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ABSTRACT

Flavan-3-ols and methylxanthines have potential beneficial effects on human health including reducing cardiovascular risk. We performed a randomized controlled cross-over intervention trial to assess the acute effects of consumption of flavan-3-ol-enriched dark chocolate, compared with standard dark chocolate and white chocolate, on the human metabolome. We assessed the metabolome in urine and blood plasma samples collected before and at 2 h and 6 h after consumption of chocolates in 42 healthy volunteers using a non-targeted metabolomics approach. Plasma samples were assessed and showed differentiation between time points with no further separation among the three chocolate treatments. Multivariate statistics applied to urine samples could readily separate the postprandial time points and distinguish between the treatments. Most of the markers responsible for the multivariate discrimination between the chocolates were of dietary origin. Interestingly, small but significant level changes were also observed for a subset of endogenous metabolites. ^{1}H NMR revealed that flavan-3-ol-enriched dark chocolate and standard dark chocolate reduced urinary levels of creatinine, lactate, some amino acids and related degradation products and increased the levels of pyruvate and 4-hydroxyphenylacetate, a phenolic compound of bacterial origin. This study demonstrates that an acute chocolate intervention can significantly affect human metabolism.

Keywords: NMR, MS, metabolomics, chocolate, metabolism, health, flavonoids, methylxanthines

INTRODUCTION

Dark chocolate contains at least two groups of bioactive substances, flavan-3-ols and methylxanthines. Well known flavan-3-ols include catechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, proanthocyanidins, theaflavins, thearubigins and they are regarded as functional food ingredients[1]. The most well-known methylxanthines are theobromine, caffeine and theophylline. Dark chocolate is enriched in theobromine, with lesser amounts of caffeine and practically no theophylline[2].

Flavan-3-ols have various organoleptic properties such as astringency, bitterness, sourness, and sweetness. They also have several beneficial health effects by acting as antioxidant, anticarcinogen, antimicrobial, anti-viral, and neuro-protective agents. They are also known to affect several measures of cardiovascular health, including blood pressure, vascular function[3], platelet function[4,5], plasma lipids and lipid peroxidation in humans.

The bioactive effects of methylxanthines are mainly mediated by the so-called adenosine receptor blockers[6]. They may improve lung function[2]. Methylxanthines increase the thermogenic capacity of adipose tissue[7]. There is also a synergistic interaction between caffeine and catechins in increasing thermogenesis on brown adipose tissue[8].

Very few studies have identified the fate of cocoa components in humans or rodents[9,10] and such information may help to identify the bioactive compounds. Recently non-targeted approaches in plasma and urine were employed to assess the changes in the host and microbial metabolome after cocoa consumption in human subjects[11,12]. Martin *et al.*[12] used Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) approaches to examine human fasted plasma and urine samples to assess the chronic effects of regular consumption of dark chocolate. This study focused on endogenous metabolites involved in energy homeostasis and stress responses but lacked a control group. Llorach *et al.*[11] on the other hand, used a non-targeted Liquid Chromatography (LC)/MS method to identify metabolites up to 24 h after ingestion of a cocoa drink in 10 subjects but the focus was on the metabolites found in the cocoa product, the excretion of the polyphenols ingested and their products.

Previously, we performed a randomized controlled human trial (clinicaltrials.gov; NCT01099150) to evaluate the effect of flavan-3-ol-enriched dark chocolate, standard dark chocolate and white chocolate on platelet function in healthy subjects[13]. Both flavan-3-ol-enriched dark chocolate and white chocolate, improved several measures of postprandial

platelet function in a gender-specific way although the specific bioactive compounds could not be determined.

In the current analysis, we employed samples from the same study to assess the effects of acute consumption of flavan-3-ol-enriched dark chocolate, in comparison with standard dark chocolate and white chocolate, on both plasma and urinary proton NMR (^{1}H NMR) profiles and urinary MS profiles in 42 healthy humans having fasted ≥ 10 h overnight. Postprandial assessment of the human metabolome by such complementary techniques provides further insights into both the metabolism of flavan-3-ols and methylxanthines in the human body and the effects these plant bioactives have on endogenous metabolism.

MATERIAL AND METHODS

Subjects and study design

Recruitment of subjects (n = 42) and details of the crossover study design were reported previously[13].The study was carried out in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice. The study was approved by the Ethics Committee of the Rowett Institute of Nutrition and Health, University of Aberdeen (reference number 09-002) following review by the North of Scotland Research Ethics Committee. This trial was registered at clinicaltrials.gov as NCT01099150. Volunteers were informed about the purpose and the risks of the study, and written informed consent was obtained from all subjects before participation.

The details are summarised in the Supporting Information Material and Methods, Figure S1 and Tables S1 and 2.

Urine and blood sampling and creatinine assay

Sampling and storage of biofluid samples was previously described[13], as was measurement of creatinine in urine. Relevant details are supplied in the Supporting Information.

Extraction and analysis of flavonoid content of the chocolate bars

First, lipids were removed from chocolate samples as described by Cooper *et al.*[14]. This included the extraction of defatted and dried chocolate powder using 70% aqueous methanol containing 0.1 mg/mL galangin (282200, Sigma-Aldrich) as internal standard. Samples were heated to 70 °C for 20 min. Supernatants were filtered and filtrates from each sample were analyzed by normal-phase HPLC as described previously. Samples were analyzed in duplicate and the resulting flavonoid contents of the chocolate bars are shown in (Table S3).

NMR procedure

Urine samples were thawed at room temperature and prepared for ^{1}H NMR spectroscopy by mixing 500 μL urine with 50 μL phosphate buffer (1.2 mmol/L K_2HPO_4 and 0.3 mmol/L KH_2PO_4, pH 7.4) made up in 100% D_2O and containing 0.005% sodium azide and 3.3 mmol/L TSP (3-trimethylsilyl propionate) as chemical shift reference. The sample was shaken and 500 μL transferred into a 5 mm NMR tube for spectral acquisition. ^{1}H NMR spectra were recorded at 600 MHz on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) running TOPSPIN 2.0 software and fitted with a cryoprobe and a 60 slot autosampler. The acquisition order was randomized with respect to collection date and sample codes. Each ^{1}H NMR spectrum was acquired with 128 scans, 90^0 pulses (14 μs), spectral width 8012.8 Hz, acquisition time 2.04 s and relaxation delay 2.0 s. The noesygppr1d pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the relaxation delay and mixing time (0.01s). Spectra were transformed with zero filling and 0.3 Hz line broadening, manually phased, baseline corrected and referenced by setting the TSP methyl signal to 0 ppm.

Metabolites were identified using information found in the literature [15-17], on the web (Human Metabolome Database, http://www.hmdb.ca/), using Chenomx software, and by use of the 2D-NMR methods COSY, HSQC, and HMBC.

Plasma (200 µL) was mixed with 400 μL saline solution (0.9% w/v NaCl in 90%/10% H_2O/D_2O) and 500 µL were transferred to an NMR tube. Proton NMR spectra were measured at 300 K using the same NMR equipment. Spectra were acquired using the Bruker cpmgpr1d water suppression pulse sequence, with 32 k data points, spectral width 20.8 ppm, acquisition time 1.36 s, relaxation delay 2 s, 90º pulses (11 µs), spin-echo delay 0.4 ms, loop number 300 (giving a total echo time = 240 ms) and 128 transients per spectrum. Free induction decays were zero filled and exponential line broadening of 1 Hz was applied prior to Fourier transformation. Proton NMR spectra were referenced to the anomeric α-glucose signal (δ 5.234 ppm).

NMR data analysis

NMR Spectra were prepared for statistical analysis using the Bruker AMIX software v3.9. The spectra were divided along the horizontal axis into variable width "buckets" (or bins) using the AMIX graphical editor to draw buckets that include, where possible, recognizably complete individual peaks or multiplets. The intensities within each bucket were summed, divided by the bucket width and the bucket intensities were normalized to the same total intensity for each sample to give the final bucket table. Regions with only background noise, the urea peak and the water resonance were not included in the buckets. This gave matrices of 368 samples x 380 buckets for the urine NMR spectra and 363 samples by 101 buckets for the plasma spectra.

HPLC-TOF MS analysis

A subset of 240 urine samples (0 and 6-h post chocolate) was defrosted and vortex mixed before sampling. A portion of urine (1 mL) was centrifuged for 5 min at 9600 g and 150 µL

of the supernatant was transferred to a glass vial to which MilliQ water (450 µL) was added. The sample was vortex mixed. A portion of each individual sample (35 µL) was taken and pooled to make a quality control sample (PQC) representative of all samples in the sample set. Sample analysis used an Agilent 1100 HPLC coupled to a Bruker Daltonics MicroTOF. Sample (5 µL) was injected onto a Phenomenex Gemini C18 column (150 x 2.1 mm, 3.0 µm at 25 ºC) with a flow rate of 250 µL/min. A gradient of 100% A (MilliQ water + 0.1% formic acid) and 0% B (acetonitrile + 0.1% formic acid) was applied increasing B to 15% over 5 min and then to 95% over 15 min and held at 95% for 4 min. The PQC was injected for the first three runs and after each set of six injections for each batch (samples were randomized into batches of 50 samples). The TOF scan was over 90-1000 amu in positive ESI mode. Mass calibration was performed for each sample using reference masses of sodium formate ion clusters to give a mass accuracy of 5 ppm. The Bruker raw data files were converted to NetCDF and the metabolite feature data presented in the manuscript were generated entirely using the Bioconductor (http://www.bioconductor.org/software) packages XCMS (v 1.22.1) [18] running with default parameters under R (v 2.11.0). XCMS finds and integrates peaks within small time and mass windows and produces a table of integrated intensities for a set of features (identified by combined mass retention time labels) that are common to all samples. Features are reported if they occur in more than half the samples in at least one of the groups (*e.g.* control, treatment) so that intensities of features from say flavan-3-ol metabolites may be close to zero in baseline samples. Each feature has an associated median accurate mass which may be used for putative identification of species present via database search (*e.g.* MZedDB, http://maltese.dbs.aber.ac.uk:8888/hrmet/index.html [19] or from relevant literature[11]. A total of 2900 features were extracted for subsequent data analysis.

Data Analysis

Multivariate statistical analysis (Principal Component Analysis, PCA) was carried out using the PLS Toolbox v5.5 (Eigenvector Research Inc.,Wenatchee, WA) running within Matlab, v7.6 (The MathWorks Inc., Natick, MA). Autoscaling was applied to the columns of the bucket table. The same procedure was applied to the integrated intensities in the XCMS table with the mass retention time features taking the place of NMR buckets as variates.

Univariate analyses were carried out on individual variates (NMR or LC/MS) in GraphPad Prism v5.01 (GraphPad Software, San Diego, CA) using the Kruskal-Wallis test with Dunn's post-test. The same software was used to plot the results of these analyses. A comprehensive set of univariate analyses was also carried out on all buckets for the urine NMR data using an in-house Matlab routine. The comparison of metabolic responses to the three different chocolate types was performed on bucketed NMR spectra that were area normalised followed by two stages of probabilistic scaling [21]. First, the three baseline (time t = 0) spectra for an individual were scaled to their mean. Second, spectra collected at times t = 2 h and t = 6 h were scaled to their corresponding adjusted baseline. Post-ingestion difference spectra were calculated by subtraction of their corresponding adjusted baselines. For both post-ingestion time points the differences in the spectral response of each bucket were ranked by their Mack-Skillings statistic [22, 23]. The top 162 & 223 buckets were found to be significantly different at 2 h and 6 h respectively, based on a FDR of q = 0.05 [22], using p-values based on 10^7 within-block permutations [22, 23]. The significance of the differences in response at each bucket was also estimated from within-block permutations (Table S5).

RESULTS

We recruited 42 healthy subjects, each receiving three acute treatments (flavan-3-ol-enriched dark chocolate (EDC), standard dark chocolate low in flavan-3-ols (DC), or white

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chocolate (WC) containing no flavan-3-ols) in random order. The three intervention days were separated by two washout periods of at least two weeks. This crossover design provided optimal statistical power as each subject acted as their own control.

Dietary markers mainly contribute to the discrimination of the metabolite profiles

Plasma NMR spectra were assessed by PLSDA and showed differentiation between 0h and later time points but no discrimination between the three chocolate treatments (Figure S2). Differentiation was caused by elevation of lactate at 2h and aspartate, acetoacetate, acetone and β-hydroxybutyrate levels at 6 h after ingestion[24].

A series of Principal Component Analysis (PCA) models were created from urine ^{1}H NMR and LC/MS data to assess whether there was separation between dietary treatments at 0 h, 2 and 6 h post-ingestion and between the different time points for each treatment (Figure 1). LC/MS data from the 2h samples was not recorded hence only the 0h and 6h groups were analysed. Three 4 group PCA models were constructed: controls at 0 h, EDC-2h, DC-2h and WC-2h; controls at 0 h, EDC-6h, DC-6h and WC-6h both for NMR data and controls at 0 h, EDC-6h, DC-6h and WC-6h for LC/MS data. All three models showed partial overlap of dark chocolate groups (EDC and DC) but clear discrimination between dark chocolate groups, WC and the baseline group (0h) (Figure 1 a-c). Three treatment-based PCA models (EDC, DC and WC) applied to 3 groups (0, 2 and 6h postprandially) also showed a clear separation between 0 h and post-ingestion scores but the scores at 2 and 6 h post-ingestion groups overlapped (Figure 1d-f and Figure S3 a-c).

The discrimination of samples from the EDC group and to some extent the DC group versus the baseline group (0h) and WC group (seen on PC4 in Figure 1a and PC2 in Figure 2b) was largely due to an increase in the intensity of buckets containing signals from epicatechin derivatives, theobromine and its methylxanthine derivatives (Figure S3 d-e) in the EDC and DC groups.
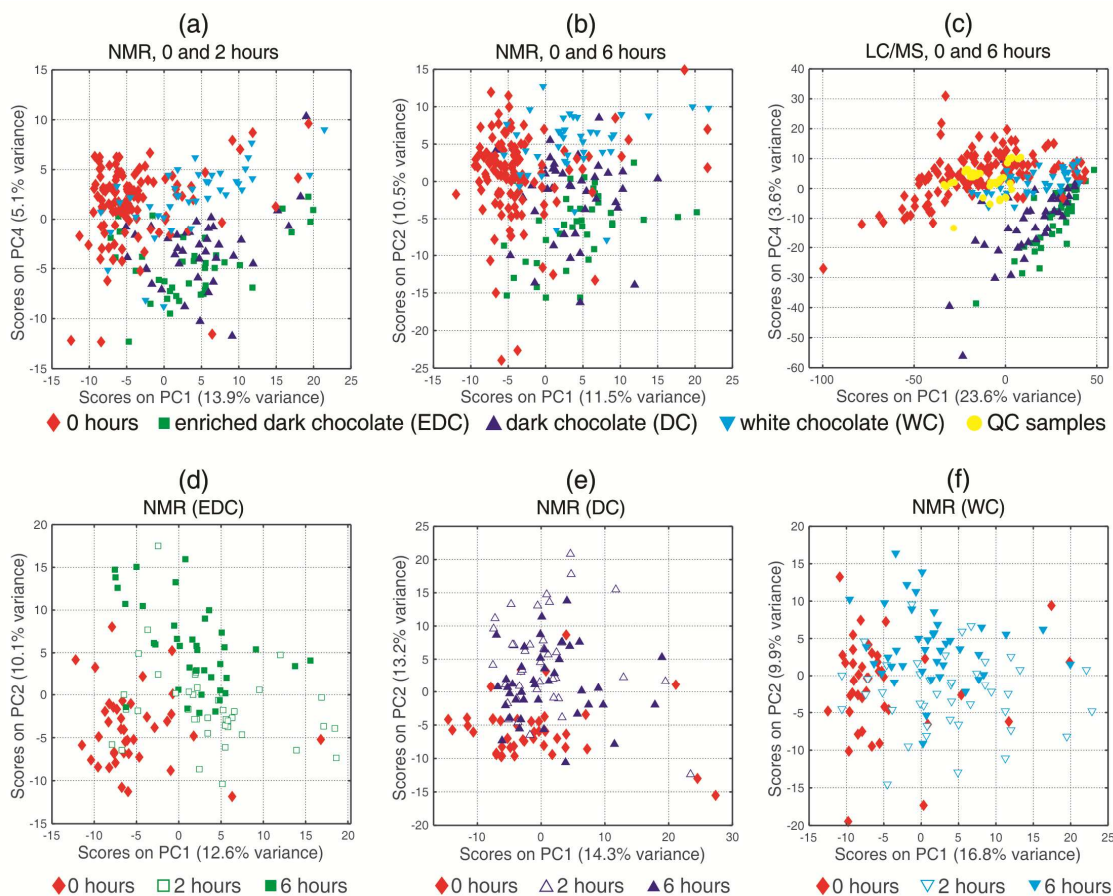
Figure 1.

Assignment of the dietary markers and their levels across the three chocolate treatments

Epicatechin derivatives and methylxanthines were detected in urine samples by both ^{1}H NMR and LC/TOF-MS. Comparison of HSQC and COSY spectra of 6h EDC and WC urines (same volunteer) revealed many new peaks in the EDC spectra that were not present in the WC. The signals were assigned as theobromine or epicatechin metabolites using information from the HMDB, literature, or via spiking (theobromine itself). Several buckets between 3.5 and 4.0 ppm containing signals arising from 3- and 7-methylxanthines and theobromine (3,7-dimethylxanthine) as well as the singlets from the corresponding xanthine purine rings at 8.03, 7.86 and 7.91 ppm were all strong markers in the urines from volunteers having ingested either DC or EDC (Figure 2 and Table 1).

Thanks to its higher sensitivity, numerous additional dietary metabolites were detected by LC/MS as reported previously[11]. Derivatives of theobromine (Figure 3) were identified in the urines after DC and EDC consumption, namely 3- and 7-methyluric acids, 3,7-dimethyluric acid, 6-amino-5[N-methylformylamino]-1-methyluracil (AMMU), as well as intact theobromine (Table 2). Several metabolites indicative of EDC and DC consumption were identified including hydroxynicotinate, caffeine and vannilloylglycine. Several metabolites of catechins were also detected, *i.e.* epicatechin monosulfate, 4-hydroxy-5-(3,4-dihydroxyphenyl)-valerate, the glucuronide and sulfate metabolites of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 3'-methoxy-4'-hydroxyphenylvalerolactone and its glucuronide. Those metabolites were all observed 6 h post-ingestion and their levels significantly increased compared with levels at 0 h. There was a decrease in hippurate levels from 0 h to 6 h postprandial after consumption of all types of chocolate (Figure 3). A set of features that were significantly enhanced in white chocolate was noticed although the identification of the compounds remains to be established (Table S4).
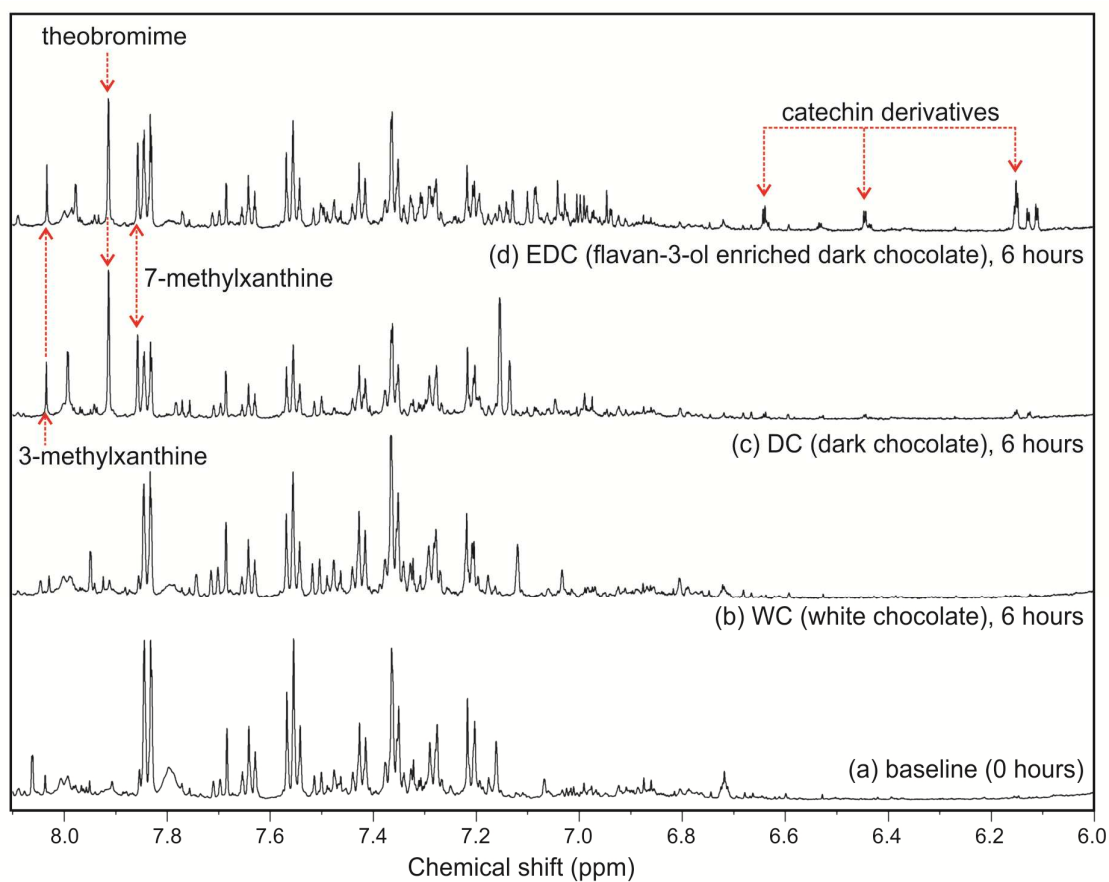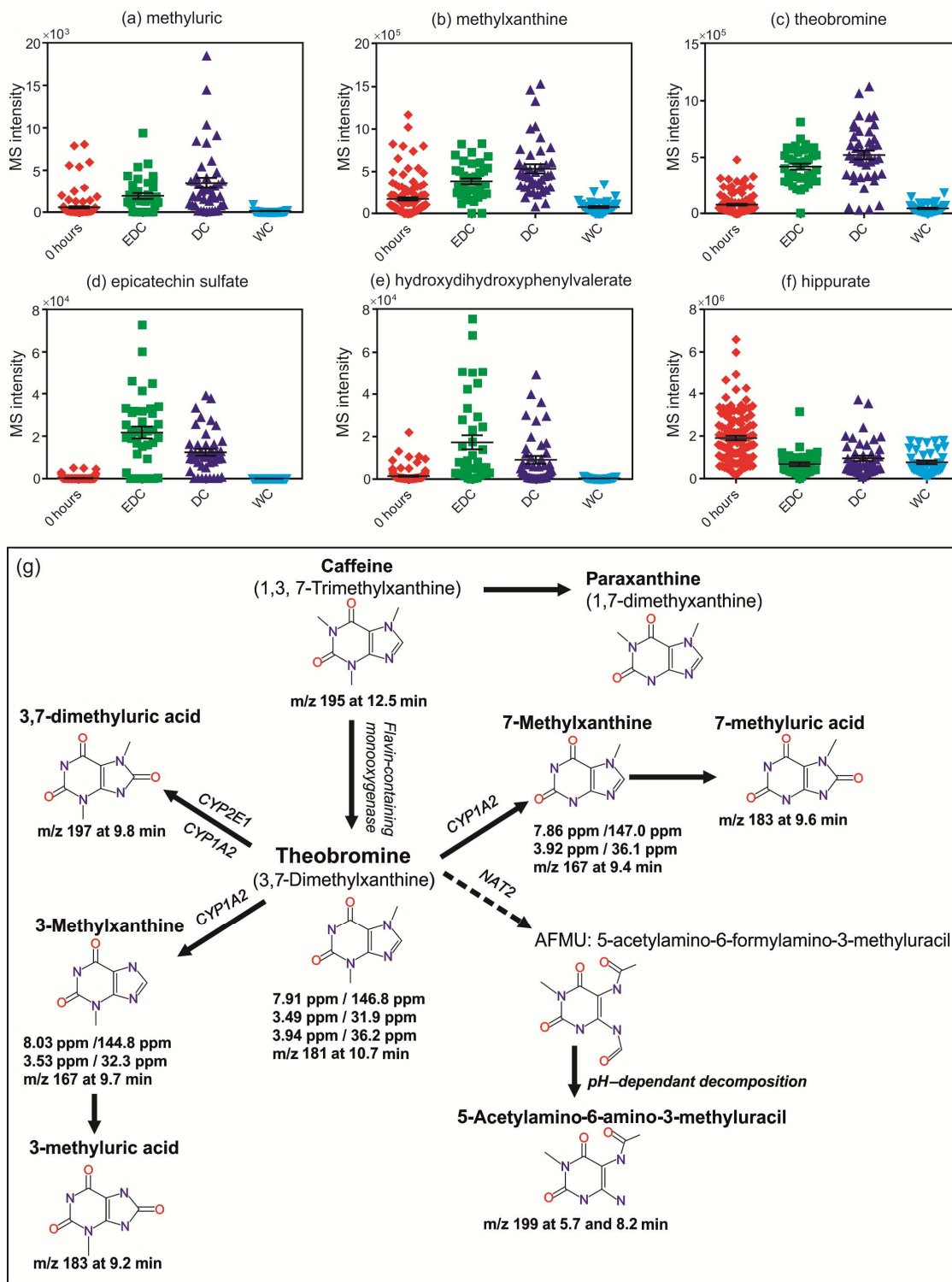
Figure 2.

Figure 3.

Concentration of flavanol-3-ols and methylxanthines in chocolate.

The combined caffeine and theobromine concentrations were quite similar in both dark chocolates. The flavanol-3-ol enriched chocolate had a combined content of 1.12% whilst the standard dark chocolate contained 1.23%. (Table S1). The enriched dark chocolate contained 1.5% total flavonoids compared to 0.64% in the standard dark chocolate (Table S3). Neither methylxanthines nor flavonoids were detected in the white chocolate.

Changes in endogenous metabolite levels across the three chocolate treatments

The urinary ^{1}H NMR spectra recorded were segmented into 380 buckets and univariate analyses were carried out on the bucket intensities. Of these buckets, 223 had a p-value lower than 0.05 when the three chocolate treatments were compared at 6 h (see Table S5). The markers with the lowest p values were assigned to catechin and xanthine derivatives. Interestingly, a subset of endogenous metabolites was affected by the chocolate intake, mainly by the two dark chocolates (Table 3). Urinary levels of several amino acids (Ala, Val, Gly) and organic acids (2- and 3-hydroxyisobutyrate, 3-hydroxyisovalerate) were significantly decreased 6 h after intake of both dark chocolates compared with white chocolate and all followed the same trend: mean level in flavan-3-ol-enriched dark chocolate < dark chocolate < white chocolate. Additionally, a few energy compounds also followed the same trend, namely creatinine, N^1-methylnicotinamide (NMA) and lactate. Interestingly pyruvate followed an inverse trend where levels were increased by flavan-3-ol-enriched dark chocolate and dark chocolate compared with white chocolate. Levels of tyrosine and its microbial product (para-hydroxyphenylacetate) were also elevated after consumption of both dark chocolates (Table 3). Trends for selected markers can be found in Figure 4.
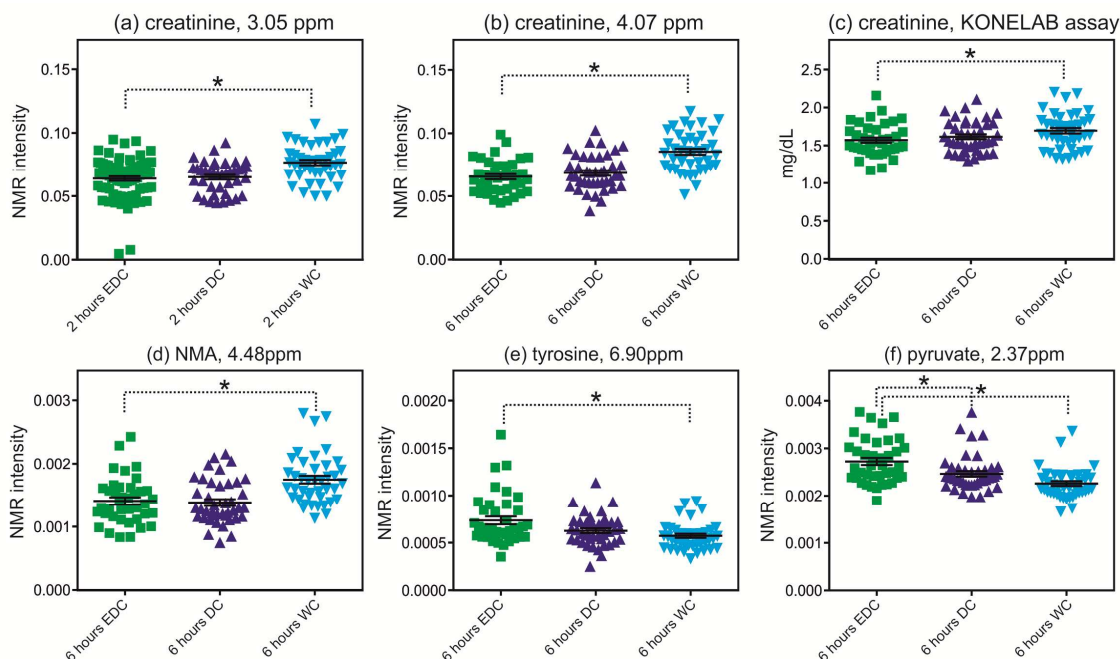
Figure 4.

Changes in metabolite levels associated with gender and age

Differences in levels of the main xanthine metabolites (3- and 7-methylxanthine and theobromine) and the buckets at 6.11, 6.13 and 6.15 ppm reflecting catechin derivatives were investigated further by subdividing the treatment groups according to gender, BMI and age, at 2- and 6-h post chocolate. Only theobromine excretion levels showed a significant difference with respect to gender, with higher levels being excreted by females compared with males. The gender differences were being observed for both dark chocolates at the 6 h and for standard dark chocolate at the 2 h time point. There was also an effect of the age on the levels of the same metabolite with a higher excretion for volunteers aged 29 or younger compared with volunteers older than 54 (Figure 5).
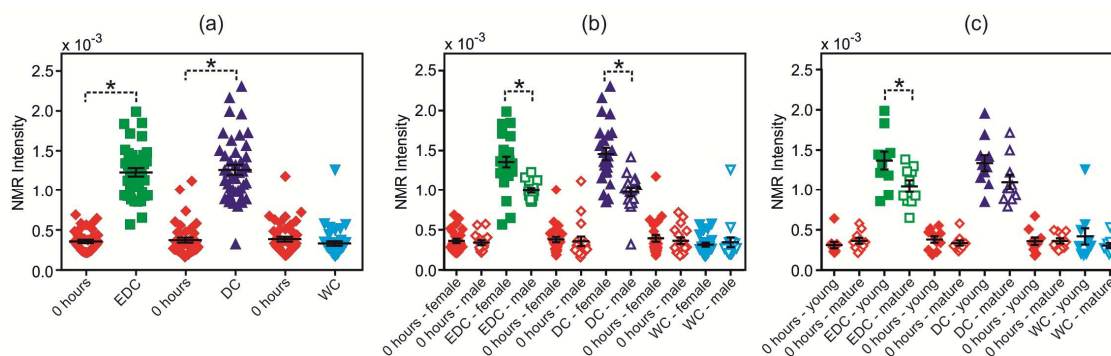
Figure 5.

DISCUSSION

In this study we assessed acute effects of consumption of flavan-3-ol-enriched dark chocolate in comparison with a standard dark and a white chocolate on plasma and urinary ^{1}H NMR profiles and urinary MS profiles in healthy humans. Both urinary and plasma profiles showed wide-scale metabolite composition changes post-chocolate intake. Multivariate and univariate analyses coupled to metabolite identification revealed that the major differences between treatments were found in urine and changes were related to both intake markers as well as markers of endogenous metabolism.

There was no differentiation between the three treatment groups in the plasma ^{1}H NMR profiles contrary to the many changes detected in the urinary profiles. No theobromine or catechin derived metabolites were found because plasma concentrations of these were under the detection limit for NMR.

Changes detected in the urinary profiles principally affected a number of NMR buckets between 3.00 and 4.00 ppm, and in the regions of 6.10 and 8.00 ppm after both dark chocolate treatments. Those signals were attributed to metabolized compounds of dietary origin (catechin [25] and methylxanthine [26] derivatives). Interestingly Martin et al[12] detected an increase in levels of two unknown singlets at 7.85 and 7.91 ppm after one week of chronic intake of dark chocolate which were most likely peaks arising from 7-methylxanthine and

theobromine (see Table 1). The levels of an unknown at 3.39 ppm detected in the present study were also increased in their study. Caffeine itself was not detected in the urinary spectra because the levels were too low to be detected. For comparison the abundance of theobromine in the dark chocolates was six times that of caffeine (supplementary Table 3) yet only theobromine and two main products (3 and 7-methyl xanthines) were detected by ^{1}H NMR. 6-amino-5[N-methylformylamino]-1-methyluracil (AMMU) is the main degradation product after caffeine intake [27]. In our study, 3-methylxanthine, 7-methylxanthine and AMMU are the main urinary products of theobromine [26] (Figure 3g). Caffeine degradation may have marginally contributed to the AMMU levels detected by LC/MS. The LC/MS on 6 h urines demonstrated changes in metabolites of polyphenols and alkaloids metabolites and also showed the occurrence of microbial metabolites. Our results are in agreement with other studies [11, ,25].

However, there was no increase in hippurate levels in our study although this metabolite of microbial origin has been recognized as the main excretion product from polyphenols such as catechins and from green and black tea consumption [28, 29].

There was an increase of para-hydroxyphenylacetate (HPA) 6 h after ingestion of both dark chocolates. Hydroxyphenylacetate is related to food intake and breakdown processes by gut microbiota [30]. It originates from dietary phenolic compounds, including the catechins contained in dark chocolate. The increase of HPA was accompanied with an increase of tyrosine, an abundant component of cacao seeds[11].

We did not observe changes in levels of citric acid cycle compounds such as succinate or citrate but, as in a study on consumption of black tea polyphenols [31], we saw an increase of pyruvate in urine. Additionally, we saw a reduction in lactate at 6 h post-intake of flavan-3-ol-enriched dark chocolate. The lactate levels increased at 6h for WC compared to 0h and to EDC or DC at 6h. Whilst we cannot elucidate the mechanisms of such changes based on metabolomics results alone, it is possible that, glycolysis and/or gluconeogenesis might be

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enhanced by the catechin-rich chocolate thus increasing levels of excreted pyruvate [32, 33]. Concomitantly, a large intake of catechins might have affected anaerobic respiration, leading to a reduced production of lactate. Furthermore, the urinary levels of four glucogenic amino acids, arginine, valine, alanine and glycine, were reduced after consumption of flavan-3-ol-enriched dark chocolate, suggesting a possible redirection of the energy metabolism towards gluconeogenesis [33] following catechin intake (Table 3).

The urinary levels of both creatinine and N^1-methylnicotinamide (NMA) were reduced following intake of both dark chocolates but creatinine was increased by WC at 6h. The levels of creatinine and NMA in WC were higher than EDC or DC at 6h as shown in Figure 4. Creatinine is a clearance product from phosphocreatine (PCr) which itself is a phosphate donor for sudden adenosine triphosphate (ATP) demand and NMA arises from dietary niacin and from tryptophan metabolism [34]. Wang *et al* [35] proposed a link between creatinine urinary levels, oxidative stress and modulation of oxidative stress by antioxidants which could be attributed to catechins in both dark chocolates. A decrease in creatinine may be associated with a better regulation of the PCr pool and ATP usage. Note that a decrease in creatinine has already been observed by Martin et al[12]. This similarity indicates that both acute and chronic intake of dark chocolate may affect the energy metabolism in a similar way.

A decrease in NMA would indicate an upregulation towards the production of NAD^+ and energy expenditure [36]. Also NMA is an activator of prostacyclin (PGI_2) production which may regulate thrombotic as well as inflammatory processes in the endothelial vascular system via anti-platelet effects [37, 38]. In our case, decreases in creatinine and NMA urinary levels may indicate effects on metabolic regulation bringing potential health benefits through the action of chocolate bioactive ingredients.

Martin *et al* [12] showed that dark chocolate had an effect on the energy metabolism and on metabolites from gut microbiota. Products from the gut microbiota are commonly found in biological fluids including urine, plasma or faecal waters [39]. These include methylamine,

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dimethylamine (DMA), trimethylamine (TMA), and trimethylamine-N-oxide (TMAO) and related metabolites (dimethylglycine) which may originate from dietary choline.

We observed a urinary decrease of DMA in urine 6 h after ingestion of both dark chocolates. Dimethylamine is a product of TMA and TMAO. It is difficult to assess if a reduction of DMA is beneficial to health. Wang *et al* [40] reported that supplementation of mice with choline, TMAO or betaine promoted upregulation of multiple macrophage scavenger receptors linked to atherosclerosis, and supplementation with choline or TMAO promoted atherosclerosis. Therefore, it appears that lower endogenous levels of methylamines may be beneficial to health.

The short chain hydroxy acids, 3-hydroxyisovalerate, 2-hydroxyisobutyrate and 3-hydroxyisobutyrate, were reduced following intake of both dark chocolates but increased from 0h to 6h after WC. These acids are normally found at low levels in urine of healthy individuals [41] and at high levels in urine and blood of patients suffering from branched-chain organic acidurias [42] or other acidosis [43]. Both 3-hydroxyisovalerate and 3-hydroxyisobutyrate are intermediates in the catabolism of branched amino acids such as leucine and valine and 2-hydroxyisobutyrate has been reported as being a gut metabolite increased in the urine of healthy humans eating a diet high in proteins [41]. Newgard et al [44] compared the blood profiles of obese and lean humans and showed a link between a branched-chain amino acid-related metabolite signature and increased catabolism of branched amino acids and correlation with insulin resistance. In our case, the reduced branched amino acids catabolism may be part of the change in energy metabolism observed after eating dark chocolate. Regulating the catabolism of these amino acids may be a positive benefit on glucose metabolism.

Caffeine and theobromine are the most abundant methylxanthines in chocolate and their physiological effects are well recognised [2]. They are extensively metabolized (urinary

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recovery of < 2%) and are known to be metabolized by cytochrome P450 (CYP450) enzymes during phase II excretion [27, 45].

In this study, females excreted on average almost twice as much theobromine as males 6 h after intake of both dark chocolates and there was also a significant dependence of theobromine excretion on age with flavan-3-ol-enriched dark chocolate treatment. Some previous studies reported no gender effect on elimination following an intake of caffeine, theobromine or theophylline [27, 46, 47] while others reported the inverse [45]. Additionally, Agarwal [48] showed that *in vitro* arginine[8]-vasopressin (AVP) levels in blood from male and female donors differed and that theophylline and a related xanthine could interfere with the platelet aggregation capacity of AVP in human platelet-rich plasma. This result points to a multiple factor effect (including gender) and/or expression of CYP450 enzymes (CYP1A2 in particular [47]) on compounds and metabolized products which in turn could affect the host metabolism.

Conclusion

These effects may have arisen from consumption of both the flavanol-3-ols and the methylxanthines. In many cases there was similar response to both dark chocolates and the increased flavonoid content did not show any dose response even though the enriched dark chocolate contained some 2.4 times the flavonoid content of the standard dark chocolate.

In summary, this NMR and MS-based metabolomic study has shown that dietary markers are traceable in the urine of healthy individuals after an acute intake of flavan-3-ol-enriched dark and standard dark chocolate. Bioactive compounds and metabolized products were also found to have an effect on the endogenous metabolism and the gut microbial activity affecting in particular several pathways relating to energy metabolism and the nicotinate pool.

Associated Content available

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29   **COMPETING FINANCIAL INTERESTS**
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42
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46   We thank the volunteers who participated in the study.
47
48   **AUTHOR CONTRIBUTIONS**
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50
51   The authors' responsibilities were as follow: LMO, GGD, PAK and BDR: designed and
52
53   conducted the nutritional intervention; GLG: designed the metabolomic analyses; MP and
54
55   SS: performed the MS analysis; LMO and GLG: performed the NMR analysis; IJC and GLG:
56
57   analyzed the NMR and MS data; EKK and HST: performed some statistical analyses; GLG:
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interpreted the NMR and MS data; GLG: wrote the manuscript and all authors: provided comments, read and approved the final manuscript.

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Table 1. Exogenous urinary NMR markers for Enriched dark (EDC) and dark chocolate (DC) 6 hours after intake
Fold: EDC compared to white chocolate (WC) the control group
underlined chemical shift: used for data analysis

Bucket group	δ_H ppm	δ_C ppm	Metabolite identification	p value t-test EDC vs WC	Fold EDC/ WC
1	2.59	.	catechin derivatives (C ring)	4E-08	1.4
2	2.79	30.1	epicatechin derivatives (C ring)	2.E-19	1.5
3	2.94, 2.95, 2.98, <u>3.00</u>, 3.01	30.3	epicatechin/polymer derivatives (C ring)	2.E-18	2.0
4	3.39	31.9	?	2.E-21	2.0
5	3.49	31.9	theobromine	3.E-11	3.0
6	3.53	32.2	3-methylxanthine	8.E-09	1.7
7	3.92	36.2	7-methylxanthine	7.E-14	2.4
8	3.94	36.2	theobromine	2.E-10	2.0
9	4.00	.	catechin derivatives (C ring)	4.E-10	0.8
10	5.08, 5.11, 5.14	80.8, 81.2	polymer catechin derivatives (C ring)	buckets not drawn	
11	6.11, 6.13, 6.15, <u>6.44</u>, 6.64	98.1, 98.8, 103.4, 105.0	all catechin derivatives (A ring mainly)	9.E-22	11.8
12	6.94 -<u>7.31</u>	.	all catechin derivatives (B ring mainly)	2.E-12	1.7
13	7.86	147.0	7-methylxanthine	5.E-12	3.5
14	7.91	146.8	theobromine	6.E-22	3.7
15	8.03	144.8	3-methylxanthine	2.E-13	2.2

Table 2. Urinary MS markers for Enriched dark (EDC) and dark chocolate (DC) 6 hours after intake
Fold: EDC compared to white chocolate (WC) the control group
* ion seen in the raw data but not detected by the deconvolution approach

Marker group	Marker ions	Metabolite identification	Retention time (min)	Theoretical mass [M+H]+	p value t-test EDC vs WC	Fold EDC/ WC
1	140.035 [M+H]+	hydroxynicotinic	4.3	140.034	2.E-07	4
2	199.084 [M+H]+			199.083	3.E-12	5
	171.087 [M+H-H2O]+	AMMU	5.7; 8.2		2.E-09	4
3	185.091	?	7.2		6.E-11	3
4	260.064	?	8.4		5.E-13	24
5	321.085 [M+H]+				2.E-12	8
	343.071 [M+Na]+	?	8.8		8.E-13	750
6	547.15 [3M+H]+				7.E-05	29
	365.101 [2M+H]+			365.095	7.E-03	2
	183.052 [M+H]+	7 and 3-methyl-uric	9.2	183.052	4.E-04	2
7	167.057 [M+H]+			167.056	5.E-11	5
	333.108 [2M+H]+	7 and 3-methylxanthine	9.5	333.105	1.E-09	9
8	197.057 [M+H]+	dimethyluric	9.9	197.0669	2.E-09	3
9	181.072 [M+H]+			181.072	2.E-17	10
	399.099 [2M+K]+	theobromine	10.7	399.093	2.E-14	8
10	420.091				1.E-05	42
	425.110				3.E-05	8
	209.082 [M+H-H_2O]+	4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid	11.8	209.081	.*	.*
11	226.073 [M+H]+			226.071	3.E-08	5
	151.042 [M+H-glycine]+	vanniloylglycine	12.1	151.040	1.E-08	3
12	568.108				2.E-08	59
	547.176	?	12.4		1.E-06	16
13	195.089 [M+H]+	caffeine	12.5	195.088	4.E-05	2
14	385.116			385.113	1.E-06	61
	407.115 [M+Na]+				9.E-10	60
	209.124 [M+H-GlcA]+	5-(3',4'-dihydroxyphenyl)-valerolactone glucuronide	13.3	209.081	1.E-06	9
14	399.135 [M+H]+			399.129	1.E-04	8
	223.096 [M+H-GlcA]+			223.096	3.E-05	8
	421.122 [M+Na]+	3'-methoxy-4'-hydrxyphenylvalerolactone glucuronide	13.4		2.E-06	19
15	369.113 [M+H]+				2.E-06	19
	391.106 [M+Na]+	O-Feruloylquinate ?	13.6	391.100	5.E-06	13

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| 16 | 289.040 [M+H]+ | 5-(3',4'-dihydroxyphenyl-valerolactone-sulfate | 17.7 | 289.038 | 2.E-05 | 23 |
| 17 | 371.044 | epicatechin-O-sulfate | 22.2 | 371.043 | 3.E-09 | 2400 |

Table 3. Changed in levels of endogenous urinary NMR markers for Enriched dark (EDC) and dark chocolate (DC) 6 hours after intake

Trends: EDC and DC compared to white chocolate (WC) the control group

underlined chemical shift: used for data analysis

* could correspond to chemical groups from amino acids such as ornithine or citrulline

Marker group	δ (ppm)	Metabolite identification	Trends	Class	p value t-test EDC vs WC	Fold EDC/WC
1	<u>3.05</u>, 4.07	creatinine	down	energy metabolism	2E-08	0.8
2	1.68, <u>1.95</u>	arginine	down	amino acid	8E-05	0.9
3	1.00, 1.<u>05</u>	valine	down	amino acid	4E-06	0.9
4	1.49	alanine	down	amino acid	4E-05	0.8
5	3.57	glycine	down	amino acid	5.E-03	0.8
6	1.78	unknown at 1.78 ppm	down	. *	2E-07	0.8
7	1.88	unknown at 1.88 ppm	down	. *	9E-05	0.9
8	<u>4.48</u>, 9.28	N-methylnicotinamide	down	pyridine, metabolite of niacin (vitB3)	2.E-04	0.8
9	2.07	N-acetylated compounds	down	(phase II and other pathways)	8E-06	0.9
10	2.73	dimethylamine	down	amine (bacterial origin)	3.E-02	0.9
11	1.28	3-hydroxyisovalerate	down	short chain hydroxy acid, leucine catabolism	2.E-04	0.8
12	1.36	2-hydroxyisobutyrate	down	short chain hydroxy acid	2.E-04	0.9
13	1.08	3-hydroxyisobutyrate	down	short chain hydroxy acid, valine catabolism	1.E-03	0.9
14	1.34	lactate	down	short chain fatty acid	2.E-04	0.8
15	2.37	pyruvate	up	glycolysis, gluconeogenesis	2E-06	1.2
16	6.86	4-hydroxyphenyl acetate	up	phenolic compound (bacterial origin)	7.E-03	1.2
17	<u>6.90</u>, 7.19	tyrosine	up	amino acid	1.E-03	1.3
18	4.4	unknown at 4.40 ppm	up	.	2.E-02	1.2

Figure Legends

Figure 1. PCA score plots from urine samples at baseline (0 h) and at the specified time for each of the three chocolate treatments (a) NMR 0 h & 2 h, (b) NMR 0 h & 6 h, (c) LC/MS 0 h & 6 h. PCA score plots from urinary NMR spectra of 0-hour, 2-hours and 6-hours samples for flavan-3-ol-enriched dark chocolate (EDC) (d), dark chocolate (DC) (e) and white chocolate (WC) (f)

Figure 2. Partial 600MHz ^{1}H NMR spectra of urines from one subject: (A) baseline and (B)-(D) after treatment indicated at 6 h

Figure 3. MS intensity for markers of enriched dark chocolate (EDC) and dark chocolate (DC) intake and hippurate 6 hours after intake. White (WC) chocolate is the control group. All have p value < 0.0001 (Kruskal-Wallis test on 4 groups). 0-hour n=126; EDC, 6-hours n=38, DC, 6-hours n=42 and WC, 6-hours n=39. Selected m/z ions were m/z 183.052, 167.057, 181.072, 371.044, 420.091, 180.066 (a to f respectively). (g) Map of the theobromine pathway. Cytochrome P450 (CYP2E1, CYP1A2) and N-acetyltransferase 2 (NAT2), are involved in the activation or detoxification of various xenobiotic compounds including alkaloid like theobromine (adapted from Arnaud [27])

Figure 4. NMR intensity/ concentration of a selection of endogenous urine metabolites for the three chocolate treatments. (all groups) n= 39-42. Plots are for the times specified from data in NMR buckets (a) creatinine at 3.05 ppm, (b) creatinine at 4.07 ppm, (c) creatinine (KONELAB assay), (d) N^1-methylnicotinamide (NMA) at 4.48 ppm, (e) tyrosine at 6.90 ppm and (f) pyruvate at 2.37 ppm. * p < 0.05 Mann-Whitney test

Figure 5. NMR intensity of theobromine at 7.91 ppm in urines at 0 h (baseline) or 6 h (treatments) showing (a) overall variation with treatment, (b) grouping by treatment and gender, (c) grouping by treatment and age (younger < 29 y, older > 54 y). (all groups) n= 14-42 n=14-42 per group. Note that because of the crossover design each treatment group has its own baseline (0 h) samples. *p < 0.05 Mann-Whitney test

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For TOC only

(a) methyluric

(b) methylxanthine

(c) theobromine

(d) epicatechin sulfate

(e) hydroxydihydroxyphenylvalerate

(f) hippurate

(g)

Caffeine
(1,3, 7-Trimethylxanthine)

m/z 195 at 12.5 min

Paraxanthine
(1,7-dimethyxanthine)

Flavin-containing monooxygenase

3,7-dimethyluric acid

m/z 197 at 9.8 min

CYP2E1
CYP1A2

Theobromine
(3,7-Dimethylxanthine)

7.91 ppm / 146.8 ppm
3.49 ppm / 31.9 ppm
3.94 ppm / 36.2 ppm
m/z 181 at 10.7 min

CYP1A2

7-Methylxanthine

7.86 ppm /147.0 ppm
3.92 ppm / 36.1 ppm
m/z 167 at 9.4 min

7-methyluric acid

m/z 183 at 9.6 min

NAT2

3-Methylxanthine

8.03 ppm /144.8 ppm
3.53 ppm / 32.3 ppm
m/z 167 at 9.7 min

CYP1A2

AFMU: 5-acetylamino-6-formylamino-3-methyluracil

pH–dependant decomposition

5-Acetylamino-6-amino-3-methyluracil

m/z 199 at 5.7 and 8.2 min

3-methyluric acid

m/z 183 at 9.2 min