Association between urinary metabolic profile and the intestinal effects of cocoa in rats

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Abbreviation list

αKMV,α-keto-β-methyl-*n*-valerate; **αKIC**,α-ketoisocaproate; **1-MX**,1-methylxanthine; **2-HIB**,2-hydroxyisobutyrate; **2-OG**,2-oxoglutarate; **3-HIB**,3-hydroxyisobutyrate; **3-IS**,3-indoxyl-sulfate;**3-MX**,3-methylxanthine; **4-GB**,4-guanidinobutanoic acid;**4-CS**,4-cresyl sulfate; **4-CG**,4-cresyl glucuronide; **HMB**,β-hydroxy-β-methylbutyrate; **4-HPA**,4-hydroxypropionic acid;**7-MX**,7-methylxanthine; **BCAA**,branched-chain amino acids; **C10**,10% cocoa diet; **CF**,cocoa fibre diet; **DF**,dietary fibre; **DMA**,dimethylamine; **DMG**,dimethylglycine; **DMU**,dimethyluric acid; **GLP-1**,glucagon-like peptide-1; **IAA**, indole-3-acetic acid; **NAG**,*N*-acetylglycoprotein; **NAMPT**, nicotinamide phophoribosyltranferase; **NMN**,nicotine mononucleotide; **NMNA**,*N*-methyl-nicotinic acid; **NMND**,*N*-methyl-nicotinamide; **NNMT,** nicontiamide *N*-methyltransferase; **OPLS-DA**,Orthogonal projection to latent structures-discriminant analysis; **PCA**, principal components analysis; **PAG**,phenylacetylglycine; **REF**,Reference diet; **RD**,recycle delay; **ROS**,reactive oxygen species; **SCFA**,short chain fatty acids; **TMAO**,trimethylamine *N*-oxide **TSP**,3-trimethylsilyl-1-[2,2,3,3-$^{2}H_4$] propionate.

Abstract

The aim of this study was to elucidate the relationship between the urinary metabolic fingerprint and the effects of cocoa and cocoa fibre on body weight, hormone metabolism, intestinal immunity and microbiota composition. To this effect, Wistar rats were fed, for 3-weeks, a diet containing 10% cocoa (C10), or two other diets with same proportion of fibres: one based on cocoa fibre (CF) and another containing inulin as a reference diet (REF). Twenty-four hour urine samples were analysed by an untargeted ^{1}H NMR spectroscopy-based metabonomic approach. Concentration of faecal IgA and plasma metabolic hormones were also quantified. The C10 diet decreased the intestinal IgA, plasma GLP-1 and glucagon concentrations and increased ghrelin levels compared to the REF group. Clear differences were observed between the metabolic profiles from the C10 group and those from the CF group. Urine metabolites derived from cocoa correlated with the cocoa effects on body weight, immunity and the gut microbiota. Overall, cocoa intake alters the host and bacterial metabolism concerning energy and amino acid pathways leading to a metabolic signature that can be used as a marker for consumption. This metabolic profile correlates with body weight, metabolic hormones, intestinal immunity and microbiota composition.

Introduction

Cocoa is considered a great source of bioactive compounds such as polyphenols and dietary fibre (DF), which have been attributed consistent positive health effects[1–5]. Cocoa contains monomeric flavonoids such as the flavanols (–)-catechin , (+)-catechin and (–)-epicatechin, and mainly its oligomers and polymers known as procyanidins. These polymeric compounds are able to pass intact through the small intestine and to reach the colon[6], where they are metabolized by the intestinal microbiota. This conversion is crucial for their absorption and also for the generation of new compounds with greater bioactivity than their precursors[6–8]. Thus, there is a growing body of evidence on the reciprocal relationship between bacteria and polyphenols that may help understand the documented benefits of polyphenols consumption[9]. In fact, it has been extensively reported that both dietary polyphenols, including those from cocoa, and the corresponding microbially-derived phenolic metabolites modulate the gut microbiota composition in *in vitro*, *in vivo* and clinical studies[8,10–13].

Similar ability to modulate the gut microbiota composition has been attributed to the DF, which has been described to modulate short chain fatty acids (SCFA) production[14–18]. Recently, cocoa fibre-enriched intake in rats has been associated with the modulation of the gut microbiota and SCFA production[17]. The DF fraction in cocoa is mainly rich in cellulose, followed by highly fermented pectic substances and hemicellulose, which is less fermentable than the former[19]. After microbial transformation, the cocoa metabolites either from flavonoids or cocoa fibre (*i.e.* SCFA) are absorbed into the bloodstream[20,21]. The microbial metabolites from flavanols, are mainly metabolized by liver phase-II enzymes to conjugated derivatives that are subsequently eliminated in urine[7].

Previous preclinical studies carried out in our laboratory have evidenced that cocoa, cocoa flavanols and cocoa fibre modify some aspects of the intestinal and systemic immune response[2,13,17,22]. On the other hand, a diet based on whole cocoa, but not based exclusively on its flavonoids or fibre, is able to reduce the body weight gain[13,17,23]. Given that this effect on weight is not associated with a lower chow intake, it is necessary to further understand the mechanism involved in such effect. In this sense, an untargeted metabonomic approach is particularly useful, since aims to determine the broadest range of metabolites present in a biological samples without *a priori* information. Thus, due to it exploratory nature, this hypothesis-generating approach provides an unbiased screening of the metabolites and can detect alterations in biological pathways to provide insight into molecular mechanism. Therefore, the

objective of this study was to correlate the urinary metabolic variation associated with cocoa intake identified by ^{1}H nuclear magnetic resonance (NMR) spectroscopy with the effects of cocoa on body weight, hormone metabolism, intestinal immunity and microbiota composition. Moreover, very limited data is available about the metabolic profile after cocoa fibre intake and even less the contribution of cocoa fibre to the above described effects ascribed to cocoa, thus it was also aimed in this study

Materials and methods

Animals and diets

Female Wistar rats (3-week-old) were obtained from Janvier (Saint-Berthevin, France) and housed in pairs under conditions of controlled temperature and humidity in a 12:12 light-dark cycle. The rats were randomly distributed into three dietary groups: cocoa (C10), cocoa fibre (CF) and reference (REF) groups (n=10/each). The C10 group received chow containing 10% cocoa that provided a final diet concentration of 0.4% of polyphenols, 0.85% soluble fibre and 2.55% of insoluble fibre; the CF group received a diet with the same cocoa soluble and insoluble fibre proportions as the C10 group but with a very low amount of polyphenols (<0.02%); and the REF group received the same amount of fibre as the C10 group, being the soluble portion (0.85%) as inulin in order to distinguish the particular effect of cocoa fibre, as has been previously reported[24]. Natural Forastero cocoa and cocoa fibre powders (provided by Idilia Foods S.L., formerly Nutrexpa S.L., Barcelona, Spain) with 4.02% and 0.35% of polyphenols, respectively, were used to elaborate the C10 and CF diets. Inulin from chicory roots (Fibruline® Instant; InnovaFood 2005, S.L., Barcelona, Spain) was used as a reference soluble fibre. The three experimental diets were elaborated on basis of the AIN-93M formula by subtracting the amount of carbohydrates, proteins, lipids and insoluble fibre provided by the corresponding supplement. The three resulting diets were isoenergetic and had the same proportion of macronutrients (carbohydrates, proteins and lipids) and fibre as has been previously stated[24]. Animals were given free access to water and food. The diets lasted for three weeks.

Body weight and food intake were monitored throughout the study. Experiments were performed according to the Guide for the Care and Use of Laboratory Animals, and experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 358/12).

Sample collection and processing

The 24 h urine samples were collected, by means of metabolic cages, at 15 days after beginning the nutritional intervention. Centrifuged urines were kept at – 80 ºC until analysis. Moreover, blood samples were collected after three weeks and plasma was kept at - 80 ºC prior to metabolic hormones determination. Faecal samples were also collected at the third week of diet and the homogenates were obtained as previously described[17] and frozen at - 20 ºC until analysis.

Sample preparation for ^{1}H NMR analysis

Urine samples were defrosted and prepared for ^{1}H NMR spectroscopy by combining 400 µL of sample with 200 µL of phosphate buffer (pH 7.4; 100% D_2O) containing 1 mM of 3-trimethylsilyl-1-[2,2,3,3-$^{2}H_4$] propionate (TSP) as an external standard and 2 mM sodium azide as a bacteriocide. Samples were vortexed and particles were removed by centrifugation (13000 *g* for 10 min) prior to transferring 550 µL to a 5 mm NMR tube. Standard one-dimensional ^{1}H NMR spectra of the urine samples were acquired on a 500 MHz Bruker NMR spectrometer using a standard noesy experiment incorporating a pre-saturation pulse to attenuate the water signal. This experiment consisted of [recycle delay (RD)-90°-t_1-90°-t_m-90°-acquire free induction decay]. The water signal was suppressed by irradiation during the RD of 2 s, with a mixing time (t_m) of 10 µs. The acquisition time was set to 2.91 s and the 90° pulse length was 15.87 µs. For each sample, 8 dummy scans were followed by 128 scans and collected in 64K data points using a spectral width of 16 ppm. Prior to data analysis, NMR spectra were phased, corrected for baseline distortions and calibrated using the reference standard TSP. ^{1}H NMR spectra (δ 0.2-10.0) were digitized into consecutive integrated spectral regions (~20,000) of equal width (0.00055 ppm) using MATLAB (Mathworks). The regions containing signals from urea (δ 5.5 – 6.0) and the residual water (δ 4-7 – 5.2) were removed to minimize baseline effects arising from imperfect water suppression. Chemical shift variation was minimized across the dataset by applying a recursive segment-wise peak alignment (RSPA) algorithm to each spectrum. Each spectrum was normalized to unit area to account for variation in sample concentration.

Quantification of metabolic hormones in plasma

Plasma concentrations of ghrelin, glucagon, glucagon-like peptide (GLP)-1 and leptin were determined in plasma using the Bio-Plex Pro™ Diabetes Assay (Bio-Rad, Madrid, Spain) according to the

manufacturer's instructions. Analysis was carried out with the Bio-Plex® MAGPIX™ Multiplex Reader and the Bio-Plex Data Pro™ software (BioRad) as in previous studies[25]. The limits of quantification can be found as Supplementary material.

Faecal IgA quantification

The concentration of IgA in faeces was quantified by ELISA following the manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, TX, USA). Absorbance was measured in a microplate photometer (LabSystems Multiskan) and data were interpolated using ASCENT version 2.6 software (Thermo Fisher Scientific, Barcelona, Spain) into the standard curves.

Statistical analysis

The number of animals used in each group for detecting a statistically significant difference among groups assuming that there is not a drop out rate and a Type I error of 0.05 (2-sided) was calculated by the Appraising Project Office's program from the Universidad Miguel Hernández de Elche (Alicante) taking into the account both the metabolic hormones, particularly the leptin and ghrelin concentrations, and the faecal IgA. Moreover we have adjusted the sample size to the minimum needed following the University Ethical Committee guidelines and trying to apply the three R's rule for experimenting in animals.

Statistical analysis for body weight, chow intake, faecal IgA and metabolic hormones was performed using the software package IBM SPSS Statistics 22.0 (SPSS, Inc. USA). Levene's and Kolmogorov–Smirnov tests were applied to assess variance equality and normal distribution, respectively. Conventional one-way ANOVA was performed when normal distribution and equality of variance existed. The Tukey's test was applied when specific cocoa intake had a significant effect on the dependent variable. Non-parametric Mann–Whitney U and Wilcoxon tests were used in order to assess significance for independent and related samples, respectively. Significant differences were established at $P<0.05$.

Multivariate modeling was performed in MATLAB using in-house scripts. This included principal components analysis (PCA) using pareto scaled data and orthogonal projection to latent structures-

discriminant analysis (OPLS-DA) using a unit variance scaling approach. Pairwise OPLS-DA models were constructed to aid model interpretation and identify discriminatory metabolites between the study groups. Here, ^{1}H NMR spectroscopic profiles served as the descriptor matrix (X) and the experimental groups (REF, C10, CF) were used pairwise as the response variable (Y). Orthogonal signal correction filters were used to remove variation in the descriptor matrix unrelated to the response variable to assist model interpretation. Loading coefficient plots were generated by back-scaling transformation where covariance is plotted between the Y-response matrix and the signal intensity of the metabolites in the NMR data (X). These plots are coloured based on the correlation coefficient (r^2) between each metabolite and the Y-response variable, with red indicating strong significance and blue indicating weak significance. The predictive performance (Q^2Y) of the model was calculated using a seven-fold cross validation approach and model validity was established by permutation testing (1000 permutations).

Clustering analysis. Unsupervised hierarchical clustering analysis (HCA) was performed to identify general patterns of metabonomic variation between samples. To do so, we used the normalized levels of metabolites identified to contribute to class separation through the OPLS-DA models. For comparative analysis across different metabolites, data were standardized as z-scores across samples for each metabolite before clustering, so that the mean is 0 and the standard deviation is 1. This standardized matrix was subsequently used in unsupervised HCA for samples and metabolites using Euclidean distance and average linkage, by means of the pdist and linkage functions in the MATLAB bioinformatics toolbox. Heatmaps and dendrograms following HCA were generated with MATLAB imagesc and dendrogram functions, respectively. In the heatmaps, a red-blue colour scale is used so that shades of red and blue represent higher and lower values, respectively, compared with the mean. Different diet groups are color-coded and shown under the dendrogram for each sample.

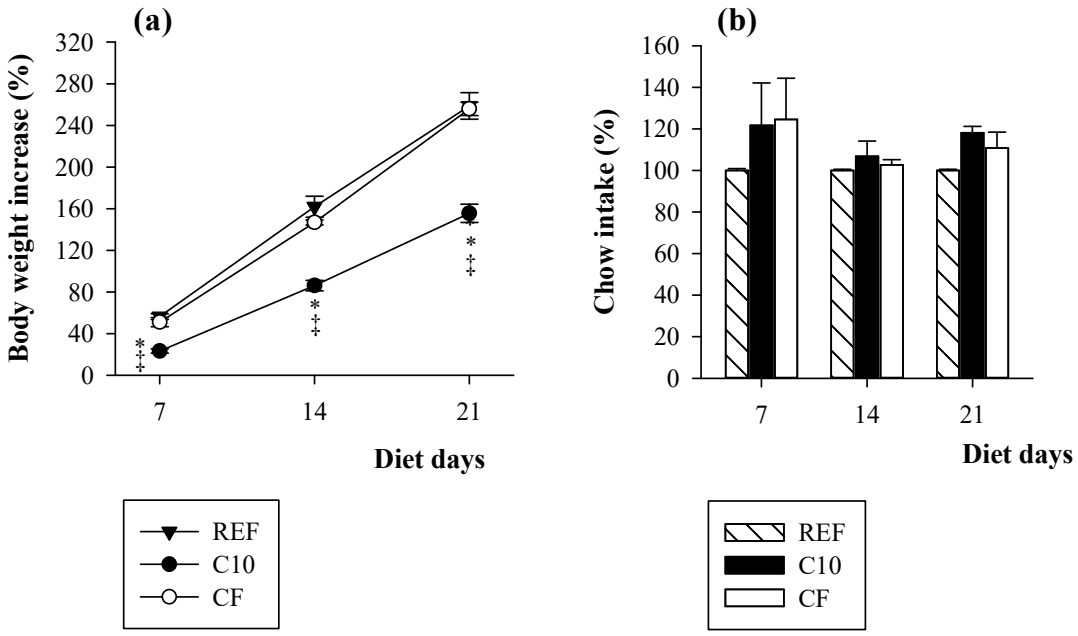
Correlation analysis. To explore the functional correlation between the changes on body weight, metabolism and intestinal immunity, metabonome perturbations and gut microbiome evaluated in a previous study by Fluorescence in situ hybridization (FISH) coupled to flow cytometry (FC). Spearman´s correlation analyses were performed on all the samples grouped together. The Benjamini-Hochberg method was used to adjust *p*-values for multiple testing considering a 5% false discovery rate (FDR).

Results

Body weight and food intake

Body weight and food intake were monitored weekly throughout the study (Fig. 1). Initially, no differences in body weight were observed between groups (mean (g) ± SEM, REF: 43.7±1.1; C10: 45.4±1.4; CF: 44.8±1.1). However, from day 7, a lower body weight gain was observed in C10 animals compared to the other groups (P<0.05) (Fig. 1(a)). This effect was not related to lower food intake, which was similar throughout the study among all experimental groups (Fig. 1(b)), being around 15 g of chow per 100 g of animal the first week of diet and around 12 g after the three weeks of intervention. No changes in body weight gain were found as a result of CF diet intake.

Fig. 1. Body weight increase (%) compared to the baseline (A) and chow intake (%) compared to the REF diet which represents 100% (B) monitored throughout the nutritional intervention. Values are expressed as mean ± SEM (n=10). * P<0.05 *vs* REF diet; and ‡ P<0.05 *vs* CF diet.



Metabolic hormones

The metabolic hormones quantified in plasma after three-week dietary intervention for all groups are summarized in Table 1. Both the C10 and CF diets increased the concentration of ghrelin compared to

224 the REF group (P<0.05). This increase was higher in the C10 group compared to the CF group
225 (P<0.05). Both diets also resulted in a lower plasma GLP-1 concentration in comparison with the REF
226 group (P<0.05). Moreover, the C10 diet reduced the glucagon concentration compared to the REF and
227 CF diets (P<0.05). The leptin concentration was not affected after the C10 diet but it was up-regulated
228 as a result of the CF diet intake compared to the rest of the groups (P<0.05) (Table 1).

Table 1. Metabolic hormones in plasma after three weeks of nutritional intervention. Results are expressed as mean ± SEM (n=7). * P<0.05 *vs* REF diet; † P<0.05 *vs* C10 diet and ‡ P<0.05 *vs* CF diet.

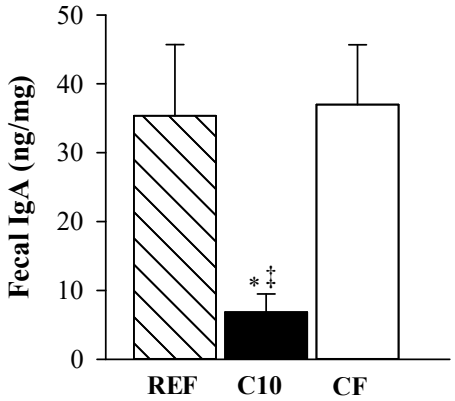
	REF	C10	CF
Ghrelin (ng/mL)	30.57 ± 3.9	98.63 ± 18.7*‡	43.59 ± 4.3*
GLP-1 (pg/mL)	29.4 ± 16.4	7.4 ± 3.1*	4.2 ± 0.2*
Glucagon (pg/mL)	180.1 ± 25.3	100.4 ± 3.1*‡	166.2 ± 26.9
Leptin (pg/mL)	647.7 ± 135.5	335.4 ± 127.5	968.7 ± 177.6*†

229

230 **Faecal IgA**

231 The C10 diet intake resulted in a decrease in the secretory IgA concentration compared to the others
232 groups (P<0.05) (Fig. 2). The CF diet did not produce any change in the secreted IgA concentration,
233 which was similar to that quantified in the REF group.

Fig. 2. Faecal IgA concentration determined after three weeks of nutritional intervention. Results are expressed as mean ± SEM (n=9-10). * P<0.05 *vs* REF diet; ‡ P<0.05 *vs* CF diet.



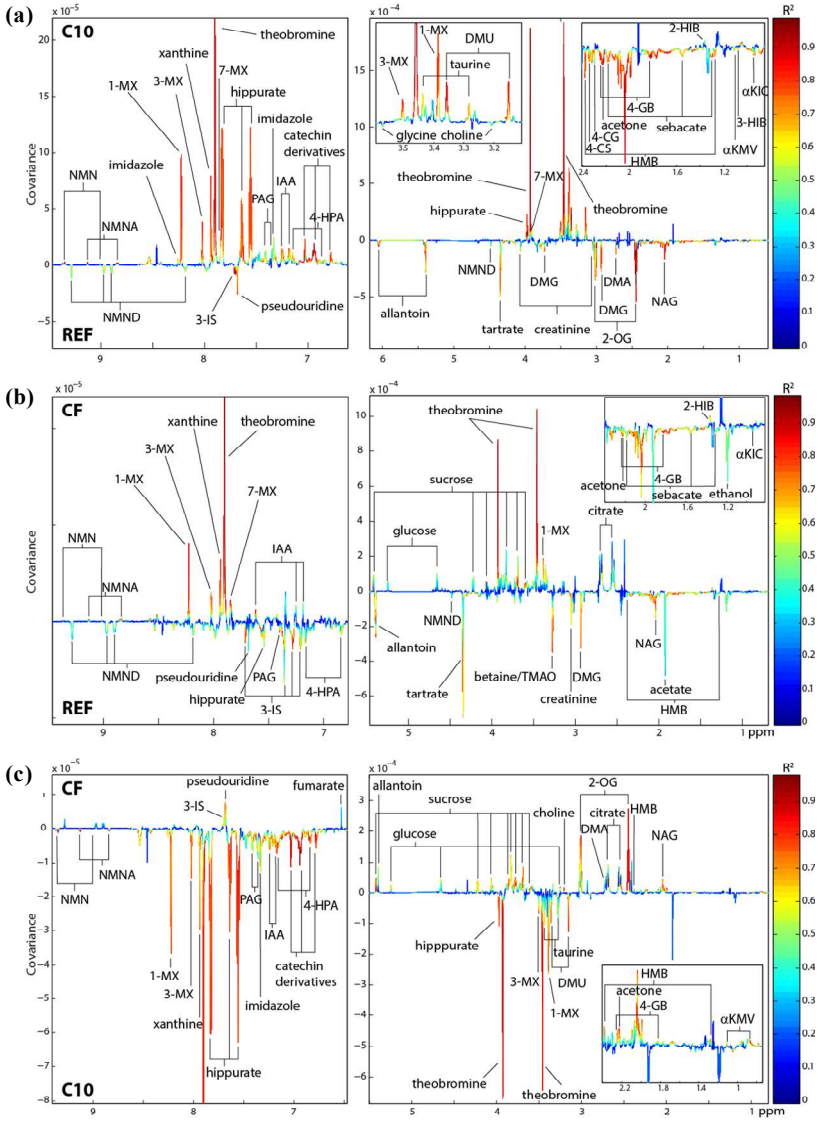
Urinary metabolic profile

An OPLS-DA model with strong predictive ability (Q^2Y=0.93; *P*=0.001) was returned comparing the metabolic profiles from rats receiving the C10 diet and the REF diet (Fig. 3(a) and 4). Rats fed the C10 diet excreted cocoa-derived metabolites such as *N*-methylnicotinic acid (NMNA) and nicotine mononucleotide (NMN), as well as methylxanthine metabolites, i.e. theobromine, xanthine, 1-methylxanthine (1-MX), 3-methylxanthine (3-MX), 7-methylxanthine (7-MX), imidazole, dimethyluric acid (DMU). In addition, urines from C10 group contained microbial-derived metabolites such as hippurate and 2-hydroxyisobutyrate (2-HIB), and 4-hydroxypropionic acid (4-HPA), phenylacetylglutamine (PAG) and indole-3-acetic acid (IAA) that could be derived from the cocoa polyphenols (epicatechin and procyanidins), aminoacids (tyrosine, phenylalanine, and trypthophan), or monoamines (tyramine, tryptamine, and 2-phenylethylamine) contained in cocoa. Moreover, rats receiving the C10 diet also excreted greater amounts of taurine compared to those receiving the REF diet. Conversely, animals receiving the C10 diet excreted lower amounts of metabolites related to energy metabolism (acetone, citrate, 2-oxoglutarate (2-OG), *N*-methylnicotinamide (NMND)), choline metabolism (dimethylamine (DMA), dimethylglycine (DMG), choline), and the metabolism of dietary components (sucrose, glucose, tartrate) compared to those receiving the REF diet. Other metabolites excreted in lower amounts by the C10 group were those related to endogenous amino acid metabolism (α-keto-isocaproate (αKIC), α-keto-β-methyl-*n*-valerate (αKMV), β-hydroxy-β-methylbutyrate (HMB), 3-hydroxyisobutyrate (3-HIB), glycine) and metabolites arising from the gut microbial-host co-metabolism of amino acids (3-indoxyl-sulfate (3-IS), 4-cresyl sulfate (4-CS) and 4-cresyl glucuronide (4-CG)). Sebacate, 4-guanidinobutanoate, creatinine, allantoin, and pseudouridine were also present in lower amounts in the urine from C10-fed animals compared to the REF group.

Regarding the CF diet, clear metabolic variation was observed in the urine from rats fed this diet compared to those fed the REF diet (Fig. 3(b) and 4; OPLS-DA model Q^2Y=0.65; *P*=0.001). Cocoa-derived metabolites such as NMNA, NMN, theobromine, xanthine, 1-MX, 3-MX, 7-MX, and DMU were found in the urine of rats receiving the CF diet but not those receiving the REF diet. Moreover, rats following the CF diet excreted higher 2-HIB, IAA, citrate, acetone, NMND, sucrose, glucose, acetate and tartrate that those following the REF diet, which are related to microbial, energetic or dietary metabolism. Lower amounts of 4-HPA, hippurate, 3-IS, PAG, αKI, DMG, sebacate,

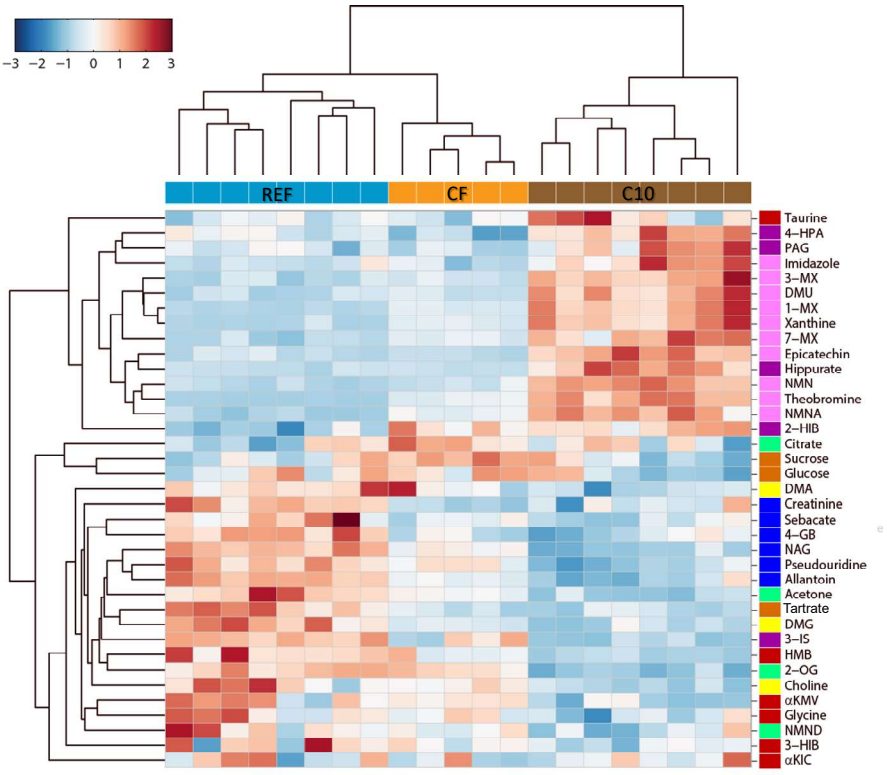
4-guanidinobutanoate, ethanol, creatinine, allantoin and pseudouridine were also measured in the urines of the CF group compared to REF group.

Fig. 3. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) comparing the urinary metabolic profiles of rats receiving different dietary regimens (n=8 for the REF and C10 groups; n=5 for the CF group). Coefficient plots extracted from the OPLS-DA models comparing rats receiving REF diet with C10 diet (A); REF diet with CF diet (B); and C10 diet with CF diet (C). **αKMV**,α-keto- β-methyl-*n*-valerate; **αKIC**, α-ketoisocaproate; **1-MX**,1-methylxanthine; **2-HIB**,2-hydroxyisobutyrate; **2-OG**,2-oxoglutarate; **3-HIB**,3-hydroxyisobutyrate; **3-IS**,3-indoxyl-sulfate; **3-MX**,3-methylxanthine; **4-GB**,4-guanidinobutanoic acid; **-CS**,4-cresyl sulfate; **4-CG**,4-cresyl glucuronide; **HMB**, β-hydroxy-β-methylbutyrate; **4-HPA**,4-hydroxypropionic acid; **7-MX**,7-methylxanthine; **C10**,10% cocoa diet; **CF**,cocoa fibre diet; **DMA**,dimethylamine; **DMG**,dimethylglycine; **DMU**,dimethyluric acid; **IAA**,indole-3-acetic acid; **NAG**,*N*-acetylglycoprotein; **NMN**,nicotine mononucleotide; **NMNA**,*N*-methyl-nicotinic acid; **NMND**,*N*-methyl-nicotinamide; **PAG**,phenylacetylglycine; **REF**,Reference diet; **TMAO**,trimethylamine *N*-oxide.



Finally, the OPLS-DA model contrasting the urinary metabolic phenotypes from rats receiving the C10 diet and those fed the CF diet (Fig. 3(c) and 4**;** Q^2Y=0.89; *P*=0.001) also showed some clear differences. As expected, rats consuming the C10 diet excreted higher levels of cocoa- (NMNA, NMN), catechin- and methylxanthine- (theobromine, xanthine, 1-MX and 3-MX, imidazole, DMU) derivatives compared to those fed the CF diet. The C10 diet fed animals also excreted higher amounts of 4-HPA, hippurate, PAG, IAA and taurine. However, compared to CF group, the C10 diet fed animals eliminated lower amounts of 3-IS, acetone, citrate, 2-OG, NMND, αKMV, HMB, DMA, choline, sucrose, glucose, sebacate, 4-guanidinobutanoate, allantoin, pseudouridine and fumarate.

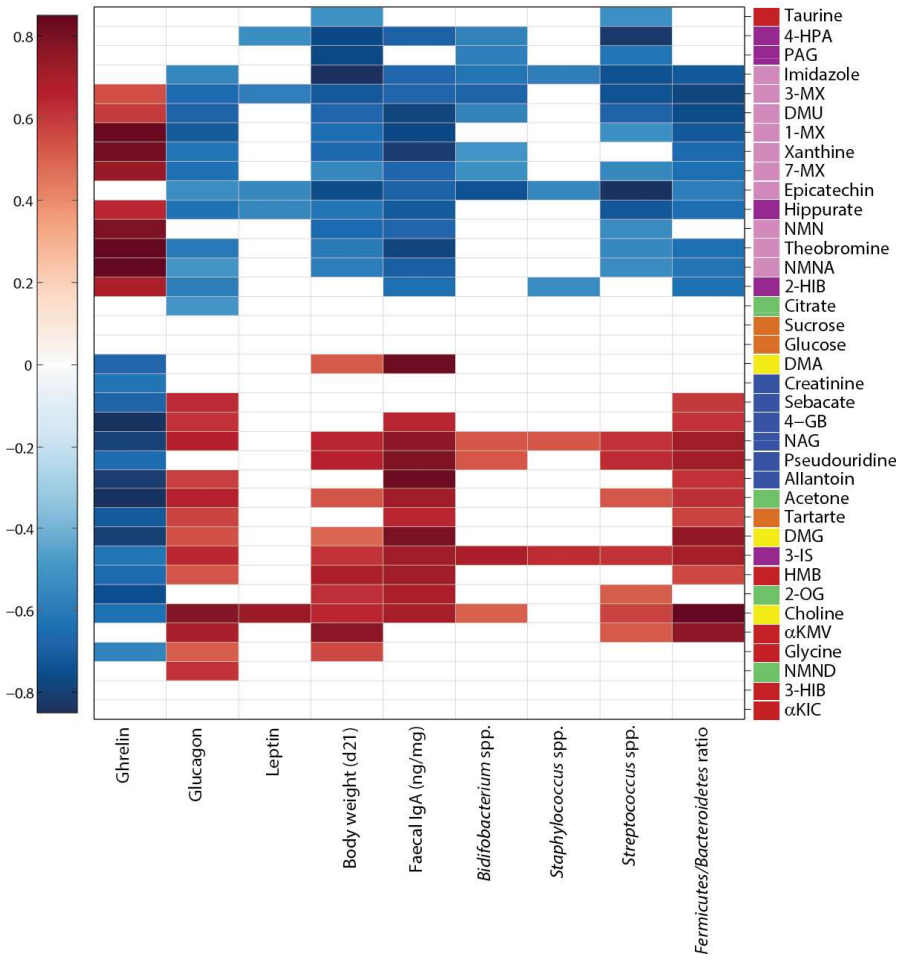
Fig. 4. Dendrogram and heatmap representation of unsupervised hierarchical clustering (HCA) of the metabonome for all rats. Each column corresponds to a single rat (n=8 for the REF and C10 groups; n=5 for the CF group), and each row corresponds to a specific metabolite. Metabolites identified to contribute to the separation between diets through OPLS-DA models were used for sample clustering. Metabolite z-score transformation was performed on the levels of each metabolite across samples, with blue denotating a lower and red a higher level compared to the mean. Metabolites and samples are clustered using correlation distance and average linkage and colour coded by diet or pathway, respectively. HCA grouped the urinary metabolic profiles from the C10-fed animals together and distinct from the other studied animals. Profiles from animals receiving the CF diet clustered together and were separated from the REF diet.



Correlations between urine metabolites and studied variables

The correlation analysis of the urine metabolic fingerprint with the effects of cocoa on metabolic hormones (ghrelin, GLP-1, glucagon, leptin), body weight, intestinal immunity (by means of intestinal IgA levels) and microbiota composition (reported previously[17,26]) was also studied (Fig. 5). Globally, whereas most of the studied variables were inversely correlated with whole cocoa metabolites, only the ghrelin concentration was positively correlated with the amount of these metabolites. In fact, the strongest correlations were found between ghrelin and NMNA, 1-MX, xanthine and theobromine. Plasma glucagon concentration correlated inversely with these metabolites derived from cocoa, whereas it has a positive correlation with choline and other metabolites. Body weight was inversely correlated with cocoa metabolites and a positive correlation with those from the amino acid metabolism. Similarly, faecal IgA concentration showed an inverse correlation with cocoa-derived metabolites whereas it was positively correlated with choline metabolites and allantoin. Urinary metabolites were also correlated with gut microbial groups previously identified to change following both the C10 and CF intake[17]. An inverse correlation was found between the *Streptococcus* genus and the excretion of epicatechin (cocoa derived) and 4-HPA (gut microbial metabolism). In addition, a strong positive correlation was observed between the *Firmicutes/Bacteroidetes* ratio and choline-related metabolites, whereas it inversely correlated with metabolites derived from cocoa.

Fig. 5. Correlations between metabolites and responses. The intensity of the colours represents the degree of correlation, with red and blue indicating positive and negative correlations, respectively. Metabolites identified to contribute to the separation between diets through OPLS-DA models were to obtain the correlations. The order of the metabolites is the same obtained in Figure 4, where metabolites have been clustered based on an unsupervised hierarchical analysis using a correlation distance and average linkage and colour coded by pathway. Only significant correlations after applying a Benjamini and Hochberg procedure for controlling for a false discovery rate of 5% are shown. Correlation coefficients were based on Spearman's correlation.

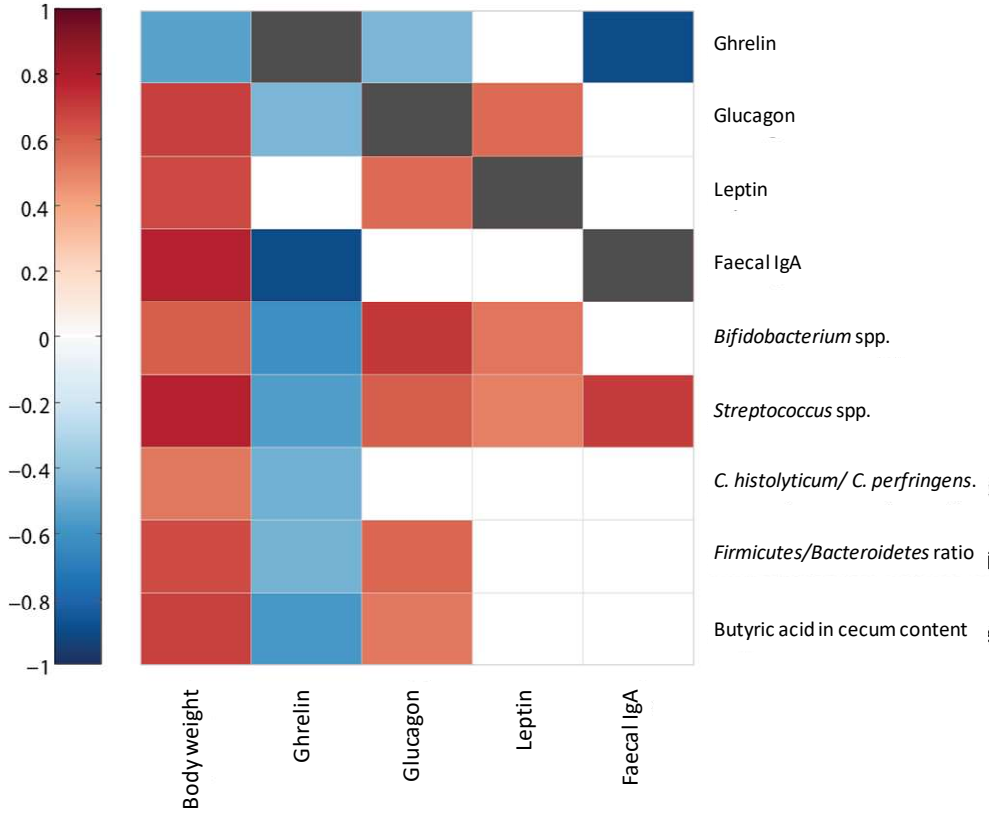


Other correlations between studied variables

The correlation analysis between the effects of cocoa on the metabolic hormones, body weight, intestinal immunity and microbiota composition and functionality[17] has been also studied (Fig. 6). When samples from all the groups were considered together, the body weight correlated positively with both plasma concentrations of glucagon and leptin, but negatively with plasma ghrelin concentration. In addition, body weight showed a positive correlation with the faecal IgA content and the *Streptococcus* genus proportion, among others.

The metabolic hormones showed significant associations with the microbiota composition and functionality (Fig. 6). Particularly, the glucagon concentration was positively correlated with the faecal counts of *Bifidobacterium* spp., whereas the ghrelin concentration correlated inversely with *Bifidobacterium* spp. proportion and the caecal butyric acid concentration. Finally, faecal IgA was positively associated with the faecal *Streptococcus* spp. counts and inversely with ghrelin concentration.

Fig. 6. Correlations between body weight, metabolic hormones, intestinal immunity and microbiota composition and functionality. The intensity of the colours represents the degree of correlation, with red and blue indicating positive and negative correlations, respectively. Only significant correlations after applying a Benjamini and Hochberg procedure for controlling for a false discovery rate of 5% are shown. Correlation coefficients were based on Spearman's correlation.



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305 **Discussion**

306 We have previously reported that a 10% cocoa diet in rats results in a lower body weight increase,
307 attenuates intestinal IgA secretion and modifies gut microbiota composition[13,17,26–29]. In the present
308 study, we demonstrate that the cocoa intake alters the metabolic hormones and results in a distinct
309 urinary metabolic pattern**.** Moreover, correlations between urinary metabolites and those variables in
310 which cocoa has an effect (*i.e.* body weight, metabolic hormones and intestinal immunity) were
311 established, as well as between these modified variables. In addition, we have also correlated all these
312 variables with the cocoa effects on microbiota composition and functionality from a previous study[17].
313 Interestingly, the effects of either whole cocoa or cocoa fibre on the metabolic hormones and urinary

314 metabolic profiles were different, which reflects the role of non-fibre cocoa compounds as well as the
315 interaction between fibre and non-fibre cocoa compounds in these effects. To strengthen this
316 conclusion, further studies testing the contribution of cocoa polyphenols to the aforementioned effects
317 related to the urinary metabolic profile and metabolic hormones should be carried out.

318 As expected, the urinary metabolic profiles of the C10 group animals showed the highest excretion of
319 cocoa-derived metabolites, which is in line with previous controlled cocoa dietary intervention
320 studies[30–32]. These metabolites include both the flavonoid and methylxanthine derivatives, some of
321 them produced by the gut microbiota. This reinforces the fact that some of these cocoa compounds
322 reach the colon intact, where they are metabolized by intestinal bacteria[6,8]. However, these metabolites
323 were found in a lower amount in the urine of animals fed the cocoa fibre, confirming the lower
324 concentrations of polyphenols and methylxanthines in the fibre powder used in the present study.

325 Rats fed a C10 diet excreted lower amounts of αKMV and αKIC, both derived from the endogenous
326 catabolism of the branched-chain amino acids (BCAA). In comparison with the CF diet, the amount of
327 glucose, sucrose and tricarboxylic acid (TCA) cycle intermediates (citrate, 2-OG, and fumarate) was
328 lower in the C10 group, indicating that the TCA cycle was down-regulated after the cocoa intake.
329 These changes, together with the lower urinary excretion of acetone, suggest an alteration to energy
330 metabolism through the modulation of ketogenesis, BCAA metabolism, and TCA cycle. This shift in
331 energy strategy, together with the lower expression of genes involved in lipid metabolism[33], may
332 contribute to the reduction of body weight in C10-fed rats, reported here and in previous studies[23,26,28].
333 Changes in NAD^+ pathway metabolism further support an alteration in energy metabolism. Thus,
334 NAD+ is required in TCA cycle and oxidative phosphorylation in the mitochondria to produce
335 energy[34]. It can be synthesized from NMN and nicotinic acid, which were excreted in higher amounts
336 by C10 rats. On the other hand, the NAD+-consuming enzymes all generate nicotinamide as by-
337 product, which can then be recycled to generate NMN by nicotinamide phophoribosyltranferase
338 (NAMPT). However, it can also be methylated by nicontiamide *N*-methyltransferase (NNMT) to
339 NMND, which was excreted in lower amounts by rats following a C10 diet. Recently, it was suggested
340 that inhibition of NNMT protected against diet-induced obesity by increasing energy expenditure[35].
341 Therefore, the lower urinary excretion of NMND could also explain the lower body weight increase
342 observed in the present study by C10 rats.

In addition, increased levels of NMND in urine were correlated with peroxisome proliferators[36], which participate in the generation of reactive oxygen species (ROS)[37]. Changes in NAD+ metabolism have been associated with oxidative stress in aging and neurodegeneration[34]. Hence, the lower excretion of NMND after the C10 diet may reflect a reduction in oxidative stress. Consistent with this, rats maintained on a C10 diet excreted lower amounts of pseudouridine, which is the oxidized form of urinary nucleosides. Elevated levels of pseudouridine have been previously identified as indicators of liver damage induced by inflammation related to oxidative stress[38]. In addition, the gut microbiota produces potentially toxic substances derived from protein metabolism[39], such as the uremic toxins 4-CS and 3-IS, derived from tyrosine and tryptophan, respectively. Both uremic toxins have shown to lead to changes in NAD metabolism, by increasing NADPH oxidase activity, and to increase ROS production[40,41]. Hence, the lower urinary excretion of 4-CS, 4-CG, and 3-IS by C10 mice strengthens the possible reduction in oxidative stress after cocoa consumption.

Microbial cocoa metabolism led to a higher excretion of 4-HPA, IAA, hippurate and PAG in the C10 group compared to both CF and REF groups, which could arise from the differences in cocoa compounds between the diets. Cocoa is rich in monoamines, such as tyramine, tryptamine and 2-phenylethylamine, which can be degraded by gut bacteria to 4-HPA, IAA, and PAG, respectively. These three metabolites can also be derived from the aminoacid catabolism of tyrosine, tryptophan, and phenylalanine by the gut microbiota, respectively. The gut microbiota can also degrade cocoa polyphenols to quinic acid and benzoic acid, which are then oxidized to hippurate and other phenol-related metabolites such as 4-HPA and PAG. Interestingly, NMND, 3-IS and pseudouridine, were excreted in lower amounts after the C10 diet compared to the CF diet. This suggests a shift in the composition and functionality of the gut microbiota towards a more favourable and less oxidative profile. This further suggests that rather than its fibre content it is the other cocoa compounds that contribute to the beneficial effects of cocoa. An inverse correlation was observed between cocoa metabolites and body weight, the highest correlation being with imidazole, one of the smallest metabolites derived from methylxanthines**.**This clearly suggests that theobromine, a methylxanthine present in cocoa in a high concentration, and its metabolites contribute to the effects of cocoa on body weight. . An inverse association was also found between body weight and the presence of metabolites derived from microbial metabolism, especially with PAG and 4-HPA. Regardless of other reported mechanisms[42–44], the contribution of microbiota composition and its activity should be considered in

the regulatory effect of cocoa on body weight. In fact, it has been shown that intake of the C10 diet decreased the abundance of the *Staphylococcus* and *Streptococcus* genera (from the *Firmicutes* phylum) and increased the *Bacteroides* spp. (*Bacteroidetes* phylum) reducing the *Firmicutes* to *Bacteroidetes* ratio[17,26]. This decreased ratio has been associated with lower body weight[45]. Consistently, an inverse correlation was also found between the counts of *Streptococcus* spp. and the excreted amounts of 4-HPA and epicatechin in urine.

The relationship between urinary metabolites and intestinal immunity has been established. While the CF diet did not affect the IgA content, the C10 diet, in accordance with findings from previous studies[2,13,27], resulted in a significantly lower faecal IgA concentration. In this sense, inverse correlations between the faecal IgA concentration and the amounts of cocoa epicatechin and methylxanthine derivatives and those from microbial metabolism were obtained. Overall, these results suggest that cocoa metabolism may lead to the formation of bioactive products that interact with the mechanisms involved in IgA secretion and/or its synthesis. Indeed, not only are the metabolites generated by the microbiota important but also the composition of the microbiota. Besides urinary metabolites and faecal IgA, metabolic hormones were determined after cocoa and the CF intakes. In line with previous studies[25], the GLP-1 and glucagon, peptide hormones involved in the glucose metabolism, were reduced by the C10 diet, but only the former was also modified after the CF intake. However, only glucagon concentration correlated inversely with the cocoa metabolites. Regarding hormones involved in regulating food intake**,** leptin helps to control the appetite and to maintain a stable body weight[46], whereas ghrelin stimulates appetite, increases fat body mass deposition and weight gain and influences glucose and lipid metabolism[47]. Although the C10 diet increased ghrelin concentration without changing leptin levels, it did not lead to changes in food intake. Therefore it is unlikely that these regulating appetite hormones contribute to cocoa influence on body weight. In fact, ghrelin concentration was inversely associated with body weight. A two-way relationship between leptin and ghrelin and the gut microbiota has been suggested[46,48]. Leptin stimulates mucin production in the intestine which could affect the composition of the microbiota[49]. It has also been suggested that the microbiota may modulate these hormones through the physiological regulation of the levels and type of autoantibodies produced against them[50]. In this sense, and in line with previous studies[46], we found that the proportions of *Bifidobacterium* and *Streptococcus* genera correlated positively with leptin and negatively with ghrelin. Moreover, it has been described that two of the main SCFA

(butyrate and propionate) regulate gut hormone release[51]. In this regard, our study demonstrates that the ghrelin concentration was inversely correlated with the butyric acid concentration in cecum content. Therefore, although hormone effects on microbiota cannot be discarded, the cocoa modulatory effect on the microbiota composition and functionality might be partially responsible for the changes observed in both hormone concentrations.

In summary, through a metabonomic approach, we have described the urinary metabolites derived from cocoa and cocoa fibre intake that can be used as consumption markers in health conditions. Moreover, the lower excretion of certain host and microbial metabolites reflected a shift in the host energy and amino acid metabolism due to cocoa compounds, but not to cocoa fibre diet. Metabolic associations were identified with body weight, intestinal IgA, the metabolic hormone profile and microbiota composition extending our understanding of the mechanisms through which cocoa may impact on healthy. Such effects were not restricted solely to the cocoa fibre and the contribution of other cocoa compounds has been demonstrated. Further studies should be carried out to evaluate the precise contribution of cocoa methylxanthines and also to study these associations in the context of weight-altering stimulus

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Author contributions

The authors' contributions were as follows: M. M. -C., À. F., F. J. P. -C and M. C. conceived and designed the research; M. M.-C. and J. M.-P. carried out the metabolomics experiments whereas M.

M.-C. carried out the rest of the experiments; J.M.-P. and J.R.S. and A.D. carried out the metabolomics data analysis and were involved in the interpretation of these data whereas M. M. -C., F. J. P.-C. and M. C. carried out the luminex and IgA data analysis and interpretation of the data; M. M. -C. and J. M.-P. contributed equally to the initial draft of the manuscript; A.C., J.R.S., À. F., F. J. P. -C and M. C. contributed to the critical revision of the manuscript; F.J.P.-C. has primary responsibility for the final content. All authors have read and approved the final version of the manuscript for publication. None of the authors has any conflict of interest to declare.

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