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The acute impact of polyphenols from *Hibiscus sabdariffa* in metabolic homeostasis: an approach combining metabolomics and gene-expression analyses†

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We explored the acute multifunctional effects of polyphenols from *Hibiscus sabdariffa* in humans to assess possible consequences on the host's health. The expected dynamic response was studied using a combination of transcriptomics and metabolomics to integrate specific functional pathways through network-based methods and to generate hypotheses established by acute metabolic effects and/or modifications in the expression of relevant genes. Data were obtained from healthy male volunteers after 3 hours of ingestion of an aqueous *Hibiscus sabdariffa* extract. The data were compared with data obtained prior to the ingestion, and the overall findings suggest that these particular polyphenols had a simultaneous role in mitochondrial function, energy homeostasis and protection of the cardiovascular system. These findings suggest beneficial actions in inflammation, endothelial dysfunction, and oxidation, which are interrelated mechanisms. Among other effects, the activation of the heme oxygenase-biliverdin reductase axis, the systemic inhibition of the renin-angiotensin system, the inhibition of the angiotensin-converting enzyme, and several actions mirroring those of the peroxisome proliferator-activated receptor agonists further support this notion. We also found concordant findings in the serum of the participants, which include a decrease in cortisol levels and a significant increase in the active vasodilator metabolite of bradykinin (des-Arg(9)-bradykinin). Therefore, our data support the view that polyphenols from *Hibiscus sabdariffa* play a regulatory role in metabolic health and in the maintenance of blood pressure, thus implying a multi-faceted impact in metabolic and cardiovascular diseases.

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1. Introduction

The notion that nutrition is associated with the preservation of health supports the challenging search for bioactive food components.[1–3] Epidemiological and intervention studies suggest that plant-derived polyphenols are correlated with beneficial health outcomes, which is probably due to their potential action as regulators of the expression of metabolically important genes and/or their intrinsic antioxidant and anti-inflammatory activities.[4–8] The emergence of unmet global clinical needs (*e.g.*, obesity and the associated conditions) may present an opportunity for the designers of functional foods to provide beneficial products, but some bioactive compounds may be incompatible with consumer acceptance (*e.g.*, due to bitterness or astringency).[9,10] The lack of a clear theoretical basis or accepted mechanisms of action also complicates the acceptance of the therapeutic potential of polyphenols. This study is based on the perspective that data obtained from different types of "omics" may be instrumental in tackling the complexity of the mechanisms of action of polyphenols from *Hibiscus sabdariffa* by integrating the outcomes of multiple effects that occur simultaneously.

It has been argued that polyphenols may act as moderate toxins (*i.e.*, *hormesis*), which is counterintuitive and contradic-

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tory with the fact that polyphenols are apparently nontoxic. The idea of *xenohormesis* was conceived to indicate that mammals are beneficiaries of phytochemicals because they may respond to "the same chemical cues" developed in plants.[11] To prove or discard this hypothesis is arduous. In addition, because the bioavailability of polyphenols is low, it is difficult to understand how foodstuffs provided in normal amounts could elicit significant effects.[12,13] We assume that polyphenols are extremely bioactive in humans and/or that the observed effects are the result of multiple beneficial and synergistic interactions. Two strategies can be used to study this aspect and both are under debate: (1) to provide polyphenol-rich extracts, usually in higher doses than those provided in current diets, as products that influence multiple molecular targets,[14,15] or (2) to manipulate the endogenous antioxidant levels by supplying weak pro-oxidants.[16] The present study was performed under the rationale that the chemical composition and conformational changes of dietary polyphenols are responsible for binding to different metabolically active enzymes and/or receptors, and consequently may have the inherent potential to exert multiple effects.[17–19] This may sound "heretical" to the pharmaceutical industries, which ignore the fact that supposedly selective drugs that are already in the market simultaneously modulate dozens of proteins and receptors.[20] To reduce the complexity found in the composition of plant foods in common diets, which include hundreds of polyphenols,[21] we studied the acute effects of a polyphenol-rich, fully characterized aqueous extract from the calyces of *Hibiscus sabdariffa* Linnaeus (Malvaceae) (HS).[15] Our aim was to assess the influence of these polyphenols on the overall metabolic host response using a combination of metabolomics and gene-expression analyses. The result is a useful tool with potential application for monitoring phytochemical exposure in humans, which may be complementary to previous efforts in the quest for nutrition biomarkers, and it may provide support to the more comprehensive concept of foodomics.[22–24]

2. Material and methods

2.1. Experimental design

All the experimental procedures were performed in accordance with protocols approved by our Ethics Committee and Institutional Review Board (EPINOLS, 12-03-29/3proj6 and OBESPAD 14-07-31/7proj3). Written informed consent was obtained from the participants prior to their entry into the study. Based on previous results,[25,26] we calculated the sample size using formulas for the 1-sample Z test with a default power of 0.90. Accordingly, to avoid possible gender and age biases, the participants comprised ten healthy male non-obese individuals, non-smokers, free of medication and any metabolic derangements, and with ages ranging from 23 to 35 years. While designing the study, we found that a case-control or case-referent design for liquid ingestion (*i.e.*, using water as a comparator) is unnecessary and is likely to provide confounders. Moreover, the basal or normal metabolic response in a control group could not be matched with the participants, and the previous exposure to different nutrients (diets) was difficult to assess. The use of the same participants on different days might also be a source of confounders, assuming that marked metabolic and hormonal differential changes can occur during the wash-up period. We thus adopted a repeated-group design (before–after study) with data collected in a short (3-hour) period, assuming that a moderate amount of water is metabolically inert and that the fasting state was clearly described and established.[27] The unit of analysis was the pair; another advantage of this design is that each pair serves as its own control, thus reducing the error and increasing the statistical power. The time of the study was limited to a few hours because the assessment of long-term effects would require chronic ingestion and a control group. Time-points for the measurements were also inferred as described in previous studies.[13,15] The importance of adhering to the fasting recommendations was repeatedly reinforced since the recruitment stage. Participants were asked to avoid strenuous physical activity the day before the experiment and were instructed to avoid ingestion of alcohol and polyphenol-rich foods or beverages (*i.e.*, coffee, tea, juice, oil, chocolate, fruits, and vegetables) during the previous 7 days. Participants remained in the fasting state during the experiment, water was not allowed, and their activity was supervised and restricted. Strict fasting was indicated 12 hours prior to experiment following standardized policies. Clinical measurements or manipulations were avoided to prevent a placebo-like effect.[15] The extract from HS calyces containing 560 mg of polyphenols per 5 g of dried material was prepared by Monteloeder S.L (Elche, Spain) dissolved in water (200 mL) and was immediately ingested to ensure that each participant received 8 mg kg^{-1} of organic acids and phenolic compounds. Details on the composition of these compounds are provided in Table S1.† Participants were asked to remain recumbent for 10 min prior to the drawing of blood samples, which took place immediately before (08:00 AM) and 3 hours after the ingestion. Serum and plasma were obtained, frozen in aliquots at −80 °C within 2 h of collection, and were then stored until batch analysis.

2.2. Laboratory measurements

In vitro antioxidant activity of HS extract was measured as previously described.[12,15] The analytical methods for the separation and identification of phenolic fraction and other soluble compounds of the HS extract have been already described.[28,29] Total and HDL-cholesterol, glucose, uric acid, bilirubin, triglycerides, cortisol and insulin were measured with standard methods (Boehringer, Mannheim, Germany). Serum aldosterone and renin activity were measured as described.[30] The homeostatic model assessment index (HOMA-IR) was calculated as an estimate of insulin resistance.[31] The ferric reducing ability of serum (FRAP)[32] and measurements of the concentrations of total polyphenols and malondialdehyde (MDA)[33] from the serum were performed essentially as described.[34] Chemokine (C–C motif) ligand 2 (CCL2), interleukin-6 (IL6),

interleukin-8 (IL8) and tumor necrosis factor-alpha (TNFα) were measured with ELISA (Invitrogen, Carlsbad, USA). High sensitivity C-reactive protein (CRP) was measured using reagents from Biokit (Barcelona, Spain). None of these measured molecules was found in the extract.

2.3. Metabolomic platform

Selected samples were outsourced to Metabolon (Research Triangle Park, Durham, NC, USA), extracted upon arrival and divided into fractions for analysis. The instrument and overall process variability were 5% and 10%, respectively. The chromatographic conditions have been previously described.[35,36] In brief, the liquid chromatography-mass spectrometry (LC-MS, LC-MS2; separately under positive mode and negative mode) platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization source and a linear ion-trap mass analyzer. The samples for the gas chromatography/mass spectrometry (GC/MS) analysis were derivatized and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. Metabolites were identified by comparing the ion data, retention time, mass (m/z), and MS or MS/MS spectra with a reference library of chemical standards.

2.4. Transcriptomic profiling

Peripheral blood mononuclear cells (PBMCs) were isolated at two time points using BD Vacutainer Cell Preparation Tubes as tentative surrogate cells for the markers of gene expression in other tissues.[37] Cells were lysed and stored at −80 °C until RNA isolation using a QIAamp RNA Blood mini kit (QIAgen, Izasa, Barcelona, Spain). The quality was checked by capillary electrophoresis and further purified using sequential DNase digestion and QIAgen RNeasy microcolumns prior to the microarray analysis. RNA samples were sent to the Center of Excellence for Fluorescent Bioanalytics (KFB, Regensburg, Germany). The RNA expression profile was analyzed using a GeneChip® High-Throughput HG-U133, which measured the gene expression of 47 000 transcripts and variants, combined with the Perfect Match array to remove possible mismatches (HT HG-U133+ PM 24-array plate, Affymetrix, Santa Clara, CA, USA). Numerical data were obtained using Affymetrix Expression Console 1.1.1 software. Gene expression was first measured using the robust multi-array average methodology, followed by quintile normalization. The quality of the data and sources of the batch effect were assessed using the affyPLM package version 1.34.0 version 2.11 and principal component analysis (PCA). Probe set annotation was downloaded from Affymetrix's website and mapped to 20 741 genes. We also measured the expression of selected genes using real-time PCR amplifications with TaqMan primers and probes obtained from validated Assays-on-Demand products (Applied Biosystems, Foster City, CA) on the 7900HT Fast Real-Time PCR system (Applied Biosystems).[17] When there were redundancies, the greatest average expression across all the samples was chosen to represent each gene.[38]

2.5. Statistical and functional association analyses

We used the sample size and power calculator from Statistical Solutions (Clearwater, FL, USA) using known μ and σ values for control variables in the population and study sample. The power was set at 0.90 to minimize Type II errors. The before-after design required the analysis of aggregated data and use of the Tukey test to decrease the probability of Type I errors. We subsequently assumed that the variation among experimental data may not be fully captured in pre-treatment predictors but would manifest itself in the outcomes.[26]

For the metabolomic analysis, we performed comparisons in the metabolomic profile with Welch's t-tests and/or Wilcoxon's rank sum tests as well as ANOVA for repeated measures. To correct for multiple testing, we used the False Discovery Rate estimated using the q-value as described earlier.[39–41] To obtain a full reconstruction of human metabolism, we considered all the biochemical reactions in the KEGG database in which humans are known to synthesize the required enzymes (or that happen spontaneously), but only main reactant pairs were considered for reconstruction.[42–45] Then, we mapped onto the reconstruction of all metabolites in the metabolomic essays with a known KEGG identifier, and then we analyzed the microarray data using different "R" packages (http://cran.r-project.org) from Bioconductor (http://www.bioconductor.org/). After controlling the quality and batch effect of the samples, we assessed the differentially expressed genes using the Limma package 3.14.4 with a linear model to test the effect of HS ingestion on gene expression. Gene-Set Enrichment Analysis (GSEA) was performed using GSEA software version 2.0.10, employing Gene Ontology (GO) as a gene-set database and gene annotation for the GO terms based on the Bioconductor package version 2.8. For GSEA calculation, we used the p-value as the statistic for ranking the gene list or the median p-value for genes with multiple probe sets. We used the GSEA Pre-Ranked tool with the classic scoring scheme, a minimum gene-set size of 15, a maximum gene-set size of 1000 and 1000 permutations. Our significant gene-set list had an FDR of 30%. We further validated the metabolomic and transcriptomic analyses using Ingenuity Pathway Analysis (IPA; Ingenuity Systems Inc., Redwood City, CA, USA; http://www.Ingenuity.com) to interpret the network functions, canonical signaling pathways and toxicity functions.

3. Results and discussion

3.1. The composition of the polyphenolic extract

The extract was a complex mixture of bioactive compounds prepared from the calyces of HS harvested in Senegal; the resulting beverage was acidic (pH = 2.8), sweet and resembled the cranberry in flavor. Small amounts of similar extracts are currently used in Western countries to market highly consumed herbal teas ("red or sour teas").[46] The beverage prepared as described, however, was considered to be of low acceptability by 40% of the participants with bitterness as a common concern. Despite polyphenols being generally

accepted as the relevant molecules in the quest for pharmacological action (Table S1†), we also measured the contribution of other compounds, including unknown proteins and/or peptides (2 mg kg^{-1}), soluble fiber (5 mg kg^{-1}), and minute quantities (<100 μg) of citric, malic, ascorbic and protocatechuic acids. We also found mucilage (not measured) and carbohydrates (3.9 mg kg^{-1}), including arabinose, galactose and glucose. According to the *in vitro* antioxidant activity of the extract, each participant received the equivalent in Trolox of 140 mg kg^{-1}, measured as FRAP.[47] Utilizing comparisons and values for bioavailability in a rat model,[13] the highest concentration of an individual polyphenol, using our design, should be 0.2 μg mL^{-1}. Employing the above-mentioned methods, polyphenols and/or their metabolites were not detectable. Nevertheless, several compounds (namely, hibiscus acid, quercetin-glucuronide and quercetin-diglucuronide) were detected using a triple quadrupole mass spectrometer, but the values remained under the limit of quantification. Bioavailability or pharmacokinetic experiments were out of the scope of this report, but these preliminary results suggest that a simple optimization of sample concentration and extraction should be used in the design of further studies.

3.2. Effects in selected laboratory variables

At the 3-hour time-point, we did not observe any changes in glucose metabolism, but lipid metabolism was affected. We also observed a significant decrease in serum cortisol and aldosterone concentration as well as a trend towards higher values in serum renin activity (Table 1). The antioxidant activity of serum measured as FRAP and MDA concentration remained unchanged. This finding probably reflects the fact that the contribution of polyphenols to serum antioxidants is relatively low (<2%). The serum concentration of other contributors to the antioxidant activity, such as proteins, ascorbate and tocopherols, did not change significantly (data not shown), which is in concordance with the lack of variation in the major contributor serum uric acid (>40%).[48] In contrast, we observed a significant increase in the serum concentration of bilirubin, another relatively minor contributor (3%–4%) of serum antioxidant activity under normal circumstances. Chromatographic values confirmed these data and provided results, which indicated the activation of the heme oxygenase-biliverdin reductase axis (*i.e.*, a simultaneous increase in the concentration of heme and bilirubin and a decrease in biliverdin concentration; Fig. 1). We also measured selected variables to check the anti-inflammatory activity, but these variables, with the exception of serum CCL2 concentration, remained unchanged.

3.3. Metabolomic changes

It is important to note that metabolic changes were qualitatively similar in all participants (*i.e.*, they followed the same trend—either a decrease or increase), suggesting that the observed results refer to the actions of the compounds in the HS extract. We detected 471 metabolites in untargeted metabolomic analyses, but we found uncertainties in the interpretation of 176 metabolites. The remaining 295 metabolites were positively identified, and a significant number (n = 107; 36%) were significantly different between groups. The final assessment was limited to 77 metabolites (25 metabolites increased and 52 metabolites decreased after the ingestion of the HS extract) after discarding marginally significant changes, which were scattered across the human metabolic network (Fig. S1†). The perturbed metabolic routes were inferred using the "network parsimony principle",[49] and acetyl-CoA was the most central metabolite in the propagation of the perturbation (Fig. 2). The HS extract significantly decreased the concentration of branched-chain amino acids (*i.e.*, isoleucine, leucine and valine) and long-chain fatty acids. The combined effect was a differential production of circulating carnitine conjugates, which suggests that cells take up these compounds to provide energy. Further analyses confirmed significant effects in the canonical pathways of amino acid metabolism (P = 0.00002) and the citric acid cycle (P = 0.000001). In addition, we found a decreased capacity to form triglycerides and an increased capacity for mitochondrial oxidation, indicating an improvement in metabolism and mitochondrial function (Fig. S2 and S3†).

The most representative metabolites that differentiate samples after the ingestion of HS extract were obtained by a random forest analysis (predictive accuracy >80%) and ranked

Table 1 Selected laboratory variables in plasma from fasting participants used to explore metabolic changes and anti-oxidative or anti-inflammatory effects prior to (0-hour) and 3 hours (3-hour) following consumption of the *Hibiscus sabdariffa* extract

	0-Hour	3-Hour	P
Glucose, $mmol \cdot L^{-1}$	5.1 (4.3–6.1)	5.2 (4.6–5.8)	n.s.
Insulin, $pmol \cdot L^{-1}$	61.2 (56.3–66.8)	63.5 (57.0–65.4)	n.s.
HOMA2-IR	1.69 (1.33–1.72)	1.65 (1.20–1.80)	n.s.
Total cholesterol, $mmol \cdot L^{-1}$	4.93 (4.59–5.24)	4.28 (4.05–4.87)	<0.001
Triglycerides, $mmol \cdot L^{-1}$	1.13 (0.84–1.45)	0.93 (0.79–1.25)	<0.001
HDL-cholesterol, $mmol \cdot L^{-1}$	1.07 (0.95–1.21)	1.08 (0.95–1.32)	n.s.
Renin activity, $mIU \cdot L^{-1}$	13.4 (5.7–17.8)	16.3 (8.4–19.0)	0.056
Aldosterone, $pmol \cdot L^{-1}$	75.4 (61.2–121.5)	66.2 (59.4–103.6)	<0.05
FRAP, $\mu mol\ TE \cdot L^{-1}$	1.29 (0.98–1.49)	1.42 (1.09–1.53)	n.s.
Malondialdehyde, $\mu mol \cdot L^{-1}$	0.15 (0.10–0.18)	0.17 (0.11–0.19)	n.s.
Polyphenols, $mmol\ GAE \cdot L^{-1}$	1.38 (1.10–1.59)	1.46 (1.31–1.52)	n.s.
Cortisol, $nmol \cdot L^{-1}$	375 (240–575)	258 (193–460)	<0.001
Bilirubin, $mmol \cdot L^{-1}$	6.2 (5.1–8.9)	11.1 (7.2–13.3)	<0.001
Uric acid, $\mu mol \cdot L^{-1}$	308 (265–350)	325 (290–357)	n.s.
Interleukin 6, $pg \cdot mL^{-1}$	0.38 (0.12–0.56)	0.45 (0.10–0.64)	n.s.
Interleukin 8, $pg \cdot mL^{-1}$	1.56 (1.35–1.75)	1.64 (1.32–1.79)	n.s.
TNF-α, $pg \cdot mL^{-1}$	5.61 (4.25–7.41)	5.49 (4.01–7.36)	n.s.
CCL2, $pg \cdot mL^{-1}$	435 (400–550)	360 (280–440)	<0.001
Hs-CRP, $\mu g \cdot L^{-1}$	0.63 (0.42–0.85)	0.72 (0.51–0.97)	n.s.

Values are expressed as the median (interquartile range); HOMA2-IR, homeostatic model assessment index; TE, trolox equivalents; GAE, gallic acid equivalents; TNF-α, tumor necrosis factor-α; CCL2, chemokine (C–C motif ligand 2); Hs-CRP, high sensitivity C-reactive protein.

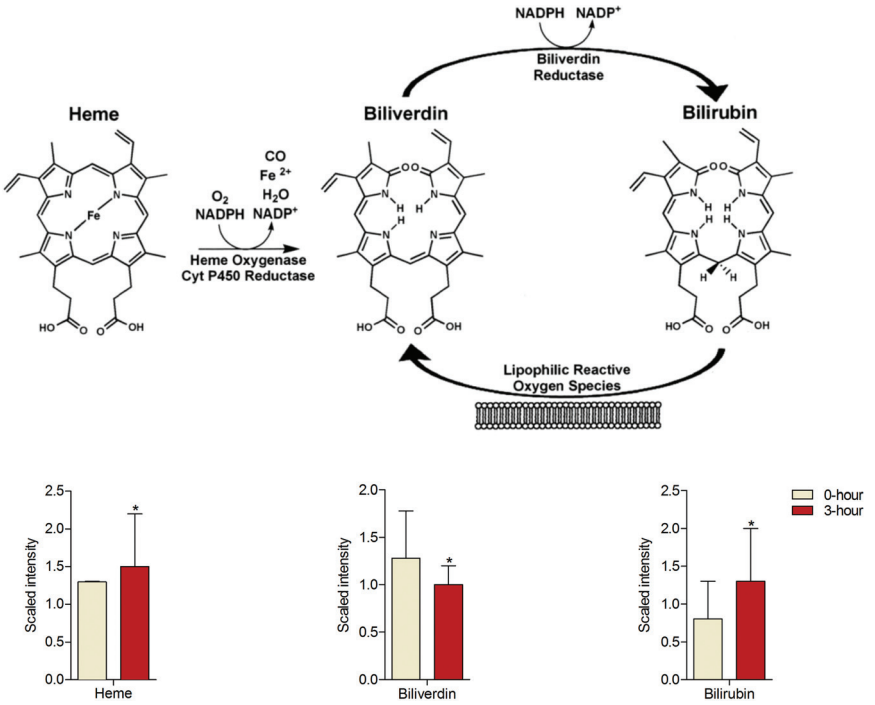

Fig. 1 The effect of polyphenols in bilirubin metabolism. The ingestion of the HS extract increased the plasma concentration of heme and bilirubin with a significant decrease in biliverdin levels, suggesting activation of the heme-oxygenase-biliverdin reductase axis; *$P < 0.05$ with respect to the 0-hour time-point.

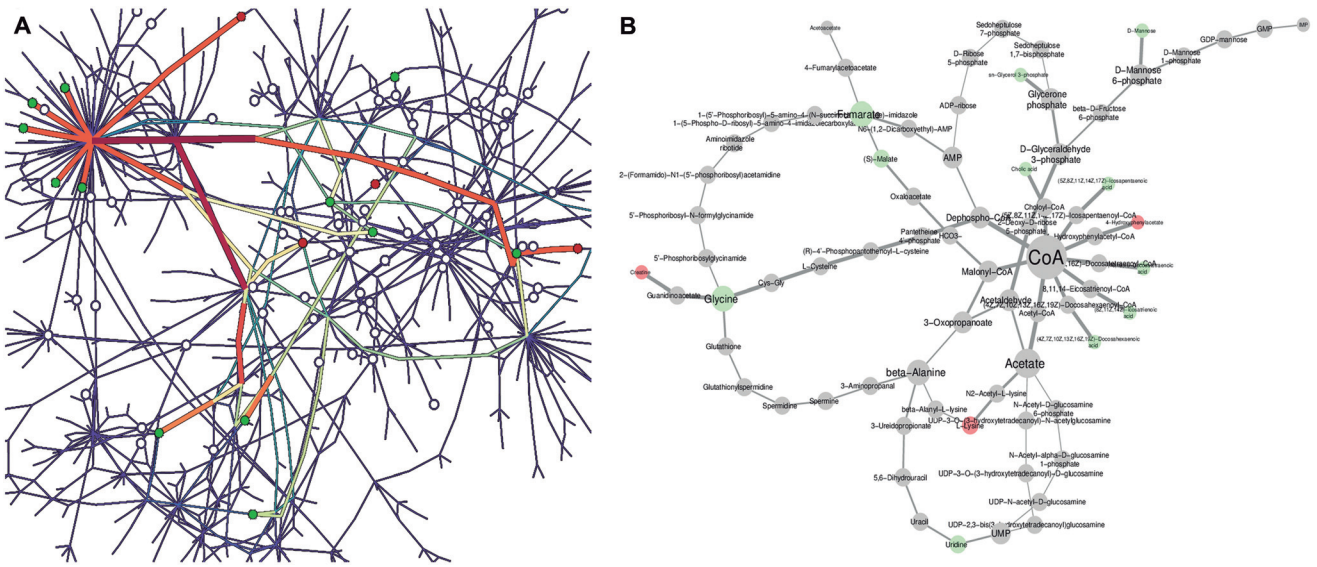

Fig. 2 Overall representation of metabolic disturbances. The affected pathways are highlighted in the metabolic network connecting altered metabolites (red nodes increased, green nodes decreased, and black nodes remain unchanged) through the shortest possible metabolic routes. The color and width of each reaction (link) represent the number of shortest paths connecting the altered metabolites (A). We inferred centrality in CoA and acetate, most likely acetyl-CoA, but this metabolite was not identified experimentally (B).

in order of their importance in the classification scheme (Fig. 3A). The application of LDA, PCA and heat-map graphic representations yielded similar results for group clustering, pattern recognition and the most perturbed pathways and sub-pathways (Fig. 3B and S4†). The HS extract acutely decreased the serum cortisone/cortisol levels (Fig. S5†) associated with changes in the expression of the SGK1 (serum/glucocorticoid regulated kinase 1) gene (Table 2). Seemingly, these changes

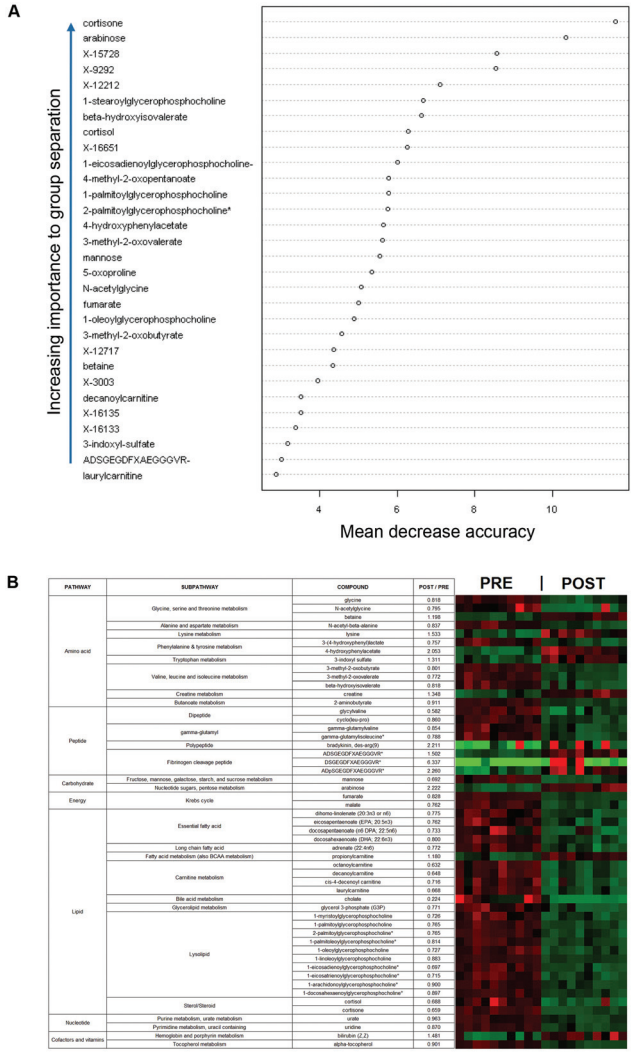

Fig. 3 Altered metabolites differ in their relative importance. Random forest analysis (A), a supervised classification technique, distinguishes between groups based on their metabolic profiles with a predictive accuracy of >80% and produces a list of primary differentiators. Heat map as a graphical representation of data (B), where the individual values contained in the metabolic profiles matrix are represented as colors. The red or green colors indicate increased or decreased plasma concentration, respectively. The represented metabolites were selected according to their relative importance to depict the fact that the actions of the HS extract are scattered across a significant number of metabolic pathways.

might have multiple beneficial effects on metabolism. The increase in serum arabinose also appears as an important differentiator (Fig. 3A). This is an intriguing finding that illustrates the possible influence of other soluble compounds present in plant-derived extracts. We also found that the HS extract significantly increased the serum concentrations of known products of gut microbiome metabolism such as catechol sulfate, 3-indoxyl sulfates, 3-phenylpropionate and 4-hydroxyphenylacetate. We also noticed a uniform and significant increase in serum concentrations of des-Arg(9)-bradykinin—the active metabolite of bradykinin. This metabolite causes blood vessels to dilate and is one of the substrates of angiotensin I-converting enzyme (ACE). Thus, this observation strongly suggests that HS extracts may act as an ACE inhibitor.[15,50–52]

3.4. Transcriptomic changes

The primary changes in the differentially expressed genes that may demonstrate an overall effect of the HS extract (some are depicted in Table 2) illustrate that PBMCs are a source of biological samples that could detect global changes with metabolic, oxidative and inflammatory implications. The GSEA *p*-value based on a ranked list of genes revealed the relative importance of the biological processes associated with the cellular response to organic substances, the immune system process, the maintenance of protein localization in organelles and the biological regulation of lipid and glucose metabolism (Fig. S6†). Similar studies on molecular functions showed an over-representation of genes related to the activity of cytokine and chemokine receptors and ligands. There was a clear and significant association between functions related to the ligand binding to vitamin D and G-protein coupled receptors (Fig. 4). The gene ontology numbers and term names, gene size and false discovery rates, as well as the list of common genes significantly involved, may be found in Table S2.† Curiously, the response to biotic stimulus (GO# 0009607) is clearly overexpressed (n = 558 genes) and the major contribution was provided by CXCL8 (interleukin 8), CCL3, CCL2, IL-6 and TNF-α, indicating the anti-inflammatory component of HS.

3.5. Inferring the routes of interacting biological macromolecules

IPA analysis further confirmed these findings, and the top associated network functions (score >40) were gene expression, post-translational modification, cell cycle, molecular transport, RNA trafficking and cellular function and maintenance. The top canonical pathway was glucocorticoid receptor signaling ($P < 0.000001$). The examination of gene expression, summarizing the differences between the 0-hour and 3-hour time-points, indicated a down-regulation in the genes involved in cholesterol and triglyceride synthesis, lipid transport, gluconeogenesis and glycolysis. Notably, the differential changes in the expression of several genes suggest a possible effect of the HS extract in energy homeostasis *via* regulatory pathways involving the mechanistic targeting of rapamycin (MTOR) and/or the AMP-activated protein kinase (AMPK) (*i.e.*, the regulation of nutrients and energy sensors[53]). Further confirmation was obtained by analyses of the metabolites and the genetic expression of acetyl-CoA carboxylases (ACC1 and ACC2), CERB-regulated transcriptional coactivator-2 (CRTC2), PPARγ coactivator-1α (Ppargc1α), ribosomal protein S6 kinase (S6K), and eukaryotic initiation factor 4E binding protein 1 (4EBP1). The genes and metabolites with known gene symbols were combined and the results on the main associated network functions and top canonical pathways did not change, except for a higher representation of the incorporation of bile acid-related

Table 2 List in alphabetical order of the top 25 differentially expressed genes that best describe transcriptomic changes after the ingestion of *Hibiscus sabdariffa* extract

Symbol	*p*-Value	FDR	Name
CCL3L3	0.001	0.24	Chemokine (C–C motif) ligand 3
CEP152	0.016	0.14	Centrosomal protein 152 kDa
CX3CR1	1.76×10^{-4}	0.20	Chemokine (C–X3–C motif) receptor 1
CXCL10	2.33×10^{-4}	0.21	Chemokine (C–X–C motif) ligand 10
CXCL8	3.73×10^{-4}	0.22	Interleukin 8
CYP2R1	4.67×10^{-4}	0.23	Cytochrome P450, family 2, subfamily R, polypeptide 1
EIF1	4.52×10^{-4}	0.23	Eukaryotic translation initiation factor 1
EIF5	1.28×10^{-4}	0.20	Eukaryotic translation initiation factor 5
ERN1	1.22×10^{-4}	0.20	Endoplasmic reticulum to nucleus signaling 1
FKBP5	2.42×10^{-4}	0.21	FK506 binding protein 5
HNRNPDL	0.001	0.06	Heterogeneous nuclear ribonucleoprotein D-like
IFRD1	1.16×10^{-4}	0.20	Interferon-related developmental regulator 1
MGAT4A	0.064	0.19	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-*N*-acetylglucosaminyl-transferase, isozyme A
MIB2	2.64×10^{-6}	0.07	Mindbomb E3 ubiquitin protein ligase 2
NID1	6.82×10^{-5}	0.19	Nidogen 1
PCMTD1	0.001	0.24	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase domain containing 1
PMAIP1	1.06×10^{-4}	0.20	Phorbol-12-myristate-13-acetate-induced protein 1
PPP1R15A	0.001	0.25	Protein phosphatase 1, regulatory subunit 15A
PTGS2	1.67×10^{-4}	0.20	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
SCAF4	1.10×10^{-4}	0.20	SR-related CTD-associated factor 4
SGK1	1.91×10^{-4}	0.20	Serum/glucocorticoid regulated kinase 1
TAGAP	0.005	0.08	T-cell activation RhoGTPase activating protein
WDR20	0.065	0.19	WD repeat domain 20
ZBTB16	2.58×10^{-4}	0.21	Zinc finger and BTB domain containing 16
ZBTB24	1.89×10^{-4}	0.20	Zinc finger and BTB domain containing 24

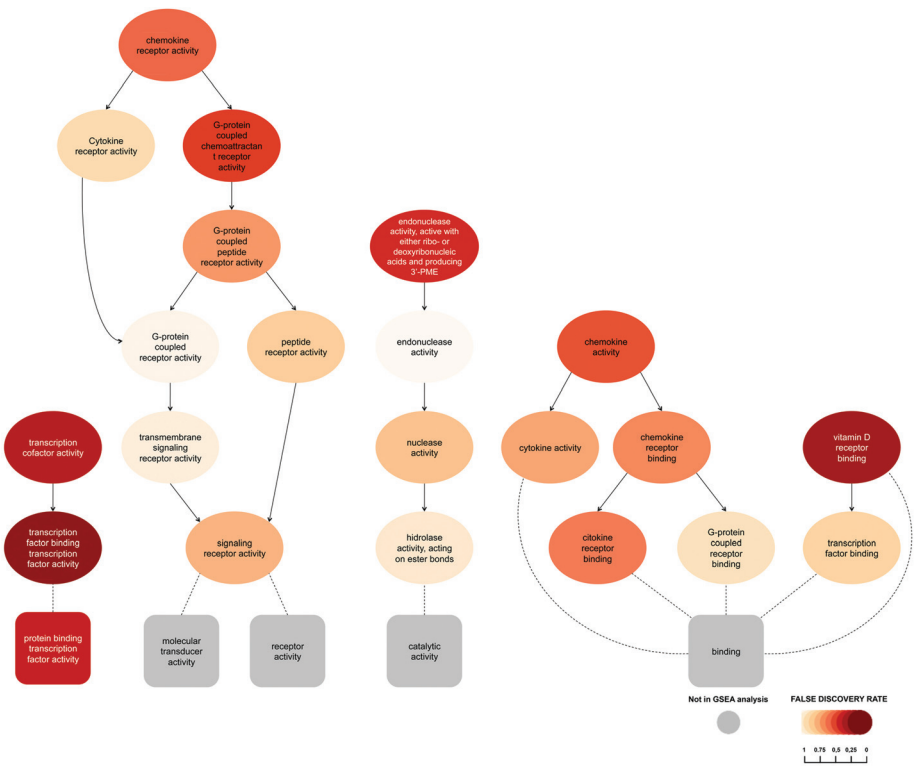

Fig. 4 Gene-Set Enrichment Analysis (GSEA) performed using Gene Ontology (GO) as the gene-set database for gene annotation. The figure depicts the significant overrepresentation of molecular function GO terms. Each node corresponds to a distinct molecular function, including gene sets with a low false discovery rate. The color scheme is at the bottom of the figure and the grey nodes correspond to terms without gene representation in the array. Dashed lines indicate missing intermediate terms between the nodes. The expression of genes with functional chemokine activity was significantly associated with the expression of genes, which indicate binding to both G-protein coupled receptors and vitamin D receptors.

functions. In this regard, the top up-regulated molecules were des-Arg-(9) bradykinin, bilirubin and CX3CR1, and the top down-regulated molecules were cholic acid, cortisone/cortisol and EGR3. Finally, we found an association between the ingestion of HS extract and a decrease in the depolarization of the mitochondrial membrane ($P = 0.0015$), mechanisms of gene regulation by peroxisome proliferators ($P = 0.009$) and p53 signaling ($P = 0.002$).

3.6. Overall discussion

The combination of metabolomic and transcriptomic analyses uncovers complex and multiple metabolic transformations following the ingestion of polyphenols and may help to predict the multiple interactions of food components on metabolic health. Inferring the routes of interacting biological macromolecules may be considered as a promising and complementary tool for capturing the metabolic complexity of phytochemical exposure.[7,15,22–24,54–57] Tissue-specific transcriptomic information requires invasive procedures, and whether changes in PBMCs are indicative of the metabolism in other tissues, although suggestive, needs confirmation. We found high serum arabinose concentrations, which may represent a cautionary note because the obvious source was the HS extract. Although human arabinose metabolism is unknown, our data may explain the effects observed in lipid metabolism because, at least in rats, arabinose reduces hepatic lipogenesis and the serum concentration of both cholesterol and triglycerides.[50]

Polyphenols are potential antioxidants *in vitro*, but it has not been unequivocally established that the consumption of polyphenols in humans evokes *in vivo* antioxidant effects.[48] Although unexpected in a short-term experiment, our data confirm that the antioxidant activity of polyphenols may be partially derived from actions in the digestive tract *via* the up-regulation of the heme-oxygenase (HO)-biliverdin reductase axis. The induction of HO expression explains the antioxidant action of serum bilirubin, contributes to the synergism with PPAR-agonists, and improves insulin sensitivity. Moreover, HO expression may suppress key steps associated with the activation of inflammatory and oxidative pathways.[58–63]

The metabolic effects of the HS extract converge on acetyl-CoA and may improve mitochondrial function *via* the transport of carbon atoms to the citric acid cycle (*i.e.*, to be oxidized for energy production). In addition, these polyphenols regulate energy sensors (the AMPK/MTOR pathway) and increase the capacity for the oxidation of conjugates derived from branched-chain amino acids and long-chain fatty acids. The metabolism of protein, fat and carbohydrates may be also affected by the HS extract as it reduces the serum concentration of cortisol. This may be a significant finding because we have previously found that the HS extract lowers blood pressure and improves endothelial function in humans,[15] which is in line with the increasingly recognized association between excess cortisol and metabolic syndrome. In particular, the link between HS polyphenols and decreased cortisol may sustain findings that indicate that the combination of oxidation, inflammation and endothelial dysfunction are interrelated mechanisms with a role in the pathogenesis of hypertension.[64,65] Excess cortisol induces hypertension,[64] and the rapid modifications induced by HS in blood pressure and serum cortisol levels confirm that the expression of the SGK1 gene may be crucial in the transport of sodium.[65] Similarly, we found significant associations between the HS extract and the expression of cytochrome P450, family 2, subfamily R, polypeptide 1 (CYP2R1), which were connected with the expression of genes affecting vitamin D receptor binding. Clinically, high serum levels of vitamin D seem to accompany a reduced risk of high blood pressure but the causality of the association remains to be ascertained.[66] Moreover, evidence presented here are concordant with our previous findings, indicating that the HS extract decreases the activity of the renin-angiotensin (RAS) system in patients with metabolic syndrome and hypertension.[15] The possible action of the HS extract as an ACE inhibitor *in vivo* might be sustained by the finding of elevated serum concentrations of the vasodilator, des-Arg(9)-bradykinin, and the interrelated effects that result in a decrease of the RAS activity may help to understand the beneficial actions of polyphenols and/or associated compounds from the HS extract. Hypertension, diabetes, obesity and cortisol stimulate RAS activity, and activated RAS is closely related to metabolic syndrome.[67–71] Conversely, the inhibition of RAS activity improves these disturbances.[72,73] We also describe that genes acting on the molecular action of G-protein-coupled receptors are differentially expressed by the HS extract. This is important because cytokines, hormones and other active components that cause the deleterious metabolic effects induced by high tissue RAS activity act through G-protein-coupled receptors.[74,75]

Conclusions

Herein, we propose that polyphenols from HS are a potential source of bioactive compounds that may provide protection for the cardiovascular system. The effects described and those provided by other authors might be used for modeling combinations that are capable of optimizing the view that polyphenols play a pivotal regulatory role in metabolic reprogramming.[76–80] In addition, investigating multiple metabolic effects and affected pathways should be considered in the assessment of therapeutic strategies.

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