column used was a Zorbax SB-C18 (15 cm × 0.46 cm; 5 µm) from Agilent Technologies with a SB-C18 precolumn from Teknokroma (Barcelona, Spain). The mobile phase consisted of A (formic acid 0.1%) and B (acetonitrile with formic acid 0.1%). The gradient elution was 1-20% B, 0-4 min, 20-95% B 4-6 min, 95% B 9-7.5 min, 95-1% B 7.5-8 min, 1% B 8-14 min. After the analyses, the column was re-equilibrated during 5 min at 1% B. The injection volume was 15 µL and the flow rate was 0.6 mL min−1. Chromatography was performed at 40 ºC. ESI conditions were as follows: gas temperature, 350 ºC; drying gas, 10 L min−1 ; nebulizer, 45 psig; capillary voltage, 3500 V; fragmentor, 175 V; and skimmer, 65 V. The instrument was set out to acquire over the m/z range 100 –2000 with an acquisition rate of 1.03 spectra s-1.

To evaluate the quality in this metabolomic study, a procedure from Gika *et al* (2007) and Llorach *et al* (2009) with some modifications was used [26-27]. Two types of quality control sample (QCs) were used: i) a standard mixture solution implemented of cytosine, L-carnitine hydrochloride, betaine, leucine, deoxyadenosine and deoxyguanosine at concentration of 1 mg/L. ii) pooled urine which was prepared by mixing equal volumes from each of the 94 samples. These samples were injected 3 times at the beginning of the run to ensure system equilibration, and then every 5 samples to further monitor stability of the analysis. Finally, samples were randomized to reduce the systematic error associated with instrumental variability and were analyzed in sets of 10-15 samples/day.

Data Processing and metabolite identification

LC-MS data were analyzed using Mass Profiler Professional 12.6.1 software (Agilent Technologies; Barcelona, Spain) to detect and align features. Alignment used a 0.15 min retention time tolerance window and a 0.002 Da mass tolerance window. For the screening of metabolites, the following filters were specified: the m/z of metabolites should appear in at least one of 94 samples. Subsequently, the detected m/z should be present in 50% of samples in only the experimental group. Then, a statistical procedure was carried out in order to detect the metabolites which discriminated the groups (further details are given in the section on statistical analysis). After that, those metabolites were identified on the basis of their exact mass, which was compared to that registered in METLIN (https://metlin.scripps.edu/index.php) within a mass accuracy below 5 mDa, as well as based on the score given by the software, the scientific literature and the metabolic pathways reported in Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), Human Metabolome Database (HMDB) (http://www.hmdb.ca/) and Lipidmaps (http://www.lipidmaps.org/). Some metabolites were confirmed by comparison with commercial standard.

Statistical analyses

The sample size was primarily calculated for the main study considering oxLDL as the main variable [21]. It was calculated taking into account the study by Khan *et al.* (2012) with a reduction of 14.1 U/L and an interquartile range of 16.3 U/L [28].With a bilateral confidence index of 95% (α=0.05) and a statistical power of 80% (β=0.80) the sample size was estimated to be 44 subjects. A possible drop-out rate of 15% was considered, establishing the final sample size of 50 subjects [21]. Data are expressed as mean (SD) and normality of the variables was assessed using the Shapiro-Wilk test. According to whether variables were normally distributed or not, comparisons between baseline and the endpoint were analysed by paired Student's t-test or the Wilcoxon signed-rank test, while comparisons between both groups were performed with and independent sample t-test or Mann–Whitney U test. Spearman correlation tests were carried out

to evaluate the relationship between metabolite changes when appropriate. $p < 0.05$ was considered significant. STATA version 12.0 (Stata- Corp, College Station, TX, USA) software was used to carry out statistical analysis.

With regard to the metabolites, a one-way ANOVA was conducted followed by a Tukey range test, and a Benjamini–Hochberg multiple correction procedure was used to statistically compare and define statistically significant metabolites ($p < 0.01$). Finally, metabolites that satisfied a fold change cut-off of 2.0 were selected. This statistical analysis was performed using Mass Profiler Professional 12.6.1 software (Agilent Technologies; Barcelona, Spain). Then, the resulting data were analyzed by principal components analysis (PCA) with Pareto scaling, using Mass Profiler Professional 12.6.1 software (Agilent Technologies; Barcelona, Spain). Finally we selected the metabolites that showed a highly significant difference between groups ($p < 0.001$) in the change from baseline to endpoint.

Results and discussion

Subjects

The principal characteristics of volunteers and the phenotypical changes after the 4 weeks intervention are reported in Table 1. Control and cocoa groups were homogeneous at baseline since no statistical differences were found at the beginning of the intervention in any of the variables assessed as reported elsewhere [23]. For this reason baseline data are shown as a collective group. From the 50 subjects enrolled in the intervention 47 volunteers completed it. Overall volunteers were men and women with a BMI of 30.3 (2.0) kg/m^2 and 57.5 (5.3) years old. As previously reported in Ibero-Baraibar *et al* (2014), BMI, blood pressure, total cholesterol and HOMA-IR significantly decreased in both groups after the 4 week intervention [21].

Urinary metabolomic profile

The PCA of urine samples was able to discriminate three groups in the negative ionization mode: the baseline group (all the volunteers before beginning the study), the control group at endpoint and the cocoa group at endpoint. The PCA in the positive ionization mode showed clear discrimination between the control and cocoa groups at the endpoint, while baseline and control group at the endpoint were not so clearly distinguished (Figure 1). Therefore, it seems that both, cocoa consumption and following a weight-loss diet influenced the excretion of metabolites in urine. This result suggests the compliance of the volunteers with the intervention and indirectly indicates the bioavailability of cocoa compounds within the ready-to-eat meals because cocoa-specific putative metabolites were identified [29]. In general, the most widely used food matrices for cocoa phytochemical consumption are chocolate bars and cocoa beverages [25, 28, 30]. In addition, when cocoa beverages or chocolate bars are consumed, they usually do not suffer additional heating process by the consumers, with the exception of hot chocolate, which is added to boiling water or milk before consumption. In the present study, test products suffered an additional heating process before being consumed by the volunteers and metabolites from cocoa consumption were still detected, which suggests that cocoa compounds were absorbed and metabolized.

Identification of putative molecules and their implication in human metabolism

In the overall urine metabolite profile 1095 features in the positive ionization mode and 1154 features in the negative ionization mode were detected. Then, predetermined cut off values were applied in order to select the metabolites that were different ($p < 0.01$) across the 3 groups. Thus, 26 putative metabolites were identified in positive ionization mode and 20 putative metabolites in negative ionization mode. From those, we focused on metabolites that showed a highly significant difference between groups ($p < 0.001$) in the change from baseline to endpoint. Thus, 8 metabolites were selected, 5 in negative ionization mode and 3 in positive mode. These metabolites are listed and grouped according to their origin (Table 2). The

remaining metabolites are reported in Supplementary Table 1 (negative ionization mode) and in Supplementary Table 2 (positive ionization mode). Tables contain information concerning mean intensity of each metabolite at baseline as well as in control and cocoa groups at the endpoint, retention time, detected mass, putative metabolite identification, assignation and mass difference.

Cocoa is a rich source of phytochemicals such as polyphenols and purine alkaloids[14]. Moreover, manufacturing procedures as well as metabolization processes may influence the content of phenolic compounds[19]. Focusing on the putative identification, cocoa related metabolites are grouped into metabolites derived from theobromine metabolism (3-methylxanthine and 3-methyluric acid), food processing (L-beta-aspartyl-L-phenylalanine), flavonoid metabolism (2,5,7,3',4'-pentahydroxyflavanone-5-O-glucoside and 7,4'-dimethoxy-6-C-methylflavanone), catecholamine metabolism (3-methoxy-4-hydroxyphenylglycol-sulphate) and endogenous metabolism (uridin monophosphate) (table 2). 3-methylxanthine and L-beta-aspartyl-L-phenylalanine were confirmed with authentic standards, while uridine monophosphate was not defined. All of these were significantly more highly excreted in the cocoa supplemented group ($p < 0.001$).

Cocoa and control groups significantly increased the excretion of theobromine metabolites such as 3-methylxanthine and 3-methyluric acid after the 4 week intervention. These metabolites were not found at baseline and they showed significantly higher excretion rates in the cocoa group (table 2). The presence of polyphenols and thebromine derivatives in urine of control group could be due to the content of those compounds in other foods consumed by the volunteers in the diet. This finding is in accordance with the results obtained by Llorach *et al* (2013) and Garcia-Aloy *et al* (2015), who found similar metabolites in human urine after cocoa intake [25, 31]. Theobromine is a 3,7-dimethylated xanthine alkaloid that is also formed during caffeine metabolism [32]. Its acute consumption stimulates heart rate and specific benefits in lipoprotein levels have been observed, specifically in high-density lipoprotein-cholesterol (HDL-c) [33-34]. Taking into account that theobromine is not degraded during cocoa processing

and that it is highly bioavailable and well absorbed in the small intestine, it can be used as an indicator of the amount of cocoa intake [14, 32]. 3-methylxanthine metabolite is produced from theobromine degradation by the P450 enzyme. Then, 3-methylxanthine is degraded by xanthine oxidase producing 3-methyluric acid [14]. These metabolites corroborate the compliance of the volunteers with the intervention, since it is firmly established that those metabolites are derived from cocoa intake [31].

With regard to food processing compounds, L-beta-aspartyl-L-phenylalanine was detected in urine. The excretion of L-beta-aspartyl-L-phenylalanine was detected in both groups after the 4 week intervention, but the excretion was significantly higher in cocoa consumers. This metabolite was not found at baseline. L-beta-aspartyl-L-phenylalanine is an isomer of aspartyl-phenylalanine, which was reported by García-Aloy *et al* (2015) in human urine after cocoa consumption [31]. The metabolite is a processing derived compound and it is produced from aspartame degradation, a sweetening agent that could present be present in the ready-to-eat meals [31]. Garcia-Aloy et al (2015) also reported the presence of a similar metabolite named cyco(asparyl-phenylalanil) which has been identified as a constituent of roasted cocoa nibs [31].

Although the principal cocoa flavonoids are flavanols, cocoa also contains other types of flavonoids in small amounts [35]. In this study, two putative metabolites derived from flavanones were detected: 2,5,7,3',4'-pentahydroxyflavanone-5-*O*-glucoside and 7,4'-dimethoxy-6-C-methylflavanone. Neither metabolite was detected at baseline. Thus, 2,5,7,3',4'-pentahydroxyflavanone-5-*O*-glucoside was identified only in the cocoa group urine samples at the endpoint, while 7,4'-dimethoxy-6-C-methylflavanone was found in both groups, showing a significantly higher excretion in the cocoa supplemented group (table 2). To our knowledge, those metabolites are derived from flavanones that could be present in the cocoa extract [35]. However, they still have not been apparently described in the scientific literature related to cocoa intake.

Interestingly, a metabolite related to catecholamine metabolism was identified, 3-methoxy-4-hydroxyphenylglycol sulphate (MHPG-sulphate). MHPG-sulphate excretion increased after the 4 week intervention in both groups, being a significantly higher excretion in the cocoa group. MPHG can be found in sulphated or in glucoronidated froms, with the sulphated form being more common in humans [36]. There are previous studies reporting that 3-methoxy-4-hydroxyphenylglycol (MHPG) levels are decreased in the brain and urine of depressive subjects and the metabolite is an indicator of brain norepinephrine metabolism [36-37]. Interestingly, the scientific literature supports that central MHPG excretion is best represented by the urinary MHPG-sulphate fraction than total MHPG levels [38]. On the other hand, there are studies suggesting that weight loss and the consumption of cocoa are beneficial for psychological disorders [39-40]. Taking into account that the excretion of the MHPG-sulphate metabolite increased in both groups showing a significantly higher excretion in the cocoa group, it could be speculated that cocoa extract supplementation could have a possible additional benefit in terms of improving depressive symptoms. Indeed, ΔMHPG-sulphate was positively correlated in the whole sample with Δ3-methylxanthine (rho= 0.552; $p<0.001$) and Δ 7,4'-dimethoxy-6-C-methylflavanone (rho= 0.447; p= 0.002) (Table 3). As previously described, 3-methylxanthine is a metabolite related to cocoa intake. Therefore, this outcome shows that higher excretion of MHPG-sulphate is associated with higher excretion of 3-methylxantine (table 3). Concerning this result, a recent investigation using a mouse model has reported that the consumption of a diet rich in theobromine, polyphenols and polyunsaturated fatty acids which was composed of cocoa, hazelnuts, polyphenols, vegetable oils and flours rich in soluble fibre and known as LMN cream, modulated the catecholaminergic and cholinergic systems of mouse brain [41]. However, this hypothesis should be viewed with caution because MHPG-sulphate in the present study is a putative metabolite. In addition, the assessment of MHPG-sulphate with other depressive markers would be needed to be established.

Finally, uridin monophosphate was identified as a putative endogenous molecule involved in nucleotide metabolism [42]. This metabolite was not excreted at baseline or in the control group

after the 4 week intervention (table 2). However, it was detected after the intervention in the cocoa group. To our knowledge, uridine monophosphate has not previously found in urine after cocoa consumption.

Some limitations can be mentioned. First, it would be of interest to perform a targeted metabolomic analysis to confirm the putative compounds and second, the inclusion of a group without a weight-loss diet consuming only the cocoa extract would be attractive option to discriminate possible interactions between weight loss metabolite changes and the changes derived from cocoa intake.

Conclusion

The untargeted metabolomics analysis was able to discriminate the intervention groups providing (clear) evidence for compliance with the cocoa intake and indirectly suggesting the availability of cocoa compounds within the ready-to-eat meals. Furthermore, the described metabolomic changes contribute to providing more information concerning the beneficial effects of cocoa intake.

Acknowledgements

This study was supported by grants from the CDTI within CENIT Program (Industrial Research of diets and food with specific characteristics for elderly –SENIFOOD), Linea Especial about Nutrition, Obesity and Health (University of Navarra LE/97), Instituto de Salud Carlos III (CIBERobn) Fisiopatología de la Obesidad y Nutrición, Centro de Investigación en Nutrición (CIN) de la Universidad de Navarra and Instituto de Investigación Sanitaria de Navarra (IdiSNA). The authors appreciate the final reading and improvements by Paul William Miller (Lecturer in the Modern Languages Department at University of Navarra). In addition, the authors wish to acknowledge the Asociación de Amigos Universidad de Navarra for the predoctoral grant given to Idoia Ibero-Baraibar.

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Tables

Table 1. General characteristics of volunteers at baseline and changes after the 4 week nutritional intervention

Variables	Baseline (n=47)	ΔControl (n=24)	ΔCocoa (n=23)	Δp
Age (years)	57.5 (5.3)	—	—	—
BMI (kg/m^2)	30.3 (2.0)	-0.9 (0.5)***	-1. (0.5)***	0.744
SBP (mmHg)	120 (19.93)	-8.7 (10.8)***	-6.7 (8.5)***	0.477
DBP (mmHg)	81 (8.43)	-7.63 (6.88)***	-5.74 (5.38)***	0.302
Total-c (mg/dL)†	240.55 (44.45)	-41.50 (24.22)***	-41.43 (34.26)***	0.509
HOMA-IR	1.75 (1.35)	-0.43 (1.38)**	-0.61 (0.90)**	0.992

Data are expressed as mean (SD). Comparisons between baseline and end of the study were analyzed by paired student t-test or Wilcoxon test (***p≤0.001; **p≤0.01; *p≤0.05).

Comparisons between both groups were performed with an independent t-test or Mann-Whitney U test (Δp).

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment insulin resistance; SBP, systolic blood pressure, Total-c, total cholesterol.

†: non-normally distributed variables

Δ: endpoint– baseline

Table 2. Putative metabolites different between baseline, control and cocoa groups.

Baseline	Control 4 weeks	Cocoa 4 weeks	Δp (Δcontrol vs. Δcocoa)	RT (min)	Detected mass (m/z)	Putative metabolites	Assignation	Mass difference (mDa)
Theobromine metabolism								
nd	5.29	21.51	<0.001	6.740	167.0583	3-Methylxanthine[a]	[M+H]+	-1.9
nd	1.72	13.10	<0.001	6.334	183.0525	3-Methyluric acid[b]	[M+H]+	-1.2
Food processing								
nd	1.72	11.04	<0.001	7.969	281.1128	L-beta-aspartyl-L-phenylalanine[a]	$[M+H]^+$	0.4
Flavonoid metabolism								
nd	nd	10.09	<0.001	8.594	465.1026	2,5,7,3',4'-Pentahydroxyflavanone 5-*O*-glucoside[b]	[M-H]-	1.2
nd	3.30	14.99	<0.001	7.995	279.1011	7,4'-Dimethoxy-6-C-methylflavanone[b]	$[M-H]^-[-H_2O]$	1.0
Catecholamine metabolism								
0.45	7.53	18.28	<0.001	8.694	263.0227	MHPG-sulphate[b]	$[M-H]^-$	0.4
Endogenous metabolism								
nd	nd	13.21	<0.001	8.666	369.0349	Uridine monophosphate[c]	[M+HCOO]-	-0.9
nd	nd	16.85	<0.001	8.696	383.0486		[M+CH3COO]-	1.2

p<0.05 was considered as significant. Δ= endopoint– baseline. Comparisons between both groups were performed with by U Mann–Whitney test.
The data in baseline, control and cocoa columns refers to mean intensity of metabolites and are presented as log 2.
Abbreviations: nd, no detected; RT, retention time; MHPG-sulphate, 3-Methoxy-4-hydroxyphenylglycol sulphate. The following comparisons were p<0.05: baseline vs. control, baseline vs. cocoa and control vs. cocoa
[a] Identification was confirmed by standard comparison
[b] Not confirmed by standard comparison
[c] Not clearly defined with standard comparison

Table 3. Correlation analysis between changes in MHPG-sulphate and metabolites related with cocoa intake

Metabolites	Δ MHPG-sulphate	
	rho	**p**
Δ 3-Methylxanthine	0.552	<0.001
Δ 3-Methyluric acid	0.265	0.072
Δ 2,5,7,3',4'-Pentahydroxyflavanone 5-*O*-glucoside	0.202	0.173
Δ 7,4'-Dimethoxy-6-C-methylflavanone	0.447	0.002

rho: Spearman correlation coefficient
$p<0.05$ was considered significant
Δ: change (endpoint– baseline)
Abbreviations: MHPG-sulphate, 3-Methoxy-4-hydroxyphenylglycol-sulphate

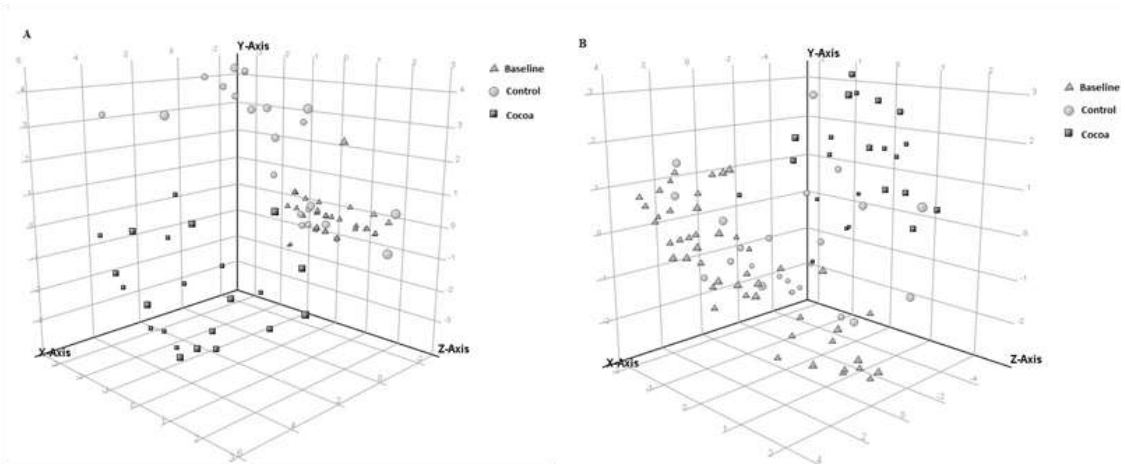

Figure 1. Principal component analysis (PCA) including baseline group (n=47), control group at the endpoint (n=24) and cocoa group at the endpoint (n=23).

A: PCA in negative ionization mode (ESI-)

B: PCA in positive ionization mode (ESI+)