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Berry fruits modulate kidney dysfunction and urine metabolome in Dahl salt-sensitive rats

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ABSTRACT

Berries are rich sources of (poly)phenols which have been associated with the prevention of cardiovascular diseases in animal models and in human clinical trials. Recently, a berry enriched diet was reported to decrease blood pressure and attenuate kidney disease progression on Dahl salt-sensitive rats. However, the relationship between kidney function, metabolism and (poly)phenols was not evaluated. We hypothesize that berries promote metabolic alterations concomitantly with an attenuation of the progression of renal disease. For that, kidney and urinary metabolomic changes induced by the berry enriched diet in hypertensive rats (Dahl salt-sensitive) were analyzed using liquid chromatography (UPLC-MS/MS) and ^{1}H NMR techniques. Moreover, physiological and metabolic parameters, and kidney histopathological data were also collected. The severity of the kidney lesions promoted in Dahl rats by a high salt diet was significantly reduced by berries, namely a decrease in sclerotic glomeruli. In addition, was observed a high urinary excretion of metabolites that are indicators of alterations in glycolysis/gluconeogenesis, citrate cycle, and pyruvate metabolism in the salt induced-hypertensive rats, a metabolic profile counteracted by berries consumption. We also provide novel insights that relates (poly)phenols consumption with alterations in cysteine redox pools. Cysteine contribute to the redox signaling that is normally disrupted during kidney disease onset and progression. Our findings provide a vision about the metabolic responses of hypertensive rats to a (poly)phenol enriched diet, which may contribute to the understanding of the beneficial effects of (poly)phenols in salt-induced hypertension.

1. Introduction

(Poly)phenols include a family of secondary metabolites that are present in plant-derived foods and their intake has been associated to a lower risk of cardiovascular diseases (CVDs), namely by reducing blood pressure [1,2]. Flavonoids are one of the main classes of (poly)phenols which have beneficial effects on renal physiology, but their effects on the progression of kidney disease are still poorly studied. Their main mechanisms of action are thought to be their impact on the inflammatory process, besides its *in vitro* antiapoptotic and antifibrotic properties (reviewed in Ref. [3]).

Recently we demonstrated that a (poly)phenol-enriched diet attenuated the progression of hypertension and renal hypertrophy in Dahl salt-sensitive (Dahl/SS) rats [4]. The berry mixture not only increased survival and maintained the body weight of the rats, but also ameliorated the systolic blood pressure (SPB), and improved cardiac and kidney function [4]. The association between hypertension and renal disease is a well-accepted condition with no effective treatment

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available. In that view, our previous work opens an avenue for the development of new preventive and/or therapeutic strategies. Moreover, a greater understanding of the cellular and metabolic alterations underlying the effect of (poly)phenols on kidney disease progression is of utmost relevance for this worldwide health crisis lacking therapeutic options.

An important aspect needed to be clarified is the identity of the metabolites resulting from (poly)phenols administration and consumption, that are responsible for their beneficial properties [3]. In that sense, in our preceding work, we demonstrated the metabolic fate of (poly)phenols in Dahl/SS rats after the ingestion of a berry-enriched diet, which was responsible for the beneficial effects observed. Moreover, we showed that berries had the ability to modulate the composition of the rat's gut microbiota [5].

Several studies based on metabolomics techniques have reported metabolite changes in serum, urine and kidney tissues in chronic kidney disease [6–8]. Metabolomics methodologies are effective for monitoring changes in small molecules, induced by internal and external factors and could, therefore, constitute an important tool for understanding the complex renal disease events. They identify biochemical signatures related to the disease pathogenesis that could be used for the diagnosis, monitoring of the disease progression and evaluation of the response to therapeutic interventions [9]. In recent years, metabolomics has been used to assess nutritional status and facilitate the discovery of new biomarkers associated with specific nutrients (food metabolome) or forms of metabolic dysfunction [10–12]. However, the relationship between (poly)phenols, kidney function, and metabolism is still not clear.

To understand the metabolic alterations associated with renal disease and the effects of diet on their progression, the metabolomic changes induced by a (poly)phenol enriched diet in urine and kidney of Dahl/SS rats were analyzed using UPLC-MS/MS and ^{1}H NMR techniques. These metabolites profile was related to phenotypic traits including physiological and metabolic parameters, as well as with kidney histopathological data. We also aimed to provide novel insights in how (poly)phenols can modulate kidney disease onset and progression by impacting the thiol pools. Despite historically defined as an imbalance of pro-oxidants and antioxidants, the current evidence supports that glutathione (GSH) redox pool might not be equilibrated with the other redox pools. In that view, oxidative stress may be better defined as a disruption of redox signaling through discrete redox pathways [13]. Comparatively to the GSH-rich liver, the kidney is an organ rich in cysteine, the glutathione precursor. In fact, the main pathway of glutathione catabolism towards cysteine replenishment (cysteinylglycine as intermediary step) is a hallmark to kidney tubular cell [14], and kidneys represent one of the tissues with the highest expression of cysteine disulfide transporters [15]. This non-essential amino acid is the most predominant thiol in plasma, mostly in its oxidized form – low molecular weight cysteine disulfides and protein bound cysteine (protein cysteinylation, CysSSP). Together with the *de novo* cysteine synthesis by the transsulfuration pathway, circulating cystine is the major source of intracellular cysteine to support several cellular processes. Overall, we evaluated changes in kidney thiols pools and relate them with the metabolic alterations induced by salt and the effect of berries consumption.

2. Material and methods

2.1. Diet preparation and animal experiments

The mixture of blueberries (*Vaccinum* spp. variety Georgia Gem), blackberries (*Rubus* L. subgenus Rubus Watson variety Karaka Black) and raspberries (*Rubus idaeus* L. variety Himbo Top) harvested at the Fataca experimental field in Odemira, Portugal; strawberry tree fruits (*Arbutus unedo* L.) harvested in the Alentejo region, Portugal, and Portuguese crowberries (*Corema album* L.) harvested in the Comporta region, Portugal, was prepared as described previously [4,5,16]. The berry mixture (poly)phenol content and the profiles before and after inclusion in rats' diets (LS and HS) was analyzed as described in the preceding papers [4,5] (supplementary tables S1 and S2) and it was in compliance with the chemical characterization already done for the fruits individually and in the mixture [16,17].

The animal trial was carried out at the platform AnimEx, which belongs to the Châtenay-Malabry Faculty of Pharmacy from the University Paris-Sud, following the experimental design and nutritional treatments as previously published in the former work [4,5]. Briefly, male Dahl-salt sensitive rats (SS/JrHsdMcwiCrl strain [18] obtained from Charles River L'Abresle, France) were maintained in a room at 22 ± 1 °C, with an artificial 12/12 h light/dark cycle. After acclimation for 1 week with access to the AIN-76a powder chow (INRA UPAE, Jouy-en Josas, France) (supplementary table S1) and water, rats were randomly divided into 4 groups. Rat powder chow (50 g) was prepared fresh every day by mixing the regular AIN-76a with or without lyophilized berry mixture: 1) low salt diet enriched with 0.26% NaCl (LS); 2) low salt diet enriched with 0.26% NaCl and supplemented with 2 g of lyophilized berry mixture (LSB); 3) hypersaline diet enriched with 8% NaCl (HS); and 4) hypersaline diet enriched with 8% NaCl, and supplemented with 2 g of lyophilized berry mixture (HSB) (Fig. 1). Food and water were available *ad libitum* in both the home and metabolic cages. At week 4 and 9 of the trial, all rats were housed in metabolic cages and body weight, food, water intake, as well as urinary volume were measured daily for three days (Fig. 1). For metabolomic analysis,

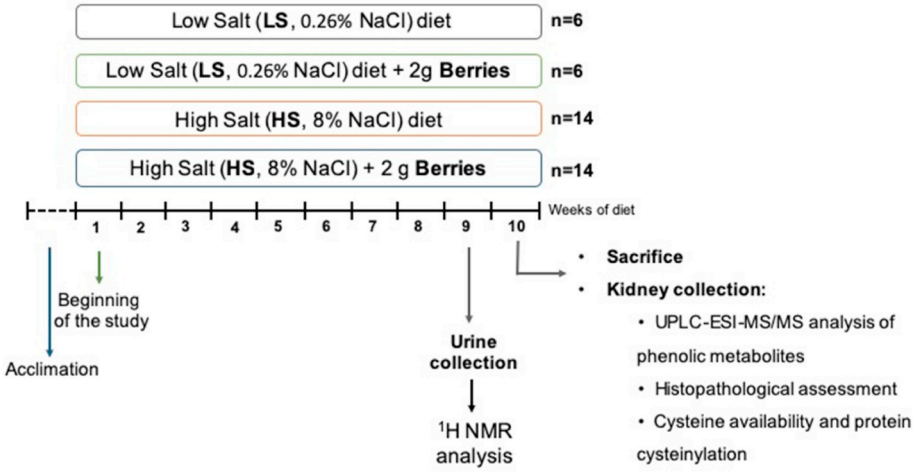

Fig. 1. Flowchart of the trial. A berry mixture was supplemented or not in the food to LS diet rats (LS and LSB) and HS diet rats (HS and HSB) for 10 weeks.

rats were fasted overnight.

All animal care and experimental procedures were performed according to the European Community guiding principles in the care and use of animals (Directive 2010/63/EU of the European Parliament) and authorizations to perform animal experiments according to this directive were obtained from the French Ministry of Agriculture, Fisheries and Food (No. D-92-283, 13 December 2012). The Ethical Committee of the University of Paris-Sud, France approved all animal research protocols (N°APAFIS#2152–2015092912128144 v3). All studies involving rats are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals.

2.2. Urine and kidney collection

For collection of urine, rats were allocated at the 9th week into metabolic cages for 4 days, with 24 h adaptation period before urine collection (Fig. 1). After, samples were centrifuged at 3000 *g* for 5 min at 7 °C. A total of 500 μL of urine were acidified with 100 μL of 50% Milli-Q water/formic acid (v/v), followed by storage at −20 °C until further analysis.

At the end of the experiment (after 9 weeks), animals were sacrificed by decapitation without any anaesthetic drug (Fig. 1). Kidneys were excised from the rats, sections of kidney were fixed in formalin for histopathological examinations, and the remaining tissue was frozen in liquid N_2 immediately after collection, freeze-dried and stored at −80 °C until further analysis.

2.3. UPLC-ESI-MS/MS analysis of phenolic metabolites in kidney

In order to clean-up the biological matrix and pre-concentrate the phenolic compounds, kidney tissues were sequentially pre-treated by a combination of liquid–solid extraction (LSE) and microelution solid-phase extraction (μSPE) [19–22]. The methodologies were followed according to previous publications [5,23].

Briefly, 50 μL of ascorbic acid (1%), and 150 μL of phosphoric acid (4%) were added to 60 mg of freeze-dried kidney. Each sample was treated four times with 400 μL of extraction solution (water/methanol/phosphoric acid 4%; 94/4.5/1.5, v/v/v). Then, the samples were sonicated (S-150D Digital SonifierR Cell Disruptor, Branson, Ultrasonidos S.A.E., Barcelona, Spain) for 30 s maintaining the sample in an ice water bath, and then centrifuged for 15 min at 8784 g at 20 °C. The supernatants were collected, and 350 μL of the extract of kidney was pre-treated by μSPE using the sample methodology previously described [20].

The analysis of phenolic metabolites in kidney was performed using a UPLC coupled to tandem MS (MS/MS). The chromatographic system is an AcQuity Ultra-Performance™ liquid chromatography and tandem MS from Waters (Milford MA, USA), as previously reported [5,22].

For the (poly)phenol metabolites identified in the kidney by UPLC-MS/MS, the non-parametric Kruskal–Wallis and Dunn's tests were applied to analyze significant differences ($p < 0.05$) between groups. The Kruskal–Wallis test was applied to determine any significant difference between the treatments and, if any were detected, Dunn's test was used to compare all the different pairs of the treatments. Data are expressed as mean concentrations (nmol/g fresh kidney) with standard error of the mean (SEM). The Prism 7 for Mac OS X, version 7.0a was used.

2.4. Histopathological assessment of the kidney

Formalin-fixed paraffin-embedded kidney sections were cut into 7-μm sections and mounted on slides. The kidney sections were subjected to the standard Masson's trichrome and McMannus periodic acid – Schiff staining protocols [24] from which glomerular, interstitial and vessels injury index were calculated to quantitate renal injury. In each group, 8 fields of each kidney were observed with 200× magnification.

Glomerular, interstitial and vessel lesions severity was graded according to the percentage of total glomeruli, interstitial and vessels affected as follows: stage 0, no lesions; stage 1, 1–25% injury; stage 2, 26–50% injury; and stage 3, 51–100% injury. In the glomeruli, lesion severity was also assessed taking into account their segmental or global aspect (all samples were blindly evaluated by a nephropathologist).

2.5. Cysteine availability and protein cysteinylation in kidney

To address total cysteine availability and cysteinylated protein pools, kidneys were excised from the rats, frozen in liquid N_2 immediately after collection freeze-dried and stored at −80 °C until further use. **Lyophilized** tissue was sonicated in PBS1x and centrifuged at 13 000 *g*, for 5 min at 4 °C. Then the supernatant was aliquoted to proceed with cysteine fraction quantification by HPLC as previously described [25]. Multiple comparisons between results from groups were done using two-way ANOVA with Bonferroni post-test (GraphPad Prism version 7.0a).

2.6. Urine ^{1}H NMR metabolomic analysis

Prior to the metabolomic analysis, the urine samples were thawed on ice and mixed using a vortex mixer. The samples were centrifuged (12 000 *g*, 15 min at 4 °C) and 400 μL of the supernatant was mixed with 300 μL of chloroform and 200 μL methanol. Samples were agitated for 15s and then incubated on ice for another 15 min. Then, samples were centrifuged (12 000 *g*, 15 min at 4 °C) and 400 μL of supernatant were dried overnight using a speed vacuum concentrator at 30 °C. Dried samples were resuspended in 200 μL phosphate buffer (100 mM sodium phosphate buffer, pH 7.4, containing 0.1–0.5 mM 3-trimethylsilylpropionic acid (TSP) and optionally 0.2% NaN_3) and 400 μL D_2O. The samples were mixed again, and finally, an aliquot of 560 μL was transferred into a 5 mm NMR tube [26].

Urine samples were analyzed with proton (^{1}H) NMR spectroscopy on an 800 MHz Bruker AvanceII + (Ettlingen, Germany) spectrometer equipped with a room temperature triple resonance HCN Z-gradient probe, at 298 K. ^{1}H 1D-NOESY spectra (spectral width: 20 ppm; mixing time: 0.01 s; relaxation delay: 4 s; acquisition time: 4 s) were collected for each urinary sample using the “noesygppr1d” pulse sequence following the parameters for profiling recommended from Chenomx NMR Suite software (Chenomx Inc., Edmonton, Canada). All spectra were processed with a line broadening (lb) of 0.5 Hz and a final number of 128 K points.

For the most concentrated sample of each group, additional spectra were collected to assist with assignment, namely J-Resolved and ^{1}H–^{1}H COSY. Spectra were processed and analyzed using TopSpin3.2 software (Bruker, Ettlingen, Germany).

Metabolite identification and quantification was carried out using Chenomx NMR Suite 8.12 software (Chenomx Inc., Edmonton, Canada), using the internal reference library (Version 10). Chemometric analysis of the raw data was also performed on binned spectra using the “nmrprocflow” platform. Bins were obtained using manually curated, intelligent binning after referencing, baseline correction, water signal removal and peak alignment.

2.7. NMR data analysis

A total of 491 NMR bin areas were analyzed using the metabolomic data processing server MetaboAnalyst 4.0 [27].

2.7.1. Multivariate statistical analysis

Multivariate statistical analysis was carried out on the extracted bin intensities. Data were pre-processed for normalization and scaling in order to remove possible bias from sample variability and preparation. Data were filtered according to the interquantile range, normalized for LS group and submitted to pareto scaling (mean-centered and divided

by the square root of the standard deviation of each variable). Normalized data were subsequentially analyzed by Principal Component Analysis (PCA) to detect intrinsic clusters and outliers within the data. To maximize separation between samples, Partial Least Squares - Discriminant Analysis (PLS-DA) with default parameters of MetaboAnalyst was applied. Models were tested using R^2 and Q^2 parameters, where R^2 provides a measure of how much variation is represented by the model and Q^2 for the goodness and prediction. After building the PLS-DA model, variable importance in projection (VIP) score of each variable was used to rank the identified distinctive features based on their significance in discriminating between the 4 groups in analysis. Variables with VIP score > 1 were selected as significant bins. The value of VIP score which is greater than 1 is the typical rule for selecting relevant variables [28,29].

2.7.2. Univariate statistical analysis

To determine if changes in NMR bin intensities were statistically significant, one-way Analysis of Variance (ANOVA) followed by post-hoc Fisher's least significant difference method (Fisher's LSD) were applied using the online Metabolomic Data Analysis with MetaboAnalyst [27]. Altered bins were considered significant when *p* values were less than 0.05.

2.7.3. Pathway analysis

The assignment of the spectral bins was done by Chenomx NMR Suite 8.12 software (Chenomx Inc., Edmonton, Canada), using the internal reference. Then, pathway analysis was conducted with MetaboAnalyst [27] uploading the assigned metabolites statistical significantly different from ANOVA analysis within all groups using the default parameters with *Rattus norvegicus* pathway library from KEGG.

3. Results

3.1. Effect of salt and a (poly)phenol enriched diet on Dahl/SS rat's metabolic parameters

We recently demonstrated the beneficial effects of a berry-enriched diet in a chronic model of hypertension-induced heart failure, the Dahl/SS rats, and assessed how the metabolic fate of (poly)phenols is affected under pathological conditions and the associated microbiota alterations [4,5]. The berry mixture provided to the hypertensive rats promoted an increased survival, maintained their body weight, ameliorated systolic blood pressure (SPB) (supplementary figure S1) and cardiac function and decreased cardiac and renal hypertrophy [4]. Moreover, alterations in metabolic fate of (poly)phenols were concomitant to gut microbiota modulation in hypertensive rats suggesting a bidirectional relationship, between metabolites and microbiota environments [5].

Irrespective of the addition of berries in the diet, from week 4, rats on HS diets started to show higher water intake and urinary volume (Table 1); this phenomenon was maintained until the end of the trial. Although not significant, there was a trend of a decreased food intake at 9 weeks by the rats in the HS diet when compared to the LS diet and berry-enriched diet tend to prevent this decline. This could be underlying in the differences observed in body weight, once HS rats present lower body weight. Interestingly, the (poly)phenol enriched diet prevented the reduction of the body weight promoted by the HS diets at 9 weeks (Table 1).

3.2. Kidney histomorphological modifications on hypertensive Dahl/SS rats are reduced by a berry-enriched diet

No histological abnormalities were found in the kidneys of the LS and LSB rats at 9 weeks (Fig. 2A-2D) rats. In contrast, kidney tissues from the HS rats, at the end of the trial, showed glomerulosclerosis (indicated with an asterisk (*) in the representative section of Fig. 2A), and inflammatory cell infiltration (indicated with an arrow in the representative section of Fig. 2A). A recovery in the kidney morphology was observed suggesting some renoprotection by the ingestion of the diet supplemented with the berries (Fig. 2A-2D).

3.3. (Poly)phenol metabolites are more retained in kidney of Dahl/SS rats

Metabolites from berry (poly)phenols were identified and quantified by UPLC-MS/MS in rats' kidneys. A total of 41 (poly)phenol metabolites and their respective concentrations were determined in rat's kidneys at the end of the trial, independently of the diet (Table 2).

Hippuric acid was (Table 2) one of the most abundant (poly)phenol metabolites in kidney samples in the 4 groups, and its concentration was significantly higher in rats that consumed a high salt diet supplemented with the berry mixture (HSB, $p < 0.01$). Protocatechuic acid sulfate was accumulated in higher amounts in HS and HSB rat kidneys. In fact, this was the metabolite which clearance was most affect if we compare LSB and HSB rat's, the retention of protocatechuic acid in the kidneys is significantly higher in HSB in relation to LSB group.

From the 41 (poly)phenol metabolites, 7 of them were only present in the kidneys of rats that consumed berries, irrespective of the salt content in the diet (Table 2). Moreover, there were 14 metabolites exclusively identified in HSB rats' kidneys (Table 2) from which 36% belonged to the flavanols derivatives class, 21% were cinnamic acid derivatives, 14% were pyrogallol derivatives, and catechol, benzoic acid, valeric acid and valerolactone derivatives represented 7.1% each. In fact, 95% of these metabolites detected are in higher concentrations in HSB rat kidneys.

3.4. Berries restore the salt-induced metabolic alterations in urine of Dahl/SS rats

3.4.1. ^1H NMR spectra analysis

The metabolic profile of urine samples from the 9th week of the trial was deduced from the spectral features of ^1H NMR. Principal Component Analysis (PCA) was performed on the profiled compound bin intensities for all groups (Fig. 3A). The bins were later identified

Table 1
Influence of high salt diet and berry-enriched diet on metabolic parameters at 4 and 9 weeks of diet.

	4th Week				9th Week			
	Food intake (g)	Body weight (g)	Water intake (mL)	Urinary volume (mL)	Food intake (g)	Body weight (g)	Water intake (mL)	Urinary volume (mL)
LS	25.8 ± 4.4	289.0 ± 5.2	32.6 ± 2.8	10.3 ± 2.0	17.0 ± 0.7	375 ± 5.6	18.2 ± 1.6	11.3 ± 1.4
LSB	23.0 ± 3.4	288.0 ± 5.1	28.3 ± 4.8	10.0 ± 1.1	19.7 ± 1.8	377 ± 5.2	17.8 ± 3.3	9.8 ± 2.0
HS	24.5 ± 2.3	286.0 ± 4.8	80.9 ± 7.5***	58.9 ± 5.1***	13.1 ± 2.4	303 ± 16.7**	62.4 ± 10.2**	42.9 ± 5.2***
HSB	23.6 ± 1.5	289.0 ± 3.4	82.9 ± 5.6$^{\$\$\$}$	60.4 ± 3.2$^{\$\$\$}$	19.8 ± 2.6	349 ± 5.2$^{\#\$\$}$	55.6 ± 5.2$^{\$\$\$}$	47.7 ± 3.8$^{\$\$\$}$

Metabolic parameters are represented as mean ± SEM of values at 4 weeks of diet for 6 rats in the LS and LSB groups, 12 rats in HS and 16 rats in HSB group. At 9 weeks, data are represented as mean ± SEM of values from 6 rats in the LS and LSB groups, from 8 rats in HS group and 12 rats in HSB group. **p < 0.01, ***p < 0.001 versus LS; $^{\$\$}$ p < 0.01, $^{\$\$\$}$ p < 0.001 vs LS Berries; #p < 0.05 vs HS.
LS – Low Salt diet; LSB – Low Salt diet supplemented with a berry mixture; HS – High Salt diet; HSB – High Salt diet supplemented with a berry mixture.

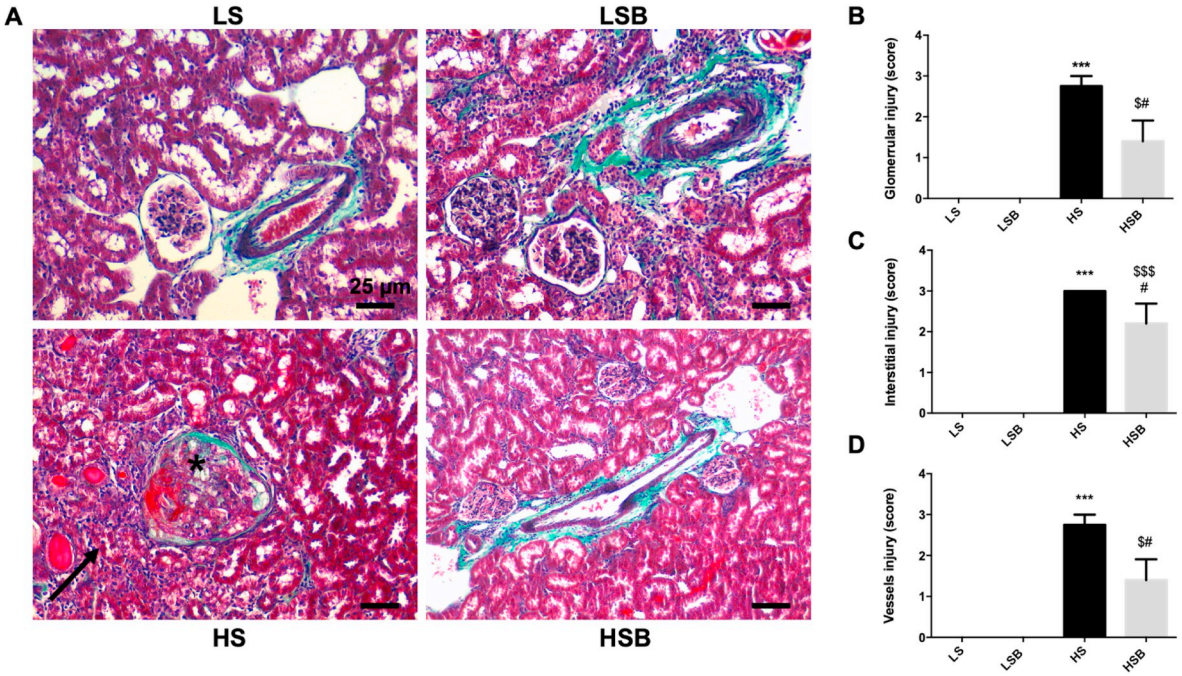

Fig. 2. Histomorphological analysis of Masson stained rats' kidney. (A) Light microscopy images of kidney stained with Masson's trichrome (200×, original magnification). Representative images are presented for each group, * indicates glomerulosclerosis and the arrow indicates inflammatory cell infiltration; (B) Glomerular injury score; (C) Interstitial injury and (D) Vessels injury score. Glomerular, interstitial and vessels lesions severity was graded according to the percentage of total glomeruli, interstitial and vessels injury involved as follows: stage 0, no lesions; stage 1, 1–25% injury; stage 2, 26–50% injury; and stage 3, 51–100% injury (all samples were blindly evaluated by a nephrologist).heat-map representing the severity of the legions: interstitial infiltrate, glomerular and vessels injuries; (C) total injuries score. N = 5 rats/group. ***p < 0.001 vs LS, $p < 0.05 and $$$p < 0.001 vs LSB, #p < 0.05 vs HS. LS, Low-salt diet; LSB, LS diet supplemented with a berry mixture; HS, high salt diet; HSB, HS diet supplemented with the berry mixture.

using database assisted spectral deconvolution. The clear separation between the LS and HS groups suggests different metabolic states, pointing to a marked alteration of urinary metabolites that could be related with the observed kidney injuries. When berries are added to the low salt diet (LSB) the change in the metabolic profile from the regular low salt samples (LS) is marginal since they overlap regions in PCA analysis. Interestingly when berries are added to the diet of the animals already with high salt (HSB group), the metabolic signature changes in a much more dramatic manner. The introduction of berries in the high salt diet seems to disrupt the clear separation between LS and HS creating an intermediate group.

Subsequent PLS-DA analysis corroborates the group separation. There is a clear separation between LS and HS samples independently of the presence of the berries (Fig. 3B) that is supported by the statistical robustness of the analysis. Our model scored high $R^2 = 0.94$ and $Q^2 = 0.49$, confirming good predictive power (Fig. 3C).

Key differentiating features (i.e. spectral bins) identified by PLS-DA analysis were sorted by increasing VIP value score (Fig. 4A, the top 30 bins with VIP > 1 are presented). Considering VIP value > 1 as cut-off, 57 features (spectral bins) were found to be significant discriminators between the 4 groups. A heatmap of relative intensity changes potentially differentiating NMR signals (from PLS-DA/VIP analysis) was then composed to visualize possible clustering (Fig. 4B). Indeed, a clear pattern emerges that shows the clustering of the samples according to the salt and or berries presence in the diets.

After this initial evaluation, we performed ANOVA followed by Fisher's LSD test ($p < 0.05$) and we were able to identify the statistically significant bins between all the groups (Supplementary Figure S2). Fisher's LSD test classified 78 spectral bins as statistically significant ($p < 0.05$; Supplementary Table S3). Correlation analysis, including the p values from the Fisher's test and the false discovery rates (FDR) is consistent with the results obtained from multivariate analysis (PLS-DA/VIP analysis), meaning that both methods identified almost the same significantly changing bins between the 4 groups.

3.4.2. Urinary metabolites identification

For a biochemical interpretation of the observed spectral differences, we attempt to assign the bins to their respective metabolites. For that, each bin was represented by its peak chemical shift value that was then related to a metabolite using the internal reference library (Version 10) from the Chenomx NMR Suite 8.12 software (Chenomx company, Edmonton, Canada).

From the statistically significant spectra bins obtained by the Fisher's test, 37 metabolites were unequivocal assigned (supplementary table S3). Almost 36% of the statistically significant spectral bins were assigned to different metabolites that include amino acids, organic acids, sugars, among others. However, a significant number of NMR signal regions could not be unequivocally assigned due to the signal overlap and/or low sensitivity. For the top 10 significantly altered metabolites ($p < 0.05$) the relative concentrations in urine of rats from all the 4 groups are presented (Fig. 5).

3.4.3. Pathway analysis

Pathway analysis was first performed for all metabolites significantly altered within all groups and with VIP values higher than 1. Significantly altered pathways ($p < 0.05$) that had also high impact values include butanoate metabolism, citrate cycle, alanine, aspartate and glutamate metabolism, synthesis and degradation of ketone bodies, D-glutamine and D-glutamate metabolism, glycolysis or gluconeogenesis metabolism and nicotinate and nicotinamide metabolism (Table 3). Pathway significance is determined from pathway enrichment analysis and based on each compound in the data set. The impact value, on the other hand, is determined by pathway topology analysis. Impact is determined based upon the importance of a metabolite within a pathway; a metabolite that is found at a junction point within a pathway may have a greater impact on the pathway function if the level

Table 2
(Poly)phenols metabolites in kidneys of the rats fed with a LS, LSB, HS and HSB at 9 weeks of trial.

(Poly)phenols metabolites in kidney (nmol/g fresh tissue)				
	LS	LSB	HS	HSB
Benzoic acid derivatives				
Vanillic acid sulfate	0.4 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	1.8 ± 1.1
Hydroxybenzoic acid[1]	0.9 ± 0.1	1.1 ± 0.2	1.2 ± 0.2	1.6 ± 0.2
Hydroxybenzoic acid sulfate[1]	0.6 ± 0.1	1.3 ± 0.3	1.0 ± 0.1	4.8 ± 0.6$^{###}$
4-*O*-methylgallic acid	n.d	0.3 ± 0.2	n.d	0.7 ± 0.1
4-*O*-methylgallic acid sulfate	n.d	0.7 ± 0.4	n.d	0.7 ± 0.1
Protocatechuic acid sulfate[1]	3.0 ± 1.3	0.2 ± 0.2	19.2 ± 4.0	17.0 ± 6.6
Syringic acid	n.d	n.d	n.d	1.2 ± 0.1
Syringic acid sulfate	n.d	0.4 ± 0.2	n.d	1.3 ± 0.1
Cinnamic acid derivatives				
Coumaric acid[1]	n.d	0.3 ± 0.2	n.d	0.2 ± 0.1
Coumaric acid sulfate[1]	0.4 ± 0.1	1.6 ± 0.4	1.6 ± 0.3	7.3 ± 1.3$^{##}$
Coumaric acid glucuronide[1]	n.d	n.d	n.d	0.1 ± 0.01
Caffeic acid sulfate[1]	n.d	0.4 ± 0.2	1.0 ± 0.2	2.6 ± 0.3 $^{\$\$\#\#}$
Caffeic acid glucuronide[1]	n.d	0.2 ± 0.1	n.d	0.1 ± 0.1
Dihydrocaffeic acid	n.d	n.d	n.d	0.4 ± 0.3
Dihydrocaffeic acid sulfate	0.7 ± 0.3	0.5 ± 0.3	1.6 ± 0.4	3.3 ± 0.4 $^{\$\$\#}$
Ferulic acid	n.d	n.d	0.5 ± 0.1	0.8 ± 0.1
Ferulic acid sulfate	n.d	0.2 ± 0.03	n.d	0.9 ± 0.1
Dihydroferulic acid sulfate	n.d	n.d	n.d	0.2 ± 0.1
Dihydroferulic acid glucuronide	0.1 ± 0.03	0.07 ± 0.4	0.4 ± 0.1	0.5 ± 0.3
Propionic acid derivatives				
3-(Phenyl)propionic acid	0.4 ± 0.1	0.4 ± 0.02	1.1 ± 0.1	1.3 ± 0.1 $^{\$\$}$
3-(hydroxyphenyl) propionic acid[1]	1.0 ± 0.7	5.2 ± 2.9	0.9 ± 0.2	1.6 ± 0.2
(Hydroxyphenyl)propionic acid sulfate[1]	0.6 ± 0.1	6.7 ± 2.1	5.8 ± 0.9	33.0 ± 5.0$^{##}$
Hippuric acid derivatives				
Hippuric acid	3.3 ± 0.7	11.7 ± 1.3	9.9 ± 1.6	46.3 ± 10.0$^{##}$
Phenylacetic acid derivatives				
2-(Phenyl)acetic acid	0.8 ± 0.1	1.6 ± 0.2 ***	2.9 ± 0.3	4.6 ± 1.4
2-(Hydroxyphenyl)acetic acid	0.9 ± 0.1	1.2 ± 0.3 **	2.9 ± 0.6	2.9 ± 0.2
2-(Trihydroxyphenyl)acetic acid[1]	n.d	0.2 ± 0.1	n.d	0.4 ± 0.2
Catechol derivatives				
Catechol	n.d	n.d	n.d	0.4 ± 0.1
Catechol sulfate	1.1 ± 0.1	1.7 ± 0.020	0.8 ± 0.05	1.4 ± 0.1$^{##}$
4-methyl catechol sulfate[1]	0.5 ± 0.05	0.4 ± 0.016	0.7 ± 0.08	0.8 ± 0.07 $^{\$}$
Hydroxytyrosol	0.9 ± 0.4	n.d	2.4 ± 0.6	2.9 ± 1.4
Pyrogallol derivatives				
Pyrogallol sulfate[1]	n.d	n.d	n.d	0.118 ± 0.0274
2-methyl pyrogallol sulfate	n.d	n.d	n.d	0.176 ± 0.0344
Flavanols derivatives				
Methylepicatechin sulfate[1]	n.d	n.d	n.d	0.232 ± 0.158
Epicatechin glucuronide[1]	n.d	n.d	n.d	0.2 ± 0.1
Methylepicatechin glucuronide[1]	n.d	n.d	n.d	0.4 ± 0.2
Catechin glucuronide[1]	n.d	n.d	n.d	0.4 ± 0.1
Methylcatechin sulfate[1]	n.d	n.d	n.d	0.9 ± 0.2
Methylcatechin glucuronide[1]	n.d	1.1 ± 0.1	0.4 ± 0.2	2 ± 0.3$^{###}$
Valeric acid derivatives				
5-(Hydroxyphenyl)valeric acid[1]	n.d	n.d	n.d	0.2 ± 0.08
Valerolactone derivatives				
5-(Dihydroxyphenyl)-δ-valerolactone[1]	n.d	n.d	n.d	0.1 ± 0.09
5-(Dihydroxyphenyl)-δ-valerolactone sulfate[1]	n.d	0.08 ± 0.05	n.d	0.3 ± 0.1

(Poly)phenols metabolites determined in kidney are represented as mean ± SEM of values at 9 weeks of diet for 6 rats in the LS and LSB groups, from 8 rats in HS group and 12 rats in HSB group. **p < 0.01, ***p < 0.001 versus LS; $$ p < 0.01, $$$ p < 0.001 vs LS Berries; #p < 0.05, ##p < 0.01, ###p < 0.001 vs HS. [1]It is possible to have more than one isomer for this compound, however since it was not confirmed by standards we cannot attribute a specific identity due to the same MS/MS fragmentation profile; n.d.: not detected; LS – Low Salt diet; LSB – Low Salt diet supplemented with a berry mixture; HS – High Salt diet; HSB – High Salt diet supplemented with a berry mixture.

is altered.

In Table 3 the results from the pathway analysis are presented. Taurine and hipotaurine metabolism result as a pathway with a high impact value, although its p value was not significant. The impact value for this pathway was high because taurine is located at a junction within the pathway. On the other hand, aminoacyl-tRNA biosynthesis pathway presented a significant p value but not a high impact value. The pathway with both significant p value and high impact was D-glutamine and D-glutamate metabolism (Table 3).

When looking in detail for the pathways affected between LS and HS (Fig. 6A), results revealed that feeding a high salt diet for 9 weeks induced significant metabolic alterations ($p < 0.05$) and also with impact in metabolites from pathways such as glycolysis or gluconeogenesis (namely ethanol, lactate and acetate), citrate cycle (citrate and succinate), and pyruvate metabolism (acetate and lactate), resulting in the increase of their relative concentrations in rats urine. Regarding taurine and hipotaurine metabolism, and glyoxylate and dicarboxylate metabolism despite being not significant ($p > 0.05$) had a high impact value, since taurine is a central metabolite in this pathway. The presence of salt in the diet decreases the relative concentration of taurine found in urine (Fig. 6E. When we compare LS and LSB (Fig. 6B) in order to understand the effect of the berries in the metabolism, we identify 5 metabolic pathways with significance of which 2 were more affected by berry mixture supplementation, namely butanoate metabolism and

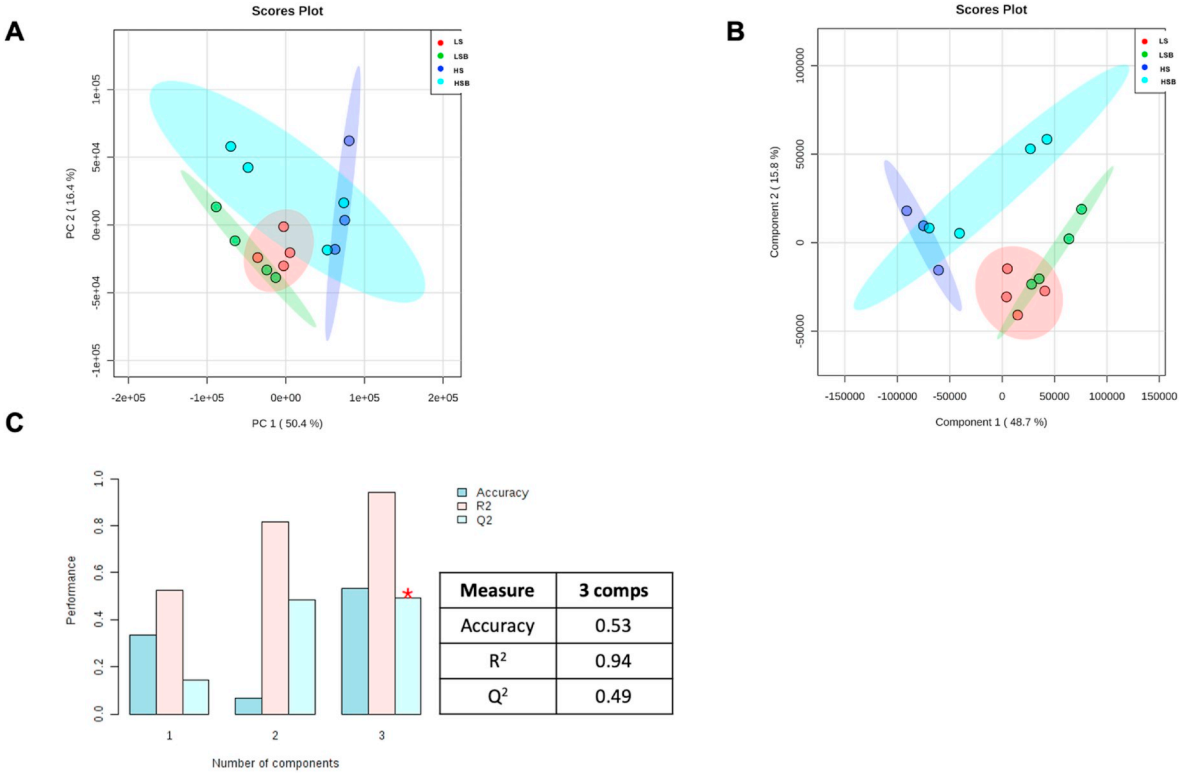

Fig. 3. Multivariate analysis for all sample groups. (A) Two-dimensional PCA score plot of all four groups at week 9 of diet intervention. (B) Two-dimensional PLS-DA score plot of all four groups at week 9 of diet intervention. (C) PLS-DA classification using different numbers of components. The red asterisk indicates the best classifier. The inset table summarizes Q^2, R^2 and accuracy of the best model. Comps means number of components. LS, Low-salt diet; LSB, LS diet supplemented with a berry mixture; HS, high salt diet; HSB, HS diet supplemented with the berry mixture.

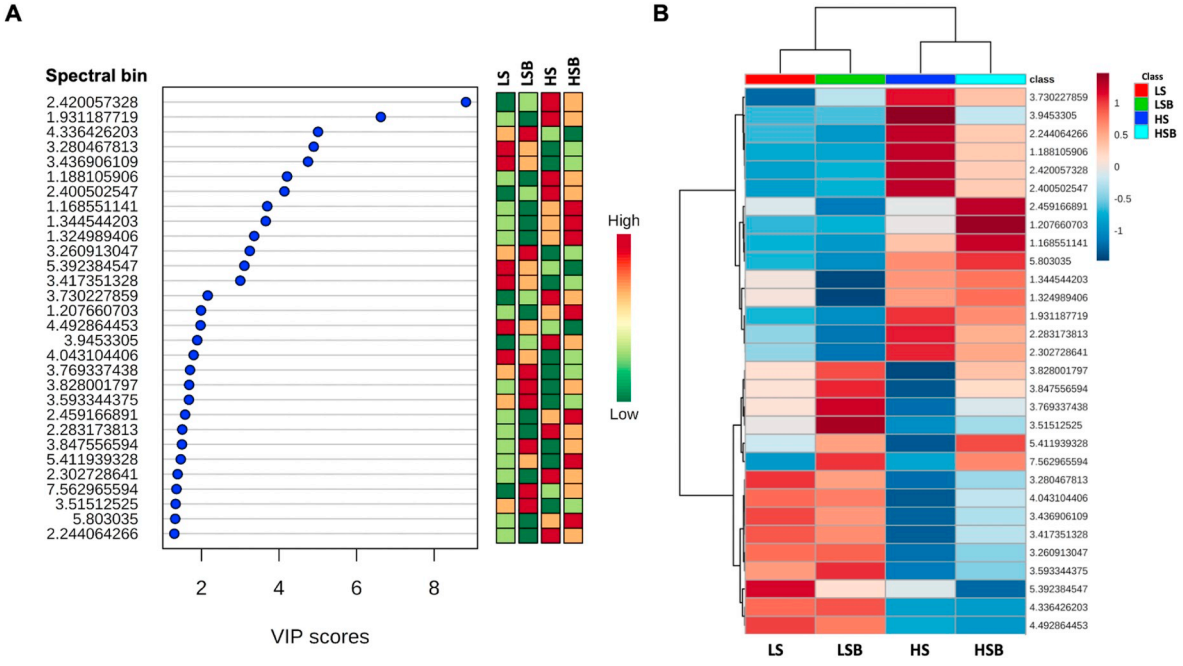

Fig. 4. Significant features (spectral bins) discriminating between all sample groups at week 9 of diet intervention. (A) Important features identified by PLS-DA and VIP scores. Only spectral bins with a significant VIP value > 1 are included as recommended by the literature. The coloured boxes on the right indicate relative bin integral for all 4 samples. Variable Importance in Projection is a weighted sum of squares of the PLS loadings taking into account the amount of explained Y-variation in each dimension. (B) Heatmap of unsupervised hierarchical clustering. The heatmap was constructed from the most significantly differing bins (features), as identified by PLS-DA and VIP scores. Each coloured cell on the map corresponds to a relative concentration value. Red and blue colour denote increased and decreased bin integrals, respectively. LS, Low-salt diet; LSB, LS diet supplemented with a berry mixture; HS, high salt diet; HSB, HS diet supplemented with the berry mixture.

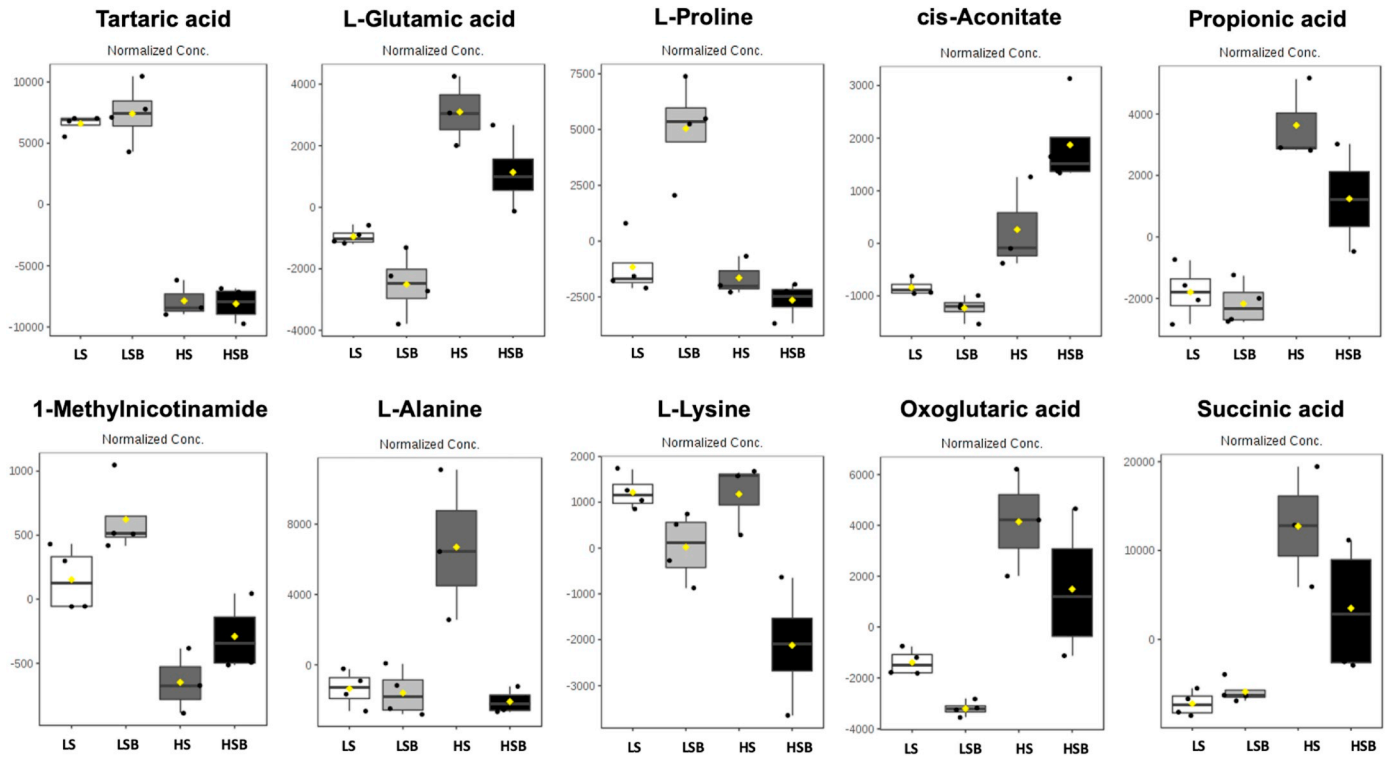

Fig. 5. Boxplot of relative concentrations for the top 10 significantly altered metabolites ($p < 0.05$) in urine of rats from all the 4 groups. The Y-axis scale is relative. Data were normalized to a pooled sample from the LS group. Due to this normalization process we obtained negative scale in the Y-axis on some of the bins (analysis performed by the online program MetaboAnalyst). The bar plots show the normalized values (mean ± standard deviation). The box ranges from 25% and the 75% percentiles; the 5% and 95% percentiles are indicated as error bars; single data points are indicated by black dots. Medians are indicated by horizontal lines within each box. LS, Low-salt diet; LSB, LS diet supplemented with a berry mixture; HS, high salt diet; HSB, HS diet supplemented with the berry mixture.

citrate cycle, since relatives concentrations of metabolites such as succinate and citrate are higher in urine of LSB rats than in LS rats. Metabolites such as lactate, acetate and acetoacetate that belong to pathways like pyruvate metabolism, glycolysis and gluconeogenesis, and synthesis and degradation of ketone body's metabolism were in lower amounts in urine of rats that consumed the LS diet supplemented with berries (LSB).

Regarding the effects of the berries in the metabolism of rats fed with a high salt diet, 3 metabolic pathways were affected such as citrate cycle, alanine, aspartate and glutamate metabolism and also taurine and hypotaurine metabolism (Fig. 6C). Concerning specific metabolite changes between these two groups, HS diet increased creatinine concentration in urine, while supplementation with the berries decreased it. By contrast, hippurate concentration increased in urine due to the berries and in less amount in urine of HS rats.

In order to understand which metabolic pathways were affected by a high salt content in diets supplemented with berries, we compared LSB and HSB rats' urine (Fig. 6D). The metabolic pathways significantly different and with more impact were related to pyruvate metabolism, starch and sucrose metabolism, glycolysis or gluconeogenesis and galactose metabolism. Starch and sucrose metabolism, and galactose metabolism were affected due to the presence of high amounts of salt in the diet, by decreasing the relative concentration of compounds (sucrose and glucose) implicated in these pathways.

3.4.4. Berry-enriched diet modulate cysteine catabolism in hypertensive rats

Some of the compounds identified by ^{1}H NMR belong to metabolic pathways related to carbon and energy metabolism, and they can result from the catabolism of cysteine. Moreover, we have previously described changes in protein cysteinylation in kidney of an animal model of hypertension and insulin resistance without histologic alterations in kidney [30]. Changes in cysteine availability and variations in cysteine dynamics were also address in the present work. A simplified metabolic flow chart illustrating the pathways of cysteine metabolism evaluated in this work are represented in Fig. 7A. Cysteine dioxygenase (CDO) catalyzes the first step in the major cysteine catabolic pathway, and shunts cysteine toward the production of pyruvate and taurine. Other two products of cysteine metabolism are glutathione (GSH) and hydrogen suphide (H_2S).

Our results showed that, while salt-induced hypertension did not increase cysteine total availability (Fig. 7B) (reduced + free oxidized + protein bound), there was a decrease in cysteine total availability in HSB group (Fig. 7B). In fact, in this group the total availability of the precursor Cysteinylglycine (CysGly, glutathione catabolism product and source of cysteine) was decreased (Fig. 7C) as well as total availability of glutathione (Fig. 7D). Regarding to the cysteine fraction bound to protein (CysSSP) it is increased in HS group and berries supplementation to this group decrease it (Fig. 7E).

4. Discussion

Berries or pure (poly)phenols-enriched diets have (direct or systemic) cardioprotective effects in various cardiovascular preclinical models as well as in humans [31–33]. We recently showed the beneficial effects of berry-(poly)phenol enriched diet ingestion in the chronic model of hypertension-induced heart failure, the Dahl-salt sensitive rats [4]. The berries mixture provided to the hypertensive rats prolonged their lifespan, maintained their body weight, ameliorated systolic blood pressure (SPB) and cardiac function, and decreased cardiac and renal hypertrophy [4]. Moreover, for the same animals, we demonstrated that the high salt diet, responsible for the development of hypertension in the rats influenced the excretion of (poly)phenol metabolites in urine and feces of the Dahl-salt sensitive rats as well as modulated the microbiota composition [5]. In the present work, we

Table 3
Pathway analysis performed for all metabolites significantly altered within all groups and with VIP values higher than 1 using the online software MetaboAnalyst.

Pathways	Total[a]	Hits[b]	*p* value	FDR[c]	Impact[d]
Butanoate metabolism	20	5	8.560E-05	0.007	0.101
Citrate cycle (TCA cycle)	20	4	0.001	0.048	0.173
Alanine, aspartate and glutamate metabolism	24	4	0.002	0.066	0.323
Synthesis and degradation of ketone bodies	5	2	0.005	0.094	0.600
D-Glutamine and D-glutamate metabolism	5	2	0.006	0.094	1.000
Aminoacyl-tRNA biosynthesis	67	5	0.023	0.289	0
Glycolysis or Gluconeogenesis	26	3	0.025	0.289	0.029
Nicotinate and nicotinamide metabolism	13	2	0.040	0.402	0.363
Glyoxylate and dicarboxylate metabolism	16	2	0.058	0.525	0.148
Propanoate metabolism	20	2	0.087	0.686	0
Arginine and proline metabolism	44	3	0.094	0.686	0.169
Pyruvate metabolism	22	2	0.102	0.686	0.055
Starch and sucrose metabolism	23	2	0.110	0.686	0.0378
Biotin metabolism	5	1	0.118	0.686	0
Glutathione metabolism	26	2	0.136	0.686	0.055
Galactose metabolism	26	2	0.136	0.686	0.077
Taurine and hypotaurine metabolism	8	1	0.184	0.850	0.429
Glycine, serine and threonine metabolism	32	2	0.189	0.850	0
Nitrogen metabolism	9	1	0.204	0.870	0
Histidine metabolism	15	1	0.317	1.000	0
Selenoamino acid metabolism	15	1	0.317	1.000	0
beta-Alanine metabolism	19	1	0.383	1.000	0
Porphyrin and chlorophyll metabolism	27	1	0.498	1.000	0
Valine, leucine and isoleucine degradation	38	1	0.622	1.000	0
Pyrimidine metabolism	41	1	0.651	1.000	0.021
Tyrosine metabolism	42	1	0.660	1.000	0
Primary bile acid biosynthesis	46	1	0.693	1.000	0.030

[a] Total – Total of compounds from the pathway.
[b] Hits – Number of metabolites identified in that pathway.
[c] FDR- False Discovery Rate.
[d] Impact - importance of a metabolite within a pathway; a metabolite that is found at a junction point within a pathway may have a greater impact on the pathway function if the level is altered.

identified unexpected differences in the retention of (poly)phenol metabolites in kidney and in the metabolic profiles of urine. Moreover, we evaluated histological alterations of kidney tissues from Dahl-salt sensitive rats either in a LS and HS diets supplemented with berries, to better understand the metabolic changes that occur in the end of the trial on the different diets. Since, many phenotypic traits that are common to hypertension in man (including sodium sensitivity of hypertension, reduced renal function, elevated urinary excretion of protein and albumin, and a low plasma renin), are described to be present in the Dahl-salt sensitive rats [34], we herein addressed the systemic effects of a berry-enriched diet on kidney that could indirectly impact heart function.

Dahl-salt sensitive rats developed renal injury after 9 weeks on a high salt diet (HS), in contrast to the rats on low salt diets supplemented or not with the berry mixture. Remarkably, on rats fed a high salt diet supplemented with berries (HSB), there was a lower extent in the severity of the kidney injuries than the lesions observed in the HS kidneys rats (Fig. 2). The renal injury consisted of mild vascular injury with some features of glomerular ischemia, glomerulosclerosis and tubulointerstitial changes with accumulation of inflammatory cells in the interstitial space [35]. Both glomerular and tubulointerstitial injuries were detected in rats with a high salt diet (HS) and were ameliorated in some extent in rats that consumed berries (HSB). Moreover, we previously showed that these rats fed with an HSB diet presented an improvement in the salt-induced cardiac dysfunction. In addition, beneficial effects of berries on kidney were observed, with a decrease in kidney weight and improved function in comparison with the rats in an HS diet as shown by differences in terms of creatinuria and renal clearance [4]. Thus, cardioprotective effects of the berry mixture may be due to their systemic effects on other organs targeted by hypertension, such as the kidney that could impact indirectly on the heart.

Beside the decreased severity of the lesions promoted by berries in kidneys of rats in a HS diet (Fig. 2)we observed a differential retention of some (poly)phenol metabolites in HSB rat kidneys, probably as consequence of functional differences between the kidneys of HS and HSB. In fact, as previously described we observed low creatinuria values in urine of HS rats, that was almost recovered to control levels (LS) when rats were fed with the diet supplemented with berries [4].

In the present study, the metabolic profiles in kidney tissues revealed that 14 (poly)phenol metabolites were accumulated exclusively in HSB rats' kidneys (Table 2). However, whether the degree of renal injuries induced by salt, namely the malfunctioning of the renal transporters promoted (poly)phenols retention in Dahl-salt sensitive rats remains unclear.

Studies suggest that kidney is a critical organ for the regulation of salt homeostasis and also to be responsible for the regulation of drug transporters, CYP-metabolizing enzymes and nuclear receptors Moreover, a decreased expression of the drug transporter Mdr1b was observed in rat kidney fed a high salt diet [36]. This fact was associated with the decreased level of aldosterone secretion or angiotensin II promoted by a high-salt diet [37]. One of the kidney transporters involved in the efflux of hydrophilic compounds, such as (poly)phenols, is the P-glycoprotein (MDR1/ABCB1) [38]. Since studies demonstrate that its expression in kidneys was decreased due to the high salt diet, the retention of (poly)phenol metabolites observed in our study, may be due to a lower Mdr1b expression. Moreover, the epithelial Na^+ channel (ENaC) in the kidney plays a key role in the regulation of blood pressure by contributing to the Na^+ reabsorption in renal tubules. In fact, in a study performed by Aoi W and co-authors they demonstrated that dietary quercetin supplementation reduces the blood pressure elevated by HS diet in Dahl-salt sensitive rats, and that this is associated with αENaC expression in the kidney [39]. These observations suggest that one of the mechanisms of the flavonoid's antihypertensive effect would be mediated through the downregulation of αENaC expression in the kidney [39], however, more studies should be done to attribute this beneficial effect to the (poly)phenols present in our berry mixture and in particular to the biological (poly)phenol metabolites accumulated in this organ.

The metabolic alterations detected in the urine analysis by ^1H NMR revealed that feeding rats with a high salt diet (HS) for 9 weeks induced significant increase of glycolysis, citrate cycle, pyruvate metabolism, alanine, aspartate and glutamate metabolism, aminoacyl-tRNA biosynthesis, and glycine, serine and threonine metabolism. Wang Le. and co-workers also demonstrated that some of these metabolic pathways were altered in kidneys of the Dahl-salt sensitive rats [40], namely five citrate cycle metabolites and pyruvate, which connects citrate cycle and glycolysis, were increased in the plasma of the animals. Their results demonstrate that abnormalities of citrate cycle in Dahl/SS rats may play a role in predisposing these rats to developing salt-sensitive hypertension. Similarly, our results showed an increase in the glycolysis and gluconeogenesis metabolites, citrate metabolism, namely citrate and succinate, and also in pyruvate metabolism in the urine of HS rats compared to rats in a LS diet. Moreover, a recent study also demonstrated that high salt diets had an impact in metabolic pathways in the kidney that were associated with high blood pressure in Dahl/SS rats [41]. Regarding the effects of the berries in the metabolism of rats fed with a high salt diet, 3 metabolic pathways were affected, such as citrate cycle, alanine, aspartate and glutamate metabolism and also taurine and hypotaurine metabolism. The diet supplementation with

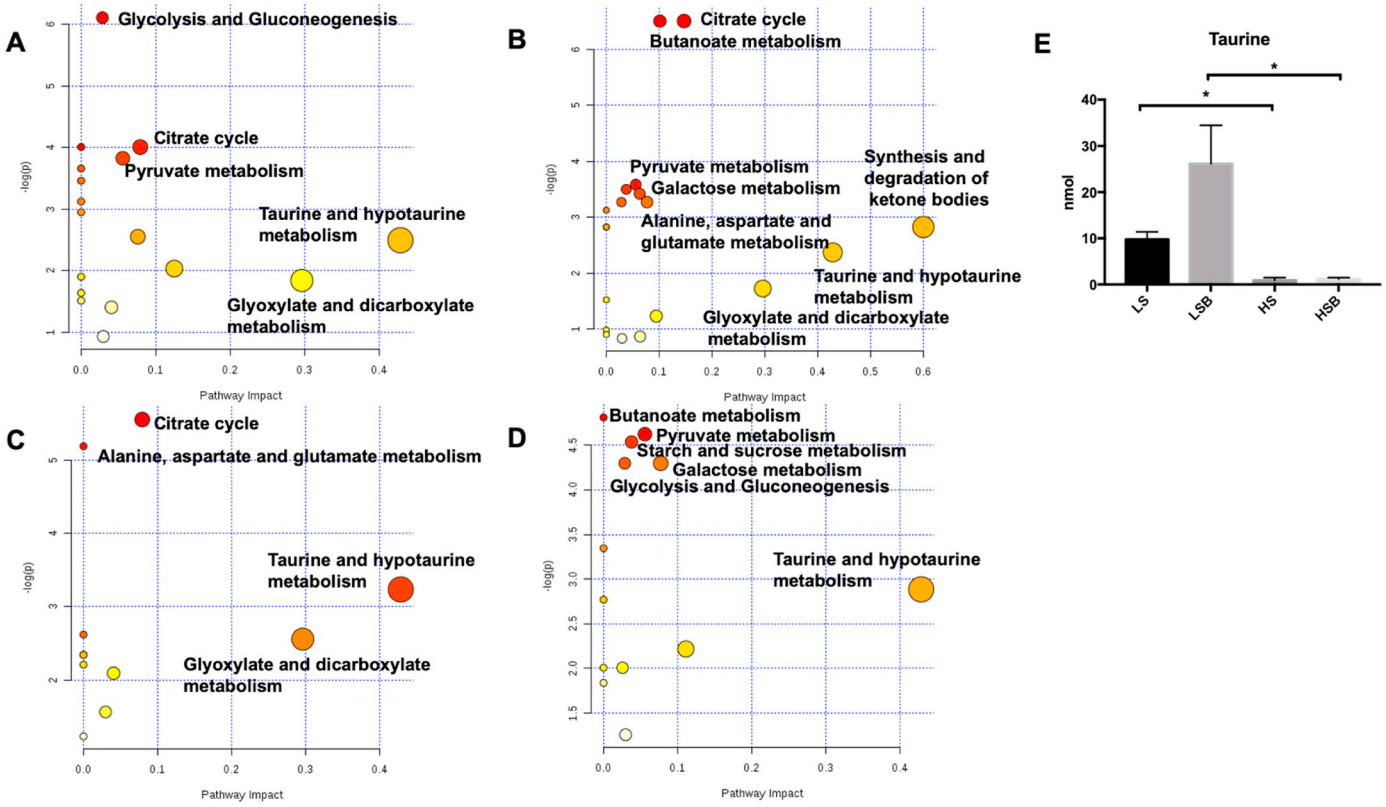

Figure 6. Metabolome view from pathway analysis performed using MetaboAnalyst (Select pathways with high impact and/or high p value). The graphics contain all the matched pathways arranged by p values (from pathway enrichment analysis) on Y-axis, and pathway impact values (from pathway topology analysis) on X-axis. The node colour is based on its p value and the node radius is determined based on their pathway impact values. The pathways that were most significantly changed are characterized by both a high-log(p) value and high impact value (top right region of the graphic). Pathway analysis results from the effects of (A) the high salt diet: LS vs HS, (B) the berries mixture: LS vs LSB, (C) the berries mixture in a high salt diet: HS vs HSB, and (D) the presence of salt in the berries mixture: LSB vs HSB. (E) Taurine ^{1}H NMR quantification (nmol) in urine at 9 weeks.

berries decreased some metabolites such as creatinine, succinic acid, citrate and alanine, which were increased due to HS diet.

He et al., demonstrate that succinic acid increases blood pressure in animals, and that the hypertensive effect of succinic acid is mediated by GPR91 through the activation of the renin–angiotensin system [42]. Moreover, in a clinical study, the measurement of estimated glomerular filtration rate (eGFR), which is considered the best available indicator of the overall function of the kidneys, was evaluated in normotensive and hypertensive patients and related with alterations in plasma free amino acids and uric acid. Alanine presented a significant positive association with reduced eGFR exclusively for the hypertension group [43]. Citrate is also a metabolic substrate in the kidneys and was also altered in urine of HS fed. Moreover urinary citrate levels were also found increased in patients with resistant hypertension [44] Thus, our study, which measured berries effect on the metabolism of hypertensive rats revealed that a (poly)phenol enriched diet had an impact in multiple metabolic pathways which dysfunction is associated with hypertension.

Some of the compounds identified in this study belong to metabolic pathways related to carbon and energy metabolism and they can also result from the catabolism of cysteine. We observe that a high salt diet did not increase cysteine total availability (Fig. 7B) (reduced + free oxidized + protein bound) but increased CysSSP (Fig. 7E), however berries promoted a decrease in cysteine total availability, CysGly glutathione and CysSSP. This might indicate that there is a metabolic switch for cysteine catabolism (Fig. 7A). It is known that the catabolism of cysteine might include the activity of cysteine dioxygenase (CDO) with production of pyruvate and taurine (Fig. 7A) [45].

In fact, pyruvate is the key initiator of the citrate cycle, one of the most significant altered pathways by berries (Fig. 6B and C). Moreover, from the top significant altered spectral bins in urine of rats, two were assigned as citrate cycle metabolites (succinic acid and oxoglutaric acid, Fig. 5), and their relative concentrations are lower in urine of hypertensive rats fed with a diet supplemented with berries. Another assigned metabolite, also with lower relative concentrations in the HSB rats urine was glutamic acid. It can be converted to oxoglutaric acid, again one of the metabolites involved in the citrate cycle. The fact that glutamic acid levels are lower could also interfere with the glutamyl cycle, which is also involved in the synthesis of cysteine.

The other byproduct from CDO activity is taurine. However, CDO modulation by berries might not be the case as taurine levels were lower in urine of this animals (Fig. 6E). Although phenolic compounds are described as natural products modulators of the nuclear factor erythroid 2-related factor 2 (Nrf2), a redox sensitive transcription factor that controls CDO1 [46,47], overall our data suggest that cysteine catabolism was not through CDO activity.

Moreover, glutamylcysteine ligase (GCL), previously known as gamma-glutamylcysteine synthetase (GCS) competes with CDO for cysteine, it controls the availability of cysteine, GSH and H_2S production [45]. However, we have a decrease in total GSH and CysGly when rats diet was supplemented with berries suggesting that cysteine is not being used for GSH metabolism.

Besides taurine and pyruvate, the degradation of cysteine generates H_2S (Fig. 7A) and, the conversion by H_2S producing enzymes (cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE)) are well documented as downregulated in kidney disease [48]. Given the cytoprotective, antioxidant and anti-inflammatory effects of H_2S an increase in the synthesis of H_2S can be one of the benefits promoted by berries

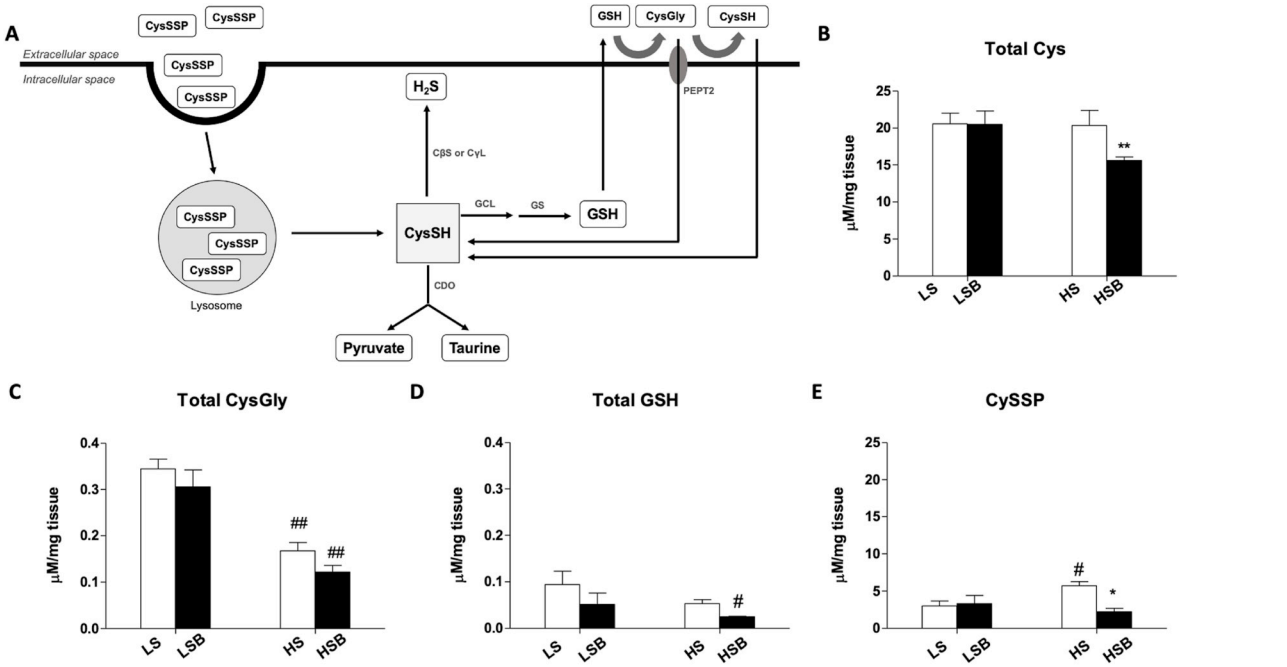

Figure 7. Cysteine dynamics in kidney. (A) A simplified metabolic flow chart illustrating the pathways of cysteine metabolism. Cysteine dioxygenase (CDO) catalyzes the first step in the major cysteine catabolic pathway, and shunts cysteine toward the production of pyruvate and taurine. Other two products of cysteine metabolism are glutathione (GSH) and hydrogen sulphide (H2S). For purposes of clarity, multistep pathways of cysteine metabolism have been condensed to single arrows. CβS, cystathionine-β-synthase; CγL, cystathionine-γ-lyase; GCL, glutamylcysteine lyase; GS, glutathione synthase; (B) Total content of cysteine in kidney; (C) Total available cysteinylglycine; (D) Total available glutathione (GSH); (E) Total S-cysteinylated (CysSSP) proteins. From 7B-2E, statistics using two-way ANOVA with Bonferroni post-test indicates *p < 0.05 and **p < 0.01 HS vs. HSB. #p < 0.05 and # < 0.01 LS vs. LSB and LSB vs. HSB.

compounds. Additionally, berries supplementation in the hypertensive rats (HSB) showed a reversion in the increased protein cysteinylation (CysSSP) levels observed in the HS fed rats (Fig. 7E) that also might involve CBS activity [49]. Interestingly, this observation is opposite to the one observed in a model of hypertension without renal impairment where low protein cysteinylation in kidney was observed [30], that might indicate an inverse relationship with protein cysteinylation at the kidney function.

Another cysteine source is homocysteine that is a precursor of cysteine upon irreversible vitamin B6 -dependent transsulfuration pathway, through CBS activity that might be decreased in kidney disease, as mentioned above [48]. Kidney is a relevant organ for homocysteine excretion and Cheng et al. (2018) have demonstrated that homocysteine was the only metabolite showing a significant association with differences in blood pressure, between low and high sodium intakes by participants of the DASH-Sodium trial, which suggests that homocysteine might have a particularly important relationship with changes in sodium intake that warrants further investigation [50]. We might hypothesize that berries might be facilitating the metabolism of homocysteine into cysteine, and cysteine further catabolism through an effect on CBS or increasing its cofactors. Accordingly, Cheng et al. (2018) showed that together with homocysteine, also urinary cystine was increased and associated with both SBP and DPB. This increased in urinary cystine might also indicate a lower capability tubular reabsorption by an impairment of one (more) of its different transporters at tubular apical membrane [15], and/or a higher increase in cystine in plasma which is an independent factor for cardiovascular morbidity [51].

The presented results suggest that berries promoted an increase in the metabolic use of cysteine by the organism, that is reflected by its decreased levels, as a strategy to better cope with kidney dysfunction observed in the hypertensive rats.

5. Conclusions

Our study demonstrates the effects of a (poly)phenol enriched diet in multiple metabolic pathways in urine and kidney associated with hypertension, namely by impacting cysteine dynamics, already described as antihypertensive. These findings support the efficacy of phytochemical-enriched diets against hypertension-associated kidney pathology. This association may have particular importance to our aging population, which has reduced intake of both fruit and vegetables.

In our work we used a dietary approach that was able to promote an increase in the metabolic use of cysteine. It have been already described that early cysteine supplementation can prevent the kidney damage associated to high salt intake [52]. By giving a (poly)phenol enriched diet we were able to demonstrate the same potential. This data opens the avenue for further exploitation of the use of a dietary intervention as a tool for the prevention of kidney disease.

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Declaration of competing interest

The authors have declared no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.freeradbiomed.2020.05.002.

References

[1] M. Ezzati, E. Riboli, Behavioral and dietary risk factors for noncommunicable diseases, N. Engl. J. Med. 369 (2013) 954–964, https://doi.org/10.1056/NEJMra1203528.
[2] X. Wang, Y. Ouyang, J. Liu, M. Zhu, G. Zhao, W. Bao, et al., Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies, BMJ 349 (2014) g4490, https://doi.org/10.1136/bmj.g4490.
[3] F. Vargas, P. Romecín, A.I. García-Guillén, R. Wangesteen, P. Vargas-Tendero, M.D. Paredes, et al., Flavonoids in kidney health and disease, Front. Physiol. 9 (2018) 394, https://doi.org/10.3389/fphys.2018.00394.
[4] C. Oudot, A. Gomes, V. Nicolas, M. Le Gall, P. Chaffey, C. Broussard, et al., CSRP3 mediates polyphenols-induced cardioprotection in hypertension, J. Nutr. Biochem. 66 (2019) 29–42, https://doi.org/10.1016/j.jnutbio.2019.01.001.
[5] A. Gomes, C. Oudot, A. Macia, A. Foito, D. Carregosa, D. Stewart, et al., Berry-enriched diet in salt-sensitive hypertensive rats: metabolic fate of (poly)phenols and the role of gut microbiota, Nutrients 11 (2019) 2634, https://doi.org/10.3390/nu11112634.
[6] Y.-Y. Zhao, J. Liu, X.-L. Cheng, X. Bai, R.-C. Lin, Urinary metabonomics study on biochemical changes in an experimental model of chronic renal failure by adenine based on UPLC Q-TOF/MS, Clin. Chim. Acta 413 (2012) 642–649, https://doi.org/10.1016/j.cca.2011.12.014.
[7] M. Posada-Ayala, I. Zubiri, M. Martin-Lorenzo, A. Sanz-Maroto, D. Molero, L. Gonzalez-Calero, et al., Identification of a urine metabolomic signature in patients with advanced-stage chronic kidney disease, Kidney Int. 85 (2014) 103–111, https://doi.org/10.1038/ki.2013.328.
[8] Z.-H. Zhang, F. Wei, N.D. Vaziri, X.-L. Cheng, X. Bai, R.-C. Lin, et al., Metabolomics insights into chronic kidney disease and modulatory effect of rhubarb against tubulointerstitial fibrosis, Sci. Rep. 5 (2015) 14472, https://doi.org/10.1038/srep14472.
[9] H. Chen, G. Cao, D.-Q. Chen, M. Wang, N.D. Vaziri, Z.-H. Zhang, et al., Metabolomics insights into activated redox signaling and lipid metabolism dysfunction in chronic kidney disease progression, Redox Biol 10 (2016) 168–178, https://doi.org/10.1016/j.redox.2016.09.014.
[10] J. Pérez-Jiménez, J. Hubert, L. Hooper, A. Cassidy, C. Manach, G. Williamson, et al., Urinary metabolites as biomarkers of polyphenol intake in humans: a systematic review, Am. J. Clin. Nutr. 92 (2010) 801–809, https://doi.org/10.3945/ajcn.2010.29924.
[11] M. Guasch-Ferré, S.N. Bhupathiraju, F.B. Hu, Use of metabolomics in improving assessment of dietary intake, Clin. Chem. 64 (2018) 82–98, https://doi.org/10.1373/clinchem.2017.272344.
[12] E. Pujos-Guillot, J. Hubert, J.-F. Martin, B. Lyan, M. Quintana, S. Claude, et al., Mass spectrometry-based metabolomics for the discovery of biomarkers of fruit and vegetable intake: citrus fruit as a case study, J. Proteome Res. 12 (2013) 1645–1659, https://doi.org/10.1021/pr300997c.
[13] D.P. Jones, Redefining oxidative stress, Antioxidants Redox Signal. 8 (2006) 1865–1879, https://doi.org/10.1089/ars.2006.8.1865.
[14] C. Gonçalves-Dias, J. Morello, M.J. Correia, N.R. Coelho, A.M.M. Antunes, M.P. Macedo, et al., Mercapturate pathway in the tubulocentric perspective of diabetic kidney disease, Nephron 143 (2019) 17–23, https://doi.org/10.1159/000494390.
[15] R. Sumayao, P. Newsholme, T. McMorrow, The role of cystinosin in the intermediary thiol metabolism and redox homeostasis in kidney proximal tubular cells, Antioxidants 7 (2018) 179, https://doi.org/10.3390/antiox7120179.
[16] R.C. Pimpão, T. Dew, P.B. Oliveira, G. Williamson, R.B. Ferreira, C.N. Santos, Analysis of phenolic compounds in Portuguese wild and commercial berries after multienzyme hydrolysis, J. Agric. Food Chem. 61 (2013) 4053–4062, https://doi.org/10.1021/jf305498j.
[17] R.C. Pimpão, T. Dew, M.E. Figueira, G.J. Mcdougall, D. Stewart, R.B. Ferreira, et al., Urinary metabolite profiling identifies novel colonic metabolites and conjugates of phenolics in healthy volunteers, Mol. Nutr. Food Res. 58 (2014) 1414–1425, https://doi.org/10.1002/mnfr.201300822.
[18] J.P. Rapp, M.R. Garrett, Will the real Dahl S rat please stand up? Am J Physiol Physiol 317 (2019) F1231–F1240, https://doi.org/10.1152/ajprenal.00359.2019.
[19] M. Suárez, M.-P. Romero, A. Macià, R.M. Valls, S. Fernández, R. Solà, et al., Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 877 (2009) 4097–4106, https://doi.org/10.1016/j.jchromb.2009.10.025.
[20] M.-P. Martí, A. Pantaleón, A. Rozek, A. Soler, J. Valls, A. Macià, et al., Rapid analysis of procyanidins and anthocyanins in plasma by microelution SPE and ultra-HPLC, J. Separ. Sci. 33 (2010) 2841–2853, https://doi.org/10.1002/jssc.201000274.
[21] L. Rubió, A. Serra, A. Macià, X. Borràs, M.P. Romero, M.J. Motilva, Validation of determination of plasma metabolites derived from thyme bioactive compounds by improved liquid chromatography coupled to tandem mass spectrometry, J Chromatogr B Anal Technol Biomed Life Sci 905 (2012) 75–84, https://doi.org/10.1016/j.jchromb.2012.07.040.
[22] A. Serra, L. Rubió, X. Borràs, A. Macià, M.-P. Romero, M.-J. Motilva, Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake, Mol. Nutr. Food Res. 56 (2012) 486–496, https://doi.org/10.1002/mnfr.201100436.
[23] J.I. Mosele, S. Martín-Peláez, A. Macià, M. Farràs, R.M. Valls, Catalán Ú, et al., Faecal microbial metabolism of olive oil phenolic compounds: in vitro and in vivo approaches, Mol. Nutr. Food Res. 58 (2014) 1809–1819, https://doi.org/10.1002/mnfr.201400124.
[24] J.G.M. Bancroft, Theory and Practice of Histological Techniques, sixth ed., Churchill Livingstone, USA, 2008.
[25] N.M. Grilo, M. João Correia, J.P. Miranda, M. Cipriano, J. Serpa, M. Matilde Marques, et al., Unmasking efavirenz neurotoxicity: time matters to the underlying mechanisms, Eur. J. Pharmaceut. Sci. 105 (2017) 47–54, https://doi.org/10.1016/j.ejps.2017.05.010.
[26] O. Beckonert, H.C. Keun, T.M.D. Ebbels, J. Bundy, E. Holmes, J.C. Lindon, et al., Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts, Nat. Protoc. 2 (2007) 2692–2703, https://doi.org/10.1038/nprot.2007.376.
[27] J. Chong, O. Soufan, C. Li, I. Caraus, S. Li, G. Bourque, et al., MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis, Nucleic Acids Res. 46 (2018) W486–W494, https://doi.org/10.1093/nar/gky310.
[28] I.-G. Chong, C.-H. Jun, Performance of some variable selection methods when multicollinearity is present, Chemometr. Intell. Lab. Syst. 78 (2005) 103–112, https://doi.org/10.1016/j.chemolab.2004.12.011.
[29] M. Farrés, S. Platikanov, S. Tsakovski, R. Tauler, Comparison of the variable importance in projection (VIP) and of the selectivity ratio (SR) methods for variable selection and interpretation, J. Chemom. 29 (2015) 528–536, https://doi.org/10.1002/cem.2736.
[30] N.R. Coelho, C.G. Dias, M. João Correia, P. Grácio, J. Serpa, E.C. Monteiro, L.N.P.S. Diogo, Cysteine oxidative dynamics underlies hypertension and kidney dysfunction Induced by chronic intermittent hypoxia, Arter Chemorecept Adv Exp Med Biol 1071 (2018) 83–88, https://doi.org/10.1007/978-3-319-91137-3_10.
[31] B.N.M. Zordoky, I.M. Robertson, J.R.B. Dyck, Preclinical and clinical evidence for the role of resveratrol in the treatment of cardiovascular diseases, Biochim. Biophys. Acta 1852 (2015) 1155–1177, https://doi.org/10.1016/j.bbadis.2014.10.016.
[32] A. Rodriguez-Mateos, C. Heiss, G. Borges, A. Crozier, Berry (poly)phenols and cardiovascular health, J. Agric. Food Chem. 62 (2014) 3842–3851, https://doi.org/10.1021/jf403757g.
[33] G. Istas, E. Wood, M. Le Sayec, C. Rawlings, J. Yoon, V. Dandavate, et al., Effects of aronia berry (poly)phenols on vascular function and gut microbiota: a double-blind randomized controlled trial in adult men, Am. J. Clin. Nutr. 110 (2019) 316–329, https://doi.org/10.1093/ajcn/nqz075.
[34] R.J. Johnson, K.L. Gordon, C. Giachelli, T. Kurth, M.M. Skelton, A.W.J. Cowley, Tubulointerstitial injury and loss of nitric oxide synthases parallel the development of hypertension in the Dahl-SS Rat, J. Hypertens. 18 (2000) 1497–1505, https://doi.org/10.1097/00004872-200018100-00019.
[35] K.A. Nath, Tubulointerstitial changes as a major determinant in the progression of renal damage, Am. J. Kidney Dis. 20 (1992) 1–17, https://doi.org/10.1016/S0272-6386(12)80312-X.
[36] H.J. Kang, I.S. Song, S.S. Lee, M.A. Yoo, J.G. Shin, Effects of dietary salt on the expression of drug transporters, cytochrome P4503a, and nuclear receptors in rats, Xenobiotica 38 (2008) 147–155, https://doi.org/10.1080/00498250701744674.
[37] M.M. Morales, M.A.M. Capella, M.V. Sanches, A.G. Lopes, W.B. Guggino, Modulation of the mdr-1b gene in the kidney of rats subjected to dehydration or a high-salt diet, Pflügers Archiv 439 (2000) 356–362, https://doi.org/10.1007/s004249900175.
[38] J. Čvorović, L. Ziberna, Stefano Fornasaro, F. Tramer, S. Passamonti, Bioavailability of flavonoids: the role of cell membrane transporters, in: S. Zibadi (Ed.), Watson, Ronald ; Preedy, Victor, second ed., Polyphenols Hum. Heal. Dis, Academic Press, 2018, pp. 295–320, , https://doi.org/10.1016/B978-0-12-398456-2.00037-2.
[39] W. Aoi, N. Niisato, H. Miyazaki, Y. Marunaka, Flavonoid-induced reduction of ENaC expression in the kidney of Dahl salt-sensitive hypertensive rat, Biochem. Biophys. Res. Commun. 315 (2004) 892–896, https://doi.org/10.1016/j.bbrc.2004.01.150.
[40] L. Wang, E. Hou, Z. Wang, N. Sun, L. He, L. Chen, et al., Analysis of metabolites in plasma reveals distinct metabolic features between Dahl salt-sensitive rats and

consomic SS.13(BN) rats, Biochem. Biophys. Res. Commun. 450 (2014) 863—869, https://doi.org/10.1016/j.bbrc.2014.06.089.
[41] Y. Wang, X. Liu, C. Zhang, Z. Wang, High salt diet induces metabolic alterations in multiple biological processes of Dahl salt-sensitive rats, J. Nutr. Biochem. 56 (2018) 133–141, https://doi.org/10.1016/j.jnutbio.2018.01.007.
[42] W. He, F.J.-P. Miao, D.C.-H. Lin, R.T. Schwandner, Z. Wang, J. Gao, et al., Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors, Nature 429 (2004) 188–193, https://doi.org/10.1038/nature02488.
[43] M.H. Mahbub, N. Yamaguchi, H. Takahashi, R. Hase, H. Yamamoto, S. Kikuchi, et al., Relationship of reduced glomerular filtration rate with alterations in plasma free amino acids and uric acid evaluated in healthy control and hypertensive subjects, Sci. Rep. 9 (2019) 10252, https://doi.org/10.1038/s41598-019-46598-7.
[44] M. Martin-Lorenzo, P.J. Martinez, M. Baldan-Martin, G. Ruiz-Hurtado, J.C. Prado, J. Segura, F. de la Cuesta, M.G. Barderas, F. Vivanco, L.M. Ruilope, G. Alvarez-Llamas, Citric acid metabolism in resistant hypertension: underlying mechanisms and metabolic prediction of treatment response, Hypertension 70 (5) (2017) 1049–1056, https://doi.org/10.1161/HYPERTENSIONAHA.117.09819.
[45] M.H. Stipanuk, M. Londono, J.-I. Lee, M. Hu, A.F. Yu, Enzymes and metabolites of cysteine metabolism in nonhepatic tissues of rats show little response to changes in dietary protein or sulfur amino acid levels, J. Nutr. 132 (2002) 3369–3378, https://doi.org/10.1093/jn/132.11.3369.
[46] Y.P. Kang, L. Torrente, A. Falzone, C.M. Elkins, M. Liu, J.M. Asara, et al., Cysteine dioxygenase 1 is a metabolic liability for non-small cell lung cancer, Elife 8 (2019) e45572, , https://doi.org/10.7554/eLife.45572.
[47] Y. Zhou, Z. Jiang, H. Lu, Z. Xu, R. Tong, J. Shi, et al., Recent advances of natural polyphenols activators for Keap1-Nrf2 signaling pathway, Chem. Biodivers. 16 (2019) e1900400, , https://doi.org/10.1002/cbdv.201900400.
[48] D. Feliers, H.J. Lee, B.S. Kasinath, Hydrogen sulfide in renal physiology and disease, Antioxidants Redox Signal. 25 (2016) 720–731, https://doi.org/10.1089/ars.2015.6596.
[49] L. Regazzoni, L. Del Vecchio, A. Altomare, K.-J. Yeum, D. Cusi, F. Locatelli, et al., Human serum albumin cysteinylation is increased in end stage renal disease patients and reduced by hemodialysis: mass spectrometry studies, Free Radic. Res. 47 (2013) 172–180, https://doi.org/10.3109/10715762.2012.756139.
[50] Y. Cheng, H. Song, X. Pan, H. Xue, Y. Wan, T. Wang, et al., Urinary metabolites associated with blood pressure on a low- or high-sodium diet, Theranostics 8 (2018) 1468—1480, https://doi.org/10.7150/thno.22018.
[51] R.S. Patel, N. Ghasemzadeh, D.J. Eapen, S. Sher, S. Arshad, Y. Ko, et al., Novel biomarker of oxidative stress is associated with risk of death in patients with coronary artery disease, Circulation 133 (2016) 361–369, https://doi.org/10.1161/CIRCULATIONAHA.115.019790.
[52] C.-N. Hsu, Y.-J. Lin, P.-C. Lu, Y.-L. Tain, Early supplementation of d-cysteine or l-cysteine prevents hypertension and kidney damage in spontaneously hypertensive rats exposed to high-salt intake, Mol. Nutr. Food Res. 62 (2018) 1700596, https://doi.org/10.1002/mnfr.201700596.