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Fecal and urinary NMR-based metabolomics unveil an aging signature in mice

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ABSTRACT

Background: Aging is characterized by derangements in multiple metabolic pathways that progressively constrict the homeostatic reserve (homeostenosis). The signature of metabolic alterations that accompany aging can be retrieved through the metabolomic profiling of biological fluids.
Objective: To characterize the age-related changes in urinary and fecal metabolic profiles of BALB/c mice through a ^{1}H nuclear magnetic resonance (NMR)-based metabolomic approach.
Methods: Young (n = 19) and old (n = 13) male BALB/c mice were fed *ad libitum* standard laboratory chow. Twenty four-hour feces and urine were collected using metabolic cages and analyzed by high-resolution ^{1}H NMR spectroscopy combined with multivariate statistical analyses.
Results: An age-related metabolic phenotype was detected both in urine and feces. The metabolic signature of aging consisted of changes in levels of metabolites associated with amino acid metabolism, tricarboxylic acid cycle, tryptophan–nicotinamide adenine dinucleotide pathway, and host–microbiota metabolic axis.
Conclusions: Our ^{1}H NMR-based metabolomic approach was able to characterize the effect of age on urinary and fecal metabotypes. The implementation of this analytical strategy may increase our understanding of the metabolic alterations involved in the aging process and assist in the design of anti-aging interventions.

1. Introduction

Aging is an inevitable phenomenon characterized by a progressive decline in multiple organismal functions, resulting in a generalized constriction of the whole body homeostatic reserve (homeostenosis) (Resnick and Marcantonio, 1997). Due to the complex nature of the aging process, the mechanisms by which senescence occurs are not yet completely understood. The recent implementation of high-throughput technologies, including metabolomics, has shed new light on the pathophysiology of aging, allowing the identification of highly specific signatures of senescence in various species (Cevenini et al., 2010; Mishur and Rea, 2012).

Notably, disease- or age-related derangements in the "multidirectional interactive chemical communication highway" coupling specific activities of microbial symbionts to critical host pathways can be retrieved through the metabolic profiling of biological fluids (Nicholson et al., 2012). As such, metabolomics have recently emerged as a valuable platform to explore the complex metabolic exchanges among biological compartments, including organs, tissues, and microbial symbionts, involved in the aging process and age-related diseases (Kristal et al., 2007; Mishur and Rea, 2012).

Metabolomic studies, based on nuclear magnetic resonance (NMR) and mass spectrometry (MS), have identified a number of metabolites associated with aging across multiple species (reviewed by Mishur and Rea, 2012). The characterization of metabolites in biological fluids and tissues of rodents suggests that the aging process might be related to mitochondrial dysfunction and lipid, glucose, and protein dysmetabolism (De Guzman et al., 2012; Houtkooper et al., 2011; Nevedomskaya et al., 2010a,b; Salek et al., 2008; Son et al., 2012; Tomás-Loba et al., 2013; Yan et al., 2009). Similar results have been reported in metabolomic studies of human biofluids (Collino et al., 2013; Lawton et al., 2008; Yu et al., 2012).

Abbreviations: 1MNA, 1-methylnicotinamide; AUROC, Area Under the ROC curve; DQ2, Discriminant Q2; LV, Latent Variable; MS, mass spectrometry; NMR, nuclear magnetic resonance; NNMT, nicotinamide N-methyltransferase; NMC, Number of Misclassification; PBS, phosphate buffered saline; PLS-DA, Partial Least Squares-Discriminant Analysis; RP, Rank Product; SAM, S-adenosylmethionine; TCA, tricarboxyl acid cycle; THP, Tamm–Horsfall protein; TSP, (3-trimethylsilyl) propionic-(2,2,3,3-d4)-acid; VIP, variable importance in projection.

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The application of metabolomic analytical strategies to feces has a great potential for assessing host–microbiome interactions and the functional status of the gastrointestinal system (Martin et al., 2010; Saric et al., 2008; Zhao et al., 2013). Recent studies suggest that integrated metabolomic analyses of urine and fecal water may provide a comprehensive readout for investigating the complex metabolic interplay between mammals and their intestinal ecosystems (Martin et al., 2007; Nicholson et al., 2012; Zheng et al., 2011). Indeed, analyses of these multiple biological samples complement each other, yielding different information with regard to the origin of the metabolites identified.

Based on these premises, the present study was undertaken to characterize the age-related changes in urinary and fecal metabolic profiles in young adult and old BALB/c mice through a ^{1}H NMR-based metabolomic approach. The study was designed to identify metabolites related to chronological age and provide the groundwork for devising and evaluating interventions aimed at preventing, delaying or alleviating age-related derangements of specific metabolic networks.

2. Materials and methods

2.1. Animals

Young (3-month-old; n = 19) and old (16-month-old; n = 13) male BALB/c mice were obtained from Charles River Laboratories (Calco, Lecco, Italy) and housed individually in a temperature- and light-controlled room, maintained at 23 °C on a 12-h light–dark cycle. Animals were acclimated for two weeks before sample collection. Mice were fed a standard complete diet (Mucedola, Settimo Milanese, Milan, Italy) and had free access to food and tap water. Body mass and food intake were recorded every other day. Mice were transferred into metabolic cages (Tecniplast, Varese, Italy) for 48 h for urine and feces collection. Twenty-four-hour urinary and fecal samples were stored at −80 °C prior to NMR spectroscopy analysis. Urinary samples were collected in a solution of 0.05% sodium azide to avoid contaminations. All experimental procedures were approved by the Italian National Health Ministry, Department of Food, Nutrition, and Animal Health.

2.2. Sample preparation

Urinary samples were thawed at room temperature and centrifuged at 11,000 ×*g* for 15 min at 4 °C. Urinary samples (400 μL) were added to 3-trimethylsilyl-propionic-2,2,3,3-d_4 acid (TSP) in cold phosphate buffered saline (PBS)-D_2O (200 μL, pH: 7.4; 2 mM final concentration), as an internal standard. Fecal water samples were prepared as described by Saric et al. (2008). Briefly, feces (320 mg) were suspended in ice-cold PBS-D_2O (1.4 mL) and centrifuged at 11,000 ×*g* for 15 min at 4 °C. The supernatant was filtered through a cell strainer (100-μm pore size), centrifuged and filtered again through a sterile syringe filter (0.2-μm pore size). PBS-D_2O (60 μL) containing 2 mM TSP (internal standard) was added to the supernatants (600 μL). Aliquots of urine and fecal water were finally transferred into 5-mm NMR tubes and analyzed by high-resolution NMR spectroscopy.

2.3. ^{1}H NMR spectroscopy

^{1}H NMR spectra of urine and fecal water were acquired at 298 K using a Bruker AVANCE 400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a magnet operating at 9.4 T and 400.13 MHz for ^{1}H frequency, as described elsewhere (Miccheli et al., 2009). Spectra were recorded using pulse sequences, including standard ^{1}H experiment detection with water suppression.

Resonance assignments were conducted by comparison with literature data (Martin et al., 2009a; Salek et al., 2007; Saric et al., 2008; Tian et al., 2012) as well as with online (www.hmdb.ca) and in-house databases. Assignments were further confirmed individually through a series of two-dimensional (TOCSY and HSQC) NMR experiments. The reader is referred to the supplementary material for a detailed description of the methodology.

Monodimensional NMR spectra were processed and quantified by using the ACD Lab 1D-NMR Manager 12.0 software (Advanced Chemistry Development, Inc., Toronto, ON, Canada), whereas the Bruker Top Spin ver. 3.1 (Bruker BioSpin GmbH) was employed for 2D-NMR spectra. The quantification of metabolites was obtained by comparison of the integrals of specific signals with the internal standard TSP integral. Data from urinary spectra were normalized to the methylene peak integral of creatinine at 4.07 ppm to account for dilution effects (Ross et al., 2007). Concentrations of metabolites are expressed in $\mu mol \cdot L^{-1}$ for fecal water and $\mu mol \cdot mmol^{-1}$ creatinine for urine.

2.4. Statistical analysis

To identify age-related changes in urinary and fecal metabolic phenotypes, Partial Least Squares-Discriminant Analysis (PLS-DA) was used (Barker and Rayens, 2003; Ståhle and Wold, 1987). PLS-DA operates by projecting the samples onto a low-dimensional space of so-called Latent Variables (LVs; linear combinations of the measured signals) and by building a regression model between these new variables (scores) and a dummy binary vector y coding for class membership. In this study, young individuals were assigned y = 0, while aged mice were attributed y = 1. Cross-validation was used to select the optimal number of LVs for PLS-DA models (Stone, 1974). Statistical validity of the calculated models and the reliability of results were assessed by permutation tests and cross-model validation (Szymanska et al., 2012). Number of Misclassification (NMC), Area Under the ROC curve (AUROC) and Discriminant Q^2 (DQ2) are detailed in the supplementary material.

The identification of the metabolites mostly contributing to the differentiation between age groups was based on bootstrapped variable importance in projection (VIP) scores (Wold et al., 1993) and Rank Product (RP) (Smit et al., 2007) in cross-model validation. Metabolites with VIP > 1 and the lowest RP were considered to be responsible for the discrimination (supplementary material).

To explore possible intercompartmental relationships between urinary and fecal metabolic profiles, a PLS2 regression model was built between urinary and fecal matrices, selected as the X and Y block, respectively (Wold et al., 1983) (supplementary material). The use of a multivariate calibration technique such as PLS allows achieving a holistic view of all of the correlations existing between two sets of variables. The PLS also provides a picture of the investigated phenomena that is more complete than the one achievable by focusing only on the binary correlations between pairs of metabolites.

All of the analyses were performed using in-house routines running under Matlab R2011b environment (The MathWorks, Natick, MA).

3. Results

3.1. ^{1}H NMR spectra identification

Typical ^{1}H NMR spectra of urine and fecal water are shown in Fig. 1. Due to technical issues with sample handling, two fecal samples from young mice could not be analyzed. Seventy-eight metabolites were detected and 71 identified (U1-U7 indicate unassigned signals) in the two biological matrices. Corresponding ^{1}H and ^{13}C data are reported in Table S1.

As depicted in Table S1, ^{1}H NMR spectra of urine and feces consisted of a vast array of small molecules representing several metabolite classes and intermediates of crucial biochemical pathways, including amino acids and derivatives (*e.g.*, α-ketoisovalerate, tyrosine, and valine), short-chain fatty acids (*e.g.*, acetate and butyrate), intermediates of choline-betaine metabolism (*e.g.* choline, dimethylglycine, and trimethylamine), nicotinate/nicotinamide metabolites [*e.g.*, 1-methylnicotinamide (1MNA) and N-methylnicotinate], tricarboxylic acid (TCA) cycle intermediates (*e.g.*, citrate, fumarate, and

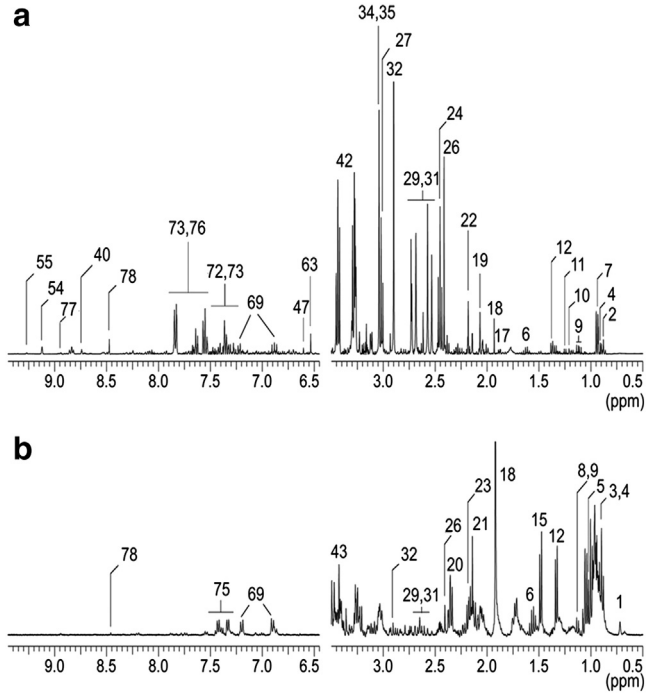

Fig. 1. ^{1}H NMR spectrum of urine (a) and fecal water (b). Spectra in the regions δ 6.45–9.95 are displayed at 6 × magnification relative to δ 0.50–3.95 regions. Numbers indicate signals corresponding to individual metabolites (Table S1).

succinate), ketone bodies (*e.g.*, acetone and β-hydroxybutyrate), carbohydrates (*e.g.*, sucrose and xylose), intermediates of nucleotide metabolism (*e.g.*, allantoin, ureidopropionate, and uridine), aromatic compounds and other compounds related to gut microbiota-host co-metabolism (*e.g.*, hippurate, indoleacetylglycine, and phenylacetylglycine).

3.2. Metabolic signature of aging in the urinary metabolome

PLS-DA was applied to analyze the urinary metabolome. The optimal complexity of the model, as assessed by 7-fold cross-validation, was found to be three LVs, resulting in 100% correct classification in all validation stages. The result can be visualized by inspecting the PLS-DA scores plot (Fig. 2), showing that urinary metabolic profiles of young and aged mice are overtly separated. The discrimination was also confirmed by the values of AUROC (1.00) and DQ2 (0.77). Permutation test and cross-model validation showed that the discrimination between age groups was statistically significant ($p < 0.001$; Fig. S1).

Table 1
Urinary metabolites responsible for the discrimination between young and old urinary metabotypes.

Metabolites	Concentration (μmol·mmol^{-1} creatinine)		VIP
	Young adult (mean ± SD; n = 19)	Aged (mean ± SD; n = 13)	
1-methylnicotinamide	99 ± 26	173 ± 58	4.24
α-ketoisovalerate	154 ± 17	108 ± 36	4.15
N-acetyl	65 ± 56	209 ± 196	3.18
Taurine	13,608 ± 2621	10,195 ± 3199	2.51
U6	74 ± 38	31 ± 36	2.17
Citrate	10,298 ± 2952	6475 ± 2639	2.02
Succinate	1826 ± 685	1021 ± 507	1.97
Tyrosine	704 ± 267	417 ± 149	1.83
Threonine	1048 ± 296	713 ± 220	1.81
β-hydroxyisovalerate	264 ± 81	192 ± 82	1.76
U4	500 ± 171	638 ± 258	1.17
Fumarate	176 ± 62	120 ± 54	1.14
Trans-aconitate	177 ± 34	183 ± 47	1.05

VIP: Variable importance in projection.

The variables responsible for the discrimination between young and old urinary metabolic profiles are reported in Table 1. The urinary metabolome of aged mice was characterized by higher levels of 1MNA, signals from N-acetyl groups of glycoproteins, and an unknown metabolite (U4, at δ 3.57).

The urinary metabolome of aged mice also showed lower levels of α-ketoisovalerate and β-hydroxyisovalerate, citrate, fumarate, succinate, taurine, threonine, tyrosine, and an unknown metabolite (U6, at δ 6.72) (Table 1).

3.3. Metabolic signature of aging in the fecal metabolome

The optimal complexity of the fecal metabolome PLS-DA model, as assessed by 7-fold cross-validation, was found to be two LVs, resulting in 100% correct classification in all validation stages (NMC = 0). The goodness of the model was also reflected by the values of AUROC (1.00) and DQ2 (0.77). Permutation test and cross-model validation

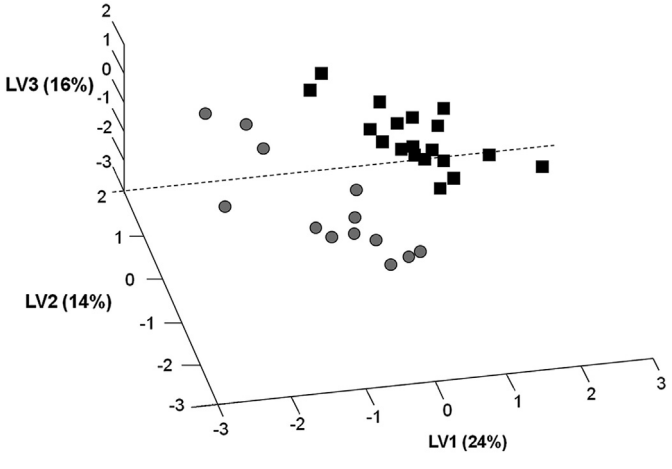

Fig. 2. PLS-DA scores plot derived from urine ^{1}H NMR spectra of young (black squares; n = 19) and old (gray circles; n = 13) mice. The model is described by three Latent Variables (LVs). $R^2X(\%)$: LV1 = 24%; LV2 = 14%; LV3 = 16%; $R^2Y(\%)$: LV1 = 29%; LV2 = 14%; LV3 = 2%. $R^2X(\%)$: percentage of the X-variance explained by the model; $R^2Y(\%)$: percentage of the Y-variance explained by the model.

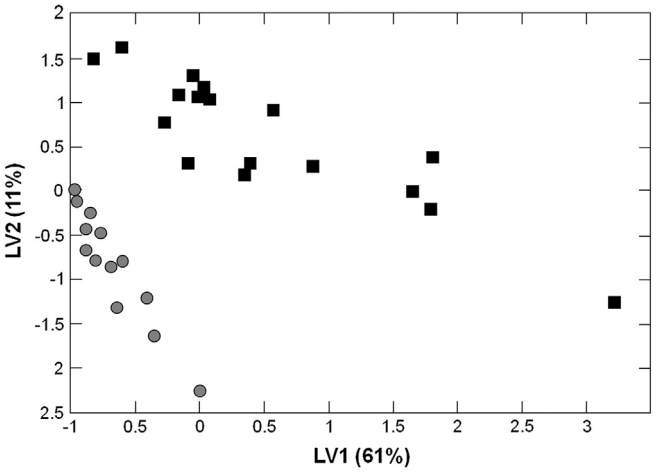

Fig. 3. PLS-DA scores plot derived from fecal water ^{1}H NMR spectra of young (black squares; n = 17) and old (gray circles; n = 13) mice. The PLS-DA model was described by two Latent Variables (LVs). $R^2X(\%)$: LV1 = 61%; LV2 = 11%; $R^2Y(\%)$: LV1 = 18%; LV2 = 27%. $R^2X(\%)$: percentage of X-variance explained by the model; $R^2Y(\%)$: percentage of the Y-variance explained by the model.

Table 2
Fecal metabolites responsible for the discrimination between young and old fecal metabotypes.

Metabolites	Concentration ($\mu mol \cdot L^{-1}$)		VIP
	Young adult (mean ± SD; n = 17)	Aged (mean ± SD; n = 13)	
Histidine	140 ± 36	180 ± 37	4.67
4-hydroxyphenylacetate	302 ± 84	394 ± 75	4.49
Bile salts	371 ± 128	153 ± 62	4.43
α-ketoisovalerate	195 ± 65	55 ± 18	4.19
β-hydroxybutyrate	749 ± 351	369 ± 58	1.58
Succinate	264 ± 168	275 ± 68	1.56
Formate	17 ± 8	29 ± 26	1.56
U3	859 ± 357	867 ± 234	1.47
Isoleucine	719 ± 276	398 ± 127	1.31
Methionine	597 ± 203	361 ± 95	1.24
α-ketoisocaproate	299 ± 103	192 ± 37	1.07

VIP: Variable importance in projection.

indicated that the discrimination between age groups was statistically significant ($p < 0.001$; Fig. S2).

PLS-DA scores plot in Fig. 3 shows that age groups are clearly separated. Metabolites responsible for the discrimination are reported in Table 2. Feces from aged mice had higher levels of 4-hydroxyphenylacetate and histidine, with lower concentrations of α-ketoisocaproate, α-ketoisovalerate, β-hydroxybutyrate, bile salts, isoleucine and methionine.

3.4. Intercompartmental metabolic correlation analysis

The optimal PLS2 model built to correlate fecal waters and urine data was found to be a 4-component model capturing more than 60% of variation in both blocks, as estimated by cross-validation. When examining the projection of the samples onto the first two LVs, a clear separation was observed between age groups (Fig. S3a). Notably, the results obtained by investigating each compartment individually are confirmed by the output of the intercompartmental PLS2 model, as evidenced by the comparison of the scores and loadings plots of the PLS-DA and the PLS2 models.

The identification of the metabolites more closely related in the two compartments was achieved by the inspection of the biplot representing both the X and Y loadings for the first two PLS LVs (Fig. S3b). In particular, a significant correlation was observed between metabolites involved in amino acid metabolism in the two matrices. This is illustrated by the positive correlation detected between urinary α-ketoisovalerate, β-hydroxyisovalerate, and fecal α-ketoisocaproate, α-ketoisovalerate, isoleucine, and valine. A positive correlation was also observed between urinary taurine and fecal bile salts. Comparison of Fig. S3b with the scores plot in Fig. S3a suggests that all these metabolites show a higher concentration in young than in aged mice. Finally, a negative correlation was found between fecal choline, the content of which was higher in young mice, and urinary dimethylamine that was more abundant in old animals.

4. Discussion

In the present study, we adopted a NMR-based metabolomics approach to simultaneously evaluate differences in urinary and fecal metabolic profiles between young and old mice, in order to identify a metabolic signature of aging. To the best of our knowledge, this is the first report investigating systemic metabolic changes associated with aging through the simultaneous metabolic screening of urine and feces. Our strategy allowed the identification of a metabolic footprint of aging in both biospecimens.

1MNA was the most important urinary metabolite in the discrimination between young and old mice (Table 1). 1MNA is a catabolic intermediate in the tryptophan-NAD pathway that supplies the liver with pyridine nucleotides and is mostly excreted with urine (Rose, 1972; Wolf, 1974). 1MNA is a product of nicotinamide N-methyltransferase (NNMT), an S-adenosyl-L-methionine-dependent cytosolic enzyme that catalyzes the N-methylation of nicotinamide and other pyridines to form pyridinium ions (Cantoni, 1951). 1MNA is involved in the regulation of intra- and extracellular levels of nicotinamide by modulating its excretion after N-methylation. Nicotinamide, in turn, is a metabolic intermediate of the coenzyme NAD, which is implicated in longevity through the activity of NAD-consuming enzymes, such as sirtuins and poly(ADP-ribose)polymerases (Imai, 2009, 2011). The increased urinary 1MNA excretion found in old mice may therefore reflect a perturbation in the "NAD World" homeostasis (Imai, 2009, 2011), which could represent a central aspect of the homeostenosis phenomenon characterizing the aging process (Resnick and Marcantonio, 1997).

The urinary and fecal metabolic signature of aging was also characterized by changes in the levels of a number of metabolites involved in amino acid metabolism (Tables 1 and 2).

Specifically, the urinary concentration of α-ketoisovalerate and β-hydroxyisovalerate (intermediates of leucine and valine metabolism), tyrosine and threonine was lower in aged mice relative to their younger counterparts. This finding is consistent with the recent observations of lower urinary levels of α-keto acids (including α-ketoisovalerate) in ERCC1$^{d/-}$ mutant mice (Nevedomskaya et al., 2010a), a model of accelerated murine aging. Our findings are also in agreement with the evidence that circulating levels of several amino acids are reduced in old mice (Houtkooper et al., 2011; Tomás-Loba et al., 2013). Moreover, alterations in blood concentrations of a number of amino acids and their derivatives have been detected in mouse models of accelerated aging and in long-lived mice (Jiang et al., 2008; Nevedomskaya et al., 2010b; Qiao-feng et al., 2011; Wijeyesekera et al., 2012). As a whole, our findings and those from others suggest that an altered amino acid metabolism may be a characteristic of the murine aging process.

The age-related metabolic signatures of feces was characterized by alterations in the concentration of amino acids (histidine, isoleucine, and methionine) and derivatives (4-hydroxyphenylacetate, α-ketoisocaproate, and α-ketoisovalerate, deriving from the catabolic degradation of tyrosine, leucine, and valine, respectively) and organic acids such as β-hydroxybutyrate, formate, and succinate (Table 2). The concentrations of these compounds and metabolic intermediates reflect the fermentation processes (mainly in the distal colon) of unabsorbed dietary substrates (*e.g.*, carbohydrates and proteins) along with other endogenous substrates, such as bile enzymes, mucus and exfoliated cells, that reach the large intestine (Jacobs et al., 2009).

The type and amount of these fermentation-derived metabolites depend on a wide range of factors, including the composition and activity of the gut microbiota, small intestinal absorption of nutrients, transit time/intestinal motility, luminal pH, and the substrates available for fermentation, which in turn derive from the combinatory effect of diet and upper intestinal digestive processes (Hamer et al., 2012; Jacobs et al., 2009).

Since no changes in food intake were observed between young and old mice (data not shown), the alterations in fecal levels of amino acids, products of food breakdown and intermediates of catabolic processes could be ascribed, at least in part, to age-related changes in gut microbiota properties (Calvani et al., 2013). The intercompartmental correlation analysis revealed significant correlations between fecal and urinary metabolites deriving from amino acid metabolism, irrespective of age. However, the net result of the metabolic exchanges between host and microbiota remains to be determined *in vivo* from both a quantitative and qualitative point of view. The application of stable isotope-labeled substrates could provide an elegant solution to study these metabolic pathways *in vivo* (de Graaf and Venema, 2008).

The finding of lower levels of bile salts in feces of aged mice may represent another clue to an age-related modification of the gut microbiota physiology. About 5–10% of bile acids produced by the liver from cholesterol are biotransformed largely through degradation

by intestinal bacteria and some are lost with feces (Ridlon et al., 2006). Establishing the exact contributions of the different physiological mediators of bile salt metabolism (*i.e.*, liver production, enterohepatic circulation, small intestine absorption, and bacterial deconjugation) in the context of aging requires further investigation. It is likely that metabolite compositional analyses of intestinal contents at specific topographical locations in different age groups, coupled with metagenomics, could provide important information on the functional status of the gastrointestinal tract (Martin et al., 2009a,b; Tian et al., 2012).

Reduced urinary concentrations of taurine represent another distinctive characteristic of the aged metabolome identified by our analysis. Taurine is a ubiquitous, multifunctional, sulfur-containing amino acid with important roles in many physiological processes, including bile acid metabolism, neuronal development and modulation, protection against oxidative stress and cellular osmoregulation (Huxtable, 1992). Circulating levels of taurine are regulated by the balance among different factors, including dietary intake, intestinal absorption, bile acid conjugation, urinary excretion, and endogenous synthesis from methionine and cysteine (Huxtable, 1992). Reduced urinary levels of taurine have been found in ERCC1$^{d/-}$ mutant mice (Nevedomskaya et al., 2010a), while increased levels of serum taurine have been retrieved in the metabolic profiles of old wild-type mice from different genetic backgrounds (Tomás-Loba et al., 2013). The PLS2 analysis of urinary and fecal metabolites revealed a positive correlation between urinary taurine levels and fecal concentrations of bile salts. Due to the complex regulation of bile salt and taurine metabolism, the biological relevance of this correlation warrants further investigation.

The intercompartmental correlation analysis also revealed a negative correlation between fecal choline and urinary dimethylamine. Choline is an essential nutrient involved in a wide range of cellular processes, such as neurotransmitter synthesis, cell membrane structure and signaling, lipid transport, and methyl group metabolism (Zeisel and da Costa, 2009). Fecal contents of choline are the resultant of different processes, mainly related to dietary intake, absorption in the small intestine and conversion by gut bacteria to methylamines (*i.e.*, trimethylamine, dimethylamine and methylamine) (Zeisel and da Costa, 2009; Zeisel et al., 1983). Some of these methylamines are then absorbed and eventually excreted with urine (Zeisel et al., 1983). Lower concentrations of choline in fecal waters ($p < 0.05$) and a trend ($p < 0.08$) towards higher urinary levels of dimethylamine were found in aged mice. Interestingly, increased plasma concentrations of dimethylamine have been found in senescence-accelerated mouse prone 8 (SAMP8) (Qiao-feng et al., 2011), while perturbations in circulating levels of choline have been described in both ERCC1$^{d/-}$ mutant mice (Nevedomskaya et al., 2010a) and different long-lived mouse models (Wijeyesekera et al., 2012).

Intermediates of the TCA cycle, including citrate, succinate and fumarate, showed lower levels in urine of aged mice compared with young animals, which might suggest an age-dependent alteration of energy metabolism. However, these compounds are involved in a large number of biochemical processes, and their changes are difficult to interpret as they may occur in response to very different stimuli (Robertson, 2005). Perturbations in circulating levels of TCA cycle intermediates are commonly reported in mouse models of accelerated aging and long-lived mice (Jiang et al., 2008; Nevedomskaya et al., 2010a; Wijeyesekera et al., 2012), but their physiologic significance may be limited at the moment to a generic "indication of altered energy metabolism"(Nevedomskaya et al., 2010a).

A significantly higher level of the N-acetyl fragment signal from glycoproteins was observed in urine of old mice. This signal may arise from the most abundant glycoprotein in mammalian urine, the Tamm–Horsfall protein (THP), also known as uromodulin, a small glycoprotein secreted by the thick ascending limb of the Henle's loop (Serafini-Cessi et al., 2003). Although the functions of this protein remain elusive, accumulating data highlight the importance of uromodulin in the pathophysiology of a number of conditions affecting the urinary apparatus (reviewed by Serafini-Cessi et al., 2003, and El-Achkar and Wu, 2012). The presence of signals from N-acetyl fragment of glycoproteins among the classifiers characterizing some mouse models of accelerated aging and long-lived mice is a common finding in metabolomic studies of aging (Nevedomskaya et al., 2010a; Qiao-feng et al., 2011; Wijeyesekera et al., 2012). Further studies are needed to establish the relationship between urinary levels of N-acetyl group signal and uromodulin concentrations and clarify the role of uromodulin in kidney aging.

5. Limitations

Given the hypothesis-generating design of our investigation and the fraction of the metabolome accessible to NMR spectroscopy, a definite interpretation of the biological significance of the individual metabolites identified is not possible. The measured perturbation in the metabolic composition of urine and fecal water results from the complex interplay among many physiological processes and, therefore, describes a systemic pattern of metabolic changes related to each other and/or occurring at overlapping timescales. This represents what we have called "metabolic signature of aging". Notwithstanding, this is the first work examining simultaneously the age-related changes in the metabolic composition of these biofluids. Our results may therefore serve as the baseline information for the design of future investigations on the pathways suggested to be relevant by the present study. The combination of different analytical platforms (*e.g.*, MS plus NMR) to obtain a more complete coverage of the metabolome, stable isotope studies, and a multivariate multicompartmental integration of the biological matrices analyzed may be needed to achieve a comprehensive characterization of age-related metabolic derangements.

Another limitation of the present study is that only two age groups were examined. Since 3-month-old mice are considered mature, albeit still young, and 16-month-old rodents aged, but not very old, the study would have been strengthened if other age classes could be analyzed. Nonetheless, we decided to select these two ages to maximize the separation between groups, while avoiding excessive interindividual variability typical of extreme ages.

Various intrinsic factors are known to affect the metabolic composition of biological samples, including diurnal variation, sex, and strain. In addition, the metabolome is influenced by a number of extrinsic agents, such as food and water intake as well environmental temperature and light intensity (Bollard et al., 2005). In the present work, we tried to cope with these sources of variability by sampling 24-h biofluids, choosing an inbred mouse strain, using only male animals, monitoring accurately food and water intake, and standardizing the environmental conditions.

Another aspect to consider is that we restricted our analyses to fecal water rather than characterizing the metabolic composition of individual intestinal segments. Further studies coupling metabolomics with next-generation high-throughput sequencing methods and *in vitro* experiments on gut model systems are warranted to evaluate the changes in intestinal microbial ecology mostly responsible for generating the host's metabotype and their contribution to the aging process.

As expected body mass was significantly higher in old (31.1 g $\pm$ 2.7) relative to young mice (27.8 g $\pm$ 1.6; $p < 0.01$). We acknowledge that this phenomenon together with changes in body composition and insulin sensitivity could have had an influence on our findings. Nevertheless, alterations in these parameters are inherent to aging across species. It could therefore be assumed that age *per se* is the main factor contributing to the metabolic trajectory, and that aging is, at least in part, characterized by metabolome variations.

6. Conclusion

The NMR-based metabolomic approach adopted in the present study identified a footprint of murine metabolic aging, revealing unique

metabolite compositional signatures in both urine and fecal water. This set of metabolic findings and the different pathways identified could be used to generate new mechanistic hypotheses on the aging process. Our findings can thus provide the baseline information for the rational design of interventions aimed at preventing or attenuating derangements in specific metabolic networks and evaluating the effects of such interventions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2013.10.010.

Conflict of interest

The authors have no conflicts of interests.

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