Aging markers in human urine:

A comprehensive, non-targeted LC-MS study

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Summary

Metabolites in human biofluids document individual physiological status. We conducted comprehensive, non-targeted, non-invasive metabolomic analysis of urine from 27 healthy human subjects, comprising 13 youths (30±3 yr) and 14 seniors (76±4 yr). Quantitative analysis of 99 metabolites revealed 55 that were linked to aging, displaying significant differences in abundance between the two groups. These include 13 standard amino acids, 5 methylated, 4 acetylated, and 9 other amino acids, 6 nucleosides, nucleobases, and derivatives, 4 sugar derivatives, 5 sugar phosphates, 4 carnitines, 2 hydroxybutyrates, 1 choline, and 1 ethanolamine derivative, and glutathione disulfide. Abundances of 53 compounds decreased, while 2 increased in elderly people. Many age-linked markers were highly correlated; 42 of 55 compounds, showed Pearson's correlation coefficients larger than 0.70. As metabolite profiles of urine and blood are quite different, age-related information in urine components offer yet more valuable insights into aging mechanisms of endocrine system and related organ systems.

Introduction

Elderly people are acutely aware of the progressive aging of different body parts, but quantifying and characterizing physiological aging is less intuitive. Nonetheless, assessment of aging and determination of aging type by analyzing metabolites in biofluids, such as blood and urine, may help us to understand broadly-featured aging of the human body (Kampmann et al., 1974) (Ames, 1989) (Short et al., 2005) (Slupsky et al., 2007) (Lawton et al., 2008) (Mishur and Rea, 2012) (Yu et al., 2012) (Menni et al., 2013) (Gonzalez-Covarrubias et al., 2013) (Auro et al., 2014) (Chaleckis et al., 2016) (Hertel et al., 2016) (Jove et al., 2016) (Rist et al., 2017) (Chak et al., 2019). Age-dependent changes of metabolite abundances may be valuable to determine the molecular causes of impaired organ functions. Technology that enables simple and rapid measurement of urinary metabolites, which can be collected non-invasively, has certain advantages over methods using blood. Urinary metabolites are promising biological samples for monitoring health parameters if the metabolic processes resulting in production of those metabolites can be fully understood. To date, few comprehensive approaches to investigate human urinary metabolites in aging have been reported (Thevenot et al., 2015) (Hertel et al., 2016) (Rist et al., 2017). Urination is a primary route by which the body eliminates water-soluble waste products. Accordingly, urine has been broadly utilized for diagnosis of renal dysfunction in diverse kidney diseases (Han et al., 2002) (Eknoyan et al., 2003) (Mishra et al., 2005) (Parikh et al., 2006) (Pisitkun et al., 2006) (Vaidya et al., 2008). Nonetheless, because urinary metabolites originate in all organ systems, they may be useful to examine human aging. In this study, we analyzed urinary metabolites

67 in elderly and young subjects, using comprehensive metabolomics to identify
68 metabolites linked to aging. Striking correlations of many urine metabolites with
69 age were found.
70

Results

Collection of urine samples

Samples of morning urine, immediately after awaking, were collected from healthy volunteer subjects (elderly, 75.8±3.9 yr, and young, 30.6±3.2 yr; **Supplementary Table s1** shows gender and BMI) in Onna Village, Okinawa, Japan. Precautions taken for sample collection are described in the Materials and Methods. Basic data analytical procedures were similar to those previously described (Chaleckis et al., 2016) (Teruya et al., 2019) (Kameda et al., 2020).

99 urine metabolites identified

Ninety-nine urinary metabolites, about half of which are amino acids and their derivatives, were identified and quantified using liquid chromatography – mass spectrometry (LC – MS) and MZmine 2 (Pluskal et al., 2010a) (Teruya et al., 2019) **(Supplemental Table s2).** These compounds were subdivided into 12 groups, containing 17 standard amino acids, 12 methylated amino acids, 6 acetylated amino acids and 15 other amino acids, 12 nucleosides, nucleobases, and derivatives, 4 sugar derivatives, 6 sugar phosphates, 3 vitamins and coenzymes (pantothenate, 4-aminobenzoate, nicotinamide), 4 choline and ethanolamine derivatives, 8 carnitines, 11 organic acids, and 1 antioxidant (oxidized form of glutathione, GSSG). Small amino acids, such as glycine and alanine, were not detected in our analysis due to the mass cutoff (100 m/z) used. Levels of individual compounds, categorized by abundance as H (high, $>10^8$), M (medium, 10^7~10^8) or L (low, $<10^7$), were estimated based upon mass spectroscopic peak area (Chaleckis et al., 2014) (Chaleckis et al.,

2016). Some compounds varied widely from one individual to the next and are denoted as H-L, H-M or M-L. According to abundance, there were 7 H, 19 H-M, 7 H-L, 5 M, 45 M-L and 16 L compounds. Twenty-six urinary metabolites were abundant (H or H-M), the great majority of which were amino acids and their derivatives, such as the methylated amino acids, betaine and dimethyl-arginine, but they also included nucleosides, such as pseudouridine and N-methyl-guanosine. In blood, sugar phosphates and methylated amino acids were enriched in red blood cells (Chaleckis et al., 2014) (Chaleckis et al., 2016). In urine, sugar phosphates are age-related, except for glucose-6-phosphate. We show below that about half of all urinary metabolites are age-related.

Most urinary age markers decreased with age

Of 99 urinary metabolites assayed in 27 subjects, 55 showed statistically significant differences between young and elderly (p-values, $0.00003 < p < 0.05$) (**Fig. 1**). Thus, about half of these compounds are affected by aging, with most becoming less abundant in elderly subjects. Two exceptions, myo-inositol and glutathione disulfide (GSSG), were more abundant in elderly samples. In other words, ~50% of all urinary metabolites decline in old age. Five of 55 metabolites, creatinine, dimethyl guanosine, decanoyl-carnitine, N-acetyl-aspartate, and tryptophan, were previously reported as urinary age markers (Kampmann et al., 1974) (Slupsky et al., 2007) (Thevenot et al., 2015) (Rist et al., 2017). myo-Inositol and GSSG increased 1.34- and 6.96-fold, respectively (**Table 1**). The increase of GSSG was striking, though its abundance in urine was rather low. Many metabolites diminished to less than half in

urine of elderly subjects [fructose-1,6-diphosphate (0.14), carnosine (0.34), glycerophosphate (0.32), 2- and 3-hydroxybutyrate (0.33, 0.27, respectively), and octanoryl-carnitine (0.40)].

Highly correlated age-linked urinary metabolites

To understand relationships among urine metabolites, Pearson's correlation coefficients were calculated from abundance data for all 55 age-linked urinary metabolites of all 27 subjects (Methods section). The highest correlation, 0.92, was obtained for isoleucine – leucine and N-methyl guanosine – dimethyl guanosine (**Fig. 2A**). This is probably due to their similar chemical structures and proximity in biochemical pathways (see below and KEGG, https://www.genome.jp/kegg/). However, correlation values 0.91 obtained for glycerol-phosphate – 3-hydroxybutyrate, pseudouridine – 2-hydroxybutyrate, and pseudouridine – isoleucine clearly have a different explanation. Fifteen urinary metabolites having correlation values >0.85 formed a network (**Fig. 2B**). The high correlation between pseudouridine and isoleucine seems to be a key connection between two groups of metabolites. Additionally, the connections of creatinine to pseudouridine and 3-hydroxybutyrate to glutamine appeared to be required for further correlation (see below). In the case of a creatinine-related network, 15 metabolites have correlation coefficients from 0.71~0.84 (**Fig. 2C**). Creatinine, a known waste product from muscle (Heymsfield et al., 1983) (Baxmann et al., 2008), is correlated with many metabolites. It even shows a negative correlation with GSSG (**Fig. 2D**).

Pairwise correlation analyses revealed 21 pairs of compounds with

correlation coefficients >0.85 (**Fig. 2A**). These show a network consisting of two groups, one centered around pseudouridine and the other around isoleucine (**Fig. 2B**). This suggests that compounds within each of these groups may be metabolically linked.

When urinary compounds with correlation coefficients larger than 0.7 were selected, the majority of age-linked metabolites formed a large network consisting of 42 metabolites (**Supplemental Fig. S1)**. Since all age-linked metabolites displayed p-values < 0.05 in dot plot profiling, the fact that the great majority (42/55 = 76%) form such a large age-linked correlation network is quite impressive.

Some age-linked metabolites were not highly correlated

On the other hand, 13 compounds, though they were age-related, showed only weak correlations (<0.7) with the other 42 age-linked urinary metabolites (**Supplemental Fig. S1**). Aspartate, a standard amino acid having an acidic side chain, showed maximal, but inverse correlation with GSSG (-0.67). Aspartate has a correlation coefficient of 0.65 with N-acetyl aspartate, indicating that structural similarity might partly explain the weak correlation. S-adenosyl-homocysteine did not show correlation values higher than 0.41 (N-methyl-adenosine). Although the correlation is rather low, the two compounds exhibit structural similarity. Similarly, lysine, dimethyl-lysine, trimethyl-lysine, and N6-acetyl-lysine did not show the correlation coefficients higher than 0.5 among them. These metabolites diminish significantly in urine of elderly people. Fructose-1,6-diphosphate showed a maximal correlation value (0.58) with pentose-phosphate and gluconate, all carbohydrates,

163 but correlations were low (**Supplemental Fig. S2**). Whether these metabolites
164 decline in elderly subjects as a consequence of aging or as a cause of it, remains to
165 be investigated.
166
167 **Heatmap analysis of urinary metabolites linked to aging**
168 We then employed a heatmap to visualize the quantitative profile of age-
169 linked urinary metabolites in individual subjects (**Fig. 3**). Since most of these
170 metabolites declined in the elderly, matrix color represents metabolite abundance for
171 individual subjects (t-score<40 white, low; 40~50 thin red, slight low; 50~60
172 moderate red, slight high; >60 deep red, high). Elderly samples to the left were
173 mostly white or pale red (lower level), except for myo-inositol and GSSG, which were
174 moderate or deep red (higher level). Thus, a heatmap of urinary metabolites
175 graphically illustrates the degree of urinary metabolite aging for individual subjects,
176 so that subjects may be compared one with another. Two subjects (elderly 11, 81F
177 and young 15, 33M) showed patterns remarkably like those of the opposite age
178 group.
179
180 **Principal component analysis of age-linked metabolites**
181 Implications of urinary compounds in aging should also be cross examined
182 using results of blood compound analyses. However, as these compounds decrease
183 in urine of elderly people, the results are consistent with actual aging. Some urinary
184 metabolites may be implicated in sustaining health and slowing aging. To integrate
185 quantitative metabolite abundance data from individual subjects, abundances of

these 55 age-related metabolites were subjected to principal component analysis (PCA) (Nakamura et al., 1988) (Kameda et al., 2020). Subjects were separated into 2 groups (red for elderly and blue for young subjects) represented by negative and positive values of principal component (PC) 1, respectively (**Fig. 4A**). One elderly subject 11 (81F) was in the middle of the young group and one young subject 15 (33M) was in the group of elderly subjects in the PCA plot (**Fig. 4A**). These are consistent with results plotted in the heatmap (**Fig. 3**). The PC1 score and chronological age of the subject showed a high correlation with r=0.80. (**Fig. 4B**).

Negative correlation between GSSG and age-linked metabolites

GSSG showed significant negative correlations with some age-linked metabolites (N-acetyl aspartate, N-methyl guanosine, dimethyl guanosine, pseudouridine, quinolinic acid, creatinine, N-methyl-histidine, etc.) (**Fig. 5A**). While the oxidized disulfide form was detected in elderly samples, but the reduced form was not detected in young or elderly samples. Thus only the inactive form of glutathione was detected in elderly samples. A possible interpretation is that the active reduced form glutathione (GSH) may be very reactive and short-lived as a metabolite (see Discussion). The abundance of GSSG in 27 subjects is shown together with N-acetyl aspartate, N-methyl guanosine, dimethyl guanosine, pseudouridine, and chronological age of subject (**Fig. 5B-F**). GSSG was low to undetectable in 5 young subjects, but accumulated in the elderly samples (**Fig. 5F**).

GSSG showed different degrees of abundance in different subjects, and was virtually absent in young subjects, but in elderly subjects, its abundance

209 increased and paralleled the abundance of creatinine in young subjects. Thus GSSG
210 abundance was inversely related to aging. The reason for this increase of GSSG in
211 elderly urine is probably due to the absence or declining effectiveness of a
212 mechanism to metabolize and reprocess GSSG by the elderly. Such a reductive
213 mechanism does exist in young subjects; however, the redox environment regarding
214 glutathione appears to be altered during aging. Inability to reduce oxidized GSSG
215 may accelerate aging.
216

Discussion

We initially investigated metabolomics of a simple model eukaryote, fission yeast (*Schizosaccharomyces pombe*) using yeast genetic technology to identify and determine metabolite profiles in wild type and mutant cell extracts (Pluskal et al., 2010b) (Pluskal et al., 2016) (Pluskal and Yanagida, 2016a) (Pluskal and Yanagida, 2016b). By adapting the software MZmine 2 for metabolite identification (Pluskal et al., 2010a) (Pluskal et al., 2012), we found that metabolomics of fission yeast and humans are surprisingly similar in regard to metabolite composition (Chaleckis et al., 2014). Metabolites detected in fission yeast and human whole blood were 75% identical. Given this unexpected similarity, we adapted our techniques to human blood metabolites to better understand health, disease, and longevity (Chaleckis et al., 2014) (Chaleckis et al., 2016) (Teruya et al., 2019) (Kameda et al., 2020).

Distinction of aging information between blood and urine.

Urinary metabolites offer a non-invasive means of obtaining aging information. The present results indicate that urinary aging information may be distinct from that obtainable from blood. Blood metabolites derived from plasma and red blood cells of elderly subjects reflect a decrease in antioxidant production and muscle activity or increasing inefficiency of nitrogen metabolism (Chaleckis et al., 2016). Aging information obtained from urine will be useful, as human aging is exceedingly complex. In the present study, we identified 55 human urinary aging markers using non-targeted, comprehensive LC-MS. Since urine contains 99 compounds, many urinary metabolites such as vitamins (pantothenate and

nicotinamide) and organic acids (citrate, malate) neither significantly decreased nor increased so that about a half of urinary metabolites are not age markers. In aged subjects, levels of protein, nucleic acid and lipid synthesis, modification, and turnover may decrease due to reduced physical activity (Hughes et al., 2002) (Pollack et al., 2002) (Maynard et al., 2015), resulting in declining metabolite abundances. Metabolite data may be considered as an overview of the physiological state of all tissues and organs. Hence these 55 metabolites constitute a panorama of human aging as seen through urine composition. Alternatively, these may not represent actual human aging, but may represent voluntary lifestyle changes that may be reversible regardless of actual age.

Myoinositol, GSSG and pseudouridine.

Two exceptional urinary age metabolites, myo-inositol and presumably inactive, oxidized GSSG increased in elderly samples (1.34- and 6.96-fold, respectively). Myo-inositol becomes an important second messenger if phosphorylated, causing changes in [Ca^{2+}] (Berridge, 1993) (Berridge, 2016). In the correlation analysis, myo-inositol was not highly correlated with any other metabolite. Glycerophosphocholine and N-methyl-adenosine showed weak, negative correlations with myo-inositol (-0.47and -0.43, respectively, **Supplemental Fig. S2**). Implications of the myo-inositol increase in elderly urine remain unclear. GSSG that we detected was the inactive, oxidized form. It increased in elderly urine. The active SH form of glutathione seems to be very unstable and it is difficult to prevent its degradation during preparation of urine as well as blood samples (Chaleckis et al.,

2016, the present study). The active SH form was rapidly oxidized to an inactive disulfide form, which accumulated in elderly subjects, whereas in young subjects the disulfide form was hardly formed or undetected possibly due to rapid decay in the young body or sample. Consistently, reduced GSSG was undetectable in urine of the five young subjects **Fig. 2D** and **Fig. 5B-F**). The abundance of GSSG was inversely related to those of pseudouridine, creatinine, and 2-hydroxybutyrate, which were most abundant in young subjects. Thus GSSG in urine appears to be appropriate as an age marker in urine of elderly subjects. In contrast, the high abundance of pseudouridine seems to be emblematic of youth.

Highly correlated urine metabolites.

Fifteen metabolites are highly correlated (correlation coefficients >0.85, **Fig. 2A, B**). They consisted of two subgroups. One comprised nucleosides pseudouridine, dimethyl guanosine, N-methyl guanosine and 2- or 3-hydroxybutyrate and the other group contained branched-chain amino acids and aromatic amino acids. Correlation within each group was high, but was not always high between the two subgroups (0.65 for pseudouridine and methionine, and 0.56 for glycerol phosphate and isoleucine; **Supplemental Fig. S2**). It remains to be determined what kind of functional distinctions exist between these two subgroups of metabolites in health and disease. In addition, a number of compounds showing correlation coefficients >0.70 are structurally only remotely related. Therefore, the high correlations of urinary compounds cannot all be easily explained. While isoleucine, leucine, and valine contain hydrophobic side chains, glutamine, having a hydrophilic sidechain,

showed correlation coefficients of 0.88-0.89 with isoleucine. Pseudouridine was highly correlated with 10 other compounds (>0.82), although we are unable to offer an explanation for this at present. The high correlation (0.83) between 2-hydroxybutyrate and 3-hydroxybutyrate may be explained by their structural relatedness, but their correlations in the range of 0.6-0.82 with 10 other compounds are difficult to explain.

Thirteen metabolites linked by low correlation.

Thirteen metabolites linked by low correlation coefficients are shown in **Supplemental Fig. S2.** They include myo-inositol, two regular amino acids (aspartate and lysine), methylated or acetylated amino acids, etc. Creatinine, an abundant aging marker (Kampmann et al., 1974) and an amino acid waste product in muscle (or other tissues) showed moderate correlations (**Fig. 2C**). Its urinary content decreased to 43% in elderly samples (**Table 1**). More than half (31) of the declining age markers in urine were amino acids (standard, methylated, acetylated, and other), and 6 more are nucleosides, nucleobases, and their derivatives. Protein degradation and nucleic acid turnover seem to cause the change in elderly urine. Pseudouridine is a tRNA component (Charette and Gray, 2000), perhaps important in catabolism of nucleosides and other compounds, as it is highly correlated with purine nucleosides (adenosine, guanosine, and inosine), muscle amino acids (isoleucine, creatinine), and organic acids (hydroxybutyrates, glycerol-phosphate). Pseudouridine is also abundant in blood, but the amount in urine is 15-fold higher. Pseudouridine showed very high correlation coefficients with 10 compounds (**Fig. 2A, B**) and its high abundance in urine may provide a convenient youth marker.

An effective overview on aging by heatmap.

We showed that heatmap analysis of age-linked metabolites provides an effective overview of aging patterns using urinary metabolite profiles of individual subjects. Heatmap patterns are convenient for visualizing differences between young and aged subjects and also individual variations within and between groups (**Fig. 3**). PCA was useful to categorize a group of subjects because of its capacity to integrate a large collection of data about various metabolites. Elderly and young subjects were clearly separated into two populations, with two exceptions (**Fig. 4**). Exceptional individuals are of considerable interest, regarding their persistent youth or premature aging, if these truly represent metabolic features. Their individuality may be worthy of further investigation. Among 55 age-related urinary markers, 13 compounds did not show correlations >0.7 (**Supplemental Fig. S1**). We determined the correlations of these 13 compounds with all metabolites. Age-related metabolites such as myo-inositol, S-adenosyl-homocysteine, N-methyl-adenosine, lysine, trimethyl-lysine, fructose-1,6-diphosphate, and glycerophosphocholine are only weakly correlated (~0.6) (**Supplemental Fig. S2**). Thus, high correlation is not necessarily required of age-related compounds. These metabolites that are only remotely correlated are of interest to better understand the metabolic breadth of human aging.

329 **Table 1. List of 55 aging markers.**

330

Category /Compound	Peak abundance	Ratio (elderly/young)
Nucleosides, nucleobases, and derivatives (6)		
Dimethyl-guanosine	H-M	0.63
N-Methyl-adenosine	H-M	0.56
Pseudouridine	H-M	0.45
N-Methyl-guanosine	M	0.51
Adenosine	M-L	0.61
Hypoxanthine	M-L	0.50
Sugar derivatives (4)		
Gluconate	H-M	0.67
N-Acetyl-glucosamine	H-M	0.70
1,5-Anhydroglucitol	M-L	0.45
myo-Inositol	M-L	1.34
Sugar phosphates (5)		
Glycerol-phosphate	H-L	0.32
Fructose-1,6-diphosphate	L	0.14
Fructose-6-phosphate	L	0.33
Pentose-phosphate	L	0.65
Phosphoglycerate	L	0.46
Choline and ethanolamine derivatives (2)		
Glycerophosphocholine	M-L	0.50
Glycerophosphoethanolamine	L	0.44
Carnitines (4)		
(iso)Butyryl-carnitine	H-M	0.61
(iso)Valeryl-carnitine	H-L	0.66
Decanoyl-carnitine	M-L	0.49
Octanoyl-carnitine	M-L	0.40
Organic acids (2)		
2-Hydroxybutyrate	M-L	0.33
3-Hydroxybutyrate	M-L	0.27
Antioxidant (1)		
Glutathione disulfide	L	6.96
Standard amino acids (13)		
Histidine	H-M	0.68
Phenylalanine	H-M	0.53
Tryptophan	H-M	0.49
Asparagine	M-L	0.73
Glutamine	M-L	0.46
Leucine	M-L	0.42
Lysine	M-L	0.50
Methionine	M-L	0.74
Serine	M-L	0.54
Threonine	M-L	0.71
Aspartate	L	0.62
Isoleucine	L	0.38
Valine	L	0.30
Methylated amino acids (5)		
Dimethyl-arginine	H	0.69
N-Methyl-histidine	H	0.64
Trimethyl-lysine	H	0.74
Dimethyl-lysine	H-M	0.52
Butyro-betaine	M-L	0.76

331 **Table 1. List of 55 aging markers.** (continued)

Category /Compound	Peak abundance	Ratio (elderly/young)
Acetylated amino acids (4)		
N-Acetyl-arginine	H-M	0.78
N2-Acetyl-lysine	M	0.62
N6-Acetyl-lysine	M	0.51
N-Acetyl-aspartate	M-L	0.50
Other amino acids (9)		
Creatinine	H	0.43
Indoxyl-sulfate	H	0.56
4-Guanidinobutanoate	H-M	0.46
Taurine	H-M	0.52
Carnosine	M-L	0.34
Keto(iso)leucine	M-L	0.36
Kynurenine	M-L	0.45
Quinolinic acid	M-L	0.44
S-Adenosyl-homocysteine	L	0.75

332

333 The list of 55 aging-linked compounds that showed a significant difference in
334 abundance between young and elderly people. The abundance of compounds (peak
335 area) displayed: H, high peak areas [>10^8 AU (arbitrary unit)]; M, medium peak areas
336 (10^8~10^7 AU); L, low peak areas (<10^7 AU). The peak ratio was calculated using the
337 median of peak abundance in elderly and young people, respectively.

338

Materials and Methods

Participants and sample collection

14 elderly (69~81 yr) and 13 young (25~38 yr) healthy people participated as subjects in this study (**Supplemental Table S1**). Measurements of metabolites in first morning urine are more consistent than random daytime sampling to monitor metabolites. Intra-individual coefficients of variation in first morning urine and 24-h collected urine are reportedly similar (Witte et al., 2009).

After collection, urine samples were brought to the laboratory within 3 hr 0.2 mL urine were immediately quenched in 1.8 mL of 55% methanol at -40°C. This quenching step stabilizes metabolites and maximizes reproducibility of metabolomic data. Two internal standards (10 nmol of HEPES and PIPES) were added to each sample. After brief vortexing, samples were transferred to Amicon Ultra 10-kDa cut-off filters (Millipore, Billerica, MA, USA) to remove proteins and cellular debris. After sample concentration by vacuum evaporation, each sample was re-suspended in 40 µL of 50% acetonitrile, and 1 µL was used for each injection into the LC-MS system, as described (Chaleckis et al., 2016) (Kameda et al., 2020).

Ethics statement

Written, informed consent was obtained from all donors, in accordance with the Declaration of Helsinki. All experiments were performed in compliance with relevant Japanese laws and institutional guidelines. All protocols were approved by the Human Subjects Research Review Committee of the Okinawa Institute of Science and Technology Graduate University (OIST).

363 **Chemicals and reagents**

364 Standards for metabolite identification were purchased from commercial sources as
365 described previously (Pluskal et al., 2010b) (Chaleckis et al., 2014) (Chaleckis et al.,
366 2016) (Teruya et al., 2019).

367

368 **LC-MS analysis and data processing**

369 Urinary metabolites were analyzed using an Ultimate 3000 DGP-3600RS HPLC
370 system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an LTQ Orbitrap
371 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), as described
372 (Chaleckis et al., 2016) (Kameda et al., 2020). Briefly, LC separation was performed
373 on a ZIC-pHILIC column (Merck SeQuant, Umea, Sweden; 150 mm x 2.1 mm, 5 µm
374 particle size). Acetonitrile (A) and 10 mM ammonium carbonate buffer, pH 9.3 (B)
375 were used as the mobile phase, with a linear gradient elution from 80-20% A over 30
376 min, at a flow rate of 100 µL mL^{-1}. The mass spectrometer was operated in full-scan
377 mode with a 100-1000 m/z scan rate and automatic data-dependent MS/MS
378 fragmentation scans. For each metabolite, we chose a singly charged, [M+H]+ or [M-
379 H]-, peak (**Supplemental Table S3**). Peak detection and identification of metabolites
380 were performed using MZmine 2 software (Pluskal et al., 2010a). Detailed data
381 analytical procedures and parameters have been described previously (Teruya et al.,
382 2019).

383

384 **Peak identification and characteristics**

385 We analyzed 99 urine metabolites that were confirmed using standards or MS/MS

386 analysis (Pluskal et al., 2010b) (Chaleckis et al., 2014) (Chaleckis et al., 2016)
387 (Teruya et al., 2019). Metabolites were classified into 3 groups (H, M, and L),
388 according to their peak areas. H denotes compounds with high peak areas (>10^8
389 AU), M with medium peak areas (10^7 ~10^8 AU) and L with low peak areas (<10^7 AU)
390 (**Supplemental Table S2**).
391
392 **Statistical analysis**
393 A non-parametric Mann–Whitney test was used to compare young and elderly
394 subjects. Statistical significance was established at $p<0.05$. Data were exported into
395 a spreadsheet and dot plots were drawn using R statistical software (http://www.r-
396 project.org). Correlation coefficients were determined for identification of metabolic
397 networks among compounds. Principal component analysis (PCA) was conducted
398 using SIMCA-P+ software (Umetrics Inc., Umea, Sweden). Pearson's correlation
399 coefficients among metabolites were calculated using Microsoft Excel.
400
401 **Data availability**
402 Raw LC-MS data in mzML format are accessible via the MetaboLights repository
403 (URL: http://www.ebi.ac.uk/metabolights). Data for the 27 volunteers are available
404 under accession number MTBLS1407.
405
406
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Declaration of Interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Dot plots of 55 compounds that showed significant differences in abundance between elderly and young people. Distributions of 11 subgroups of 55 metabolites in urine samples from 27 individuals. Pale red and azure dots represent elderly and young subjects, respectively. Bars represent medians in each group. The ratio of median value between elderly and young is shown in **Table 1.** P-values were obtained using the non-parametric Mann Whitney U test. Fifty-three of 55 compounds were more abundant in young subjects, while two (myo-inositol and GSSG) were more abundant among the elderly.

Figure 2. Correlation network among age-linked compounds. (A) Values indicate correlation coefficients between paired compounds. Highly correlated pairs of aging markers (r>0.85) are indicated with red circles. The most highly correlated pairs are indicated by green (0.92) and red arrows (0.91). (B) Interrelated compounds form a network. Correlations (r>0.90) are highlighted in yellow. (C) Fifteen metabolites with the highest correlation with creatinine are listed. (D) Scatter plot of the peak abundance (x10^6 AU) between creatinine and GSSG. GSSG was negatively correlated with creatinine.

Figure 3. Heatmap representing urinary metabolic profiles of elderly and young subjects. Standardized data for each metabolite are shown for 27 subjects using a color matrix representing relative abundance data of 55 urinary aging markers. Numerical values indicate the t-score, a kind of standardized score. The mean and

569 standard deviation are 50 and 10, respectively. Color intensity of the cells reflects the
570 t-score, indicating levels higher than average.
571
572 **Figure 4.** PCA of 55 aging markers. (A) Thirteen young and 14 elderly subjects are
573 shown in blue and red, respectively, with their subject numbers (corresponding to
574 **Fig. 3**). PC2 comprised metabolites that were not strongly correlated
575 (glycerophosphocholine, S-adenosyl-homocysteine, etc.), but that were isolated from
576 a strong correlation network. (B) PC1 score of each subject (X-axis) is plotted versus
577 subject age (Y-axis). The correlation coefficient is shown in the box.
578
579 **Figure 5.** Scatter plots between GSSG and correlated compounds or age.
580 (A) Ten metabolites with the highest correlations with GSSG are listed. Scatter plot of
581 the peak abundance (x10^6 AU) between GSSG and N-acetyl aspartate (B), N-methyl
582 guanosine (C), dimethyl guanosine (D), pseudouridine (E), and the age of the
583 subject (F).
584
585

Figure 1.

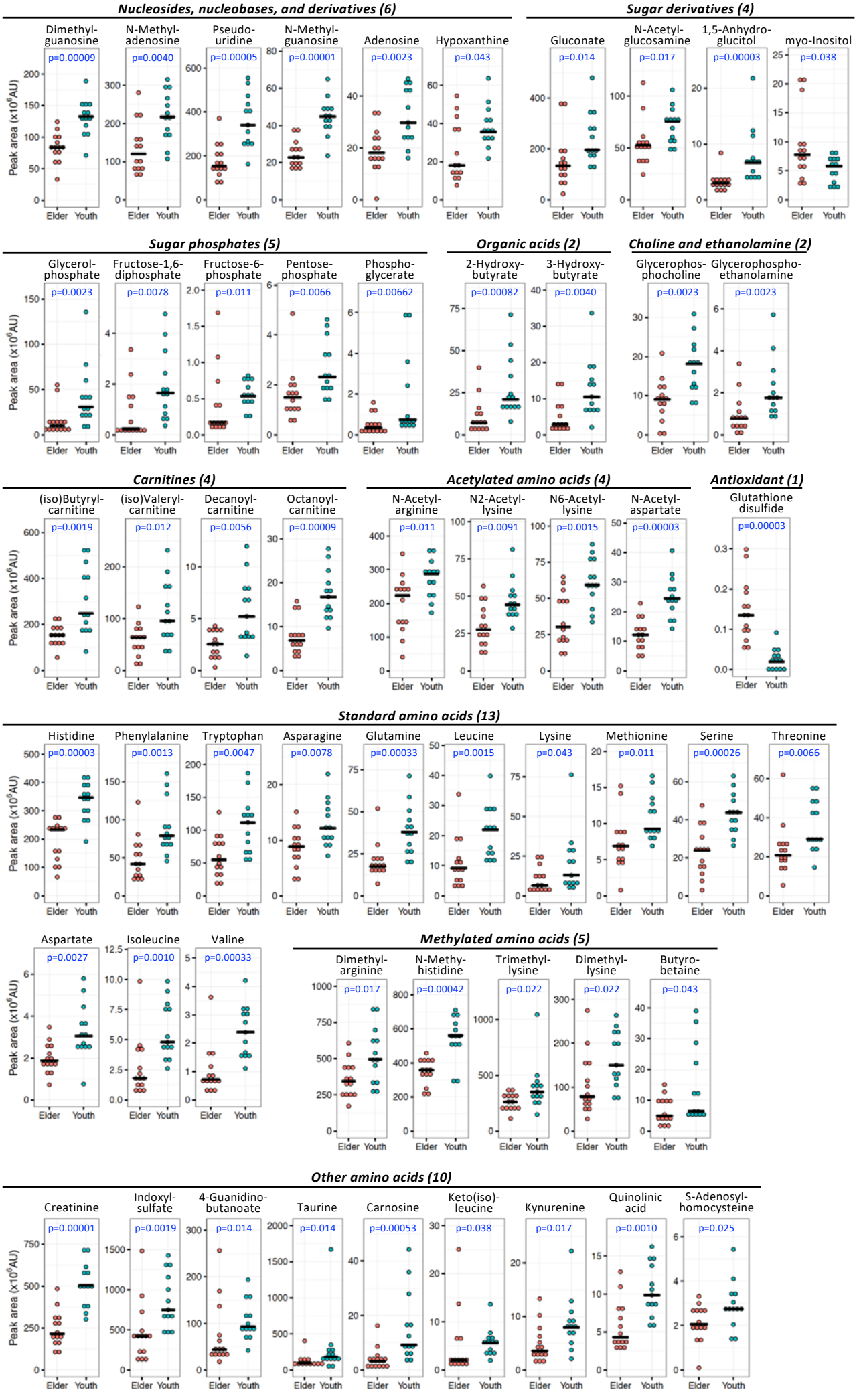
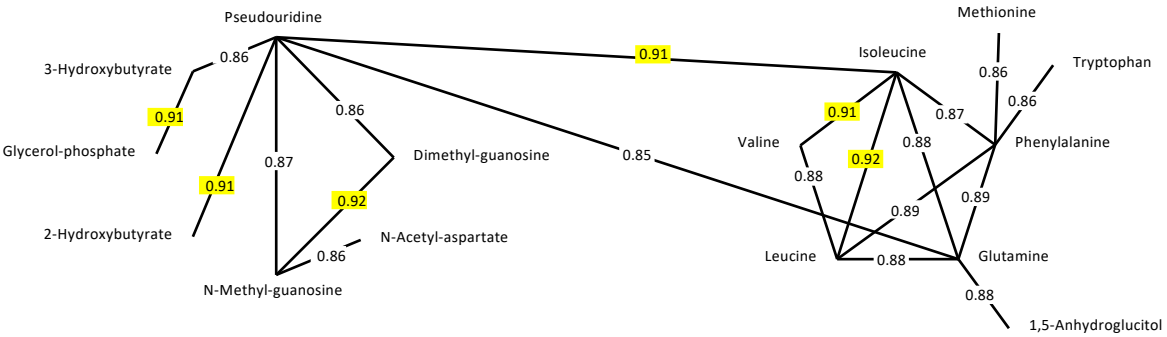


Figure 2.

A

B



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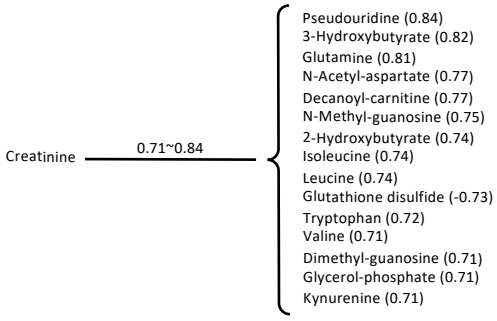

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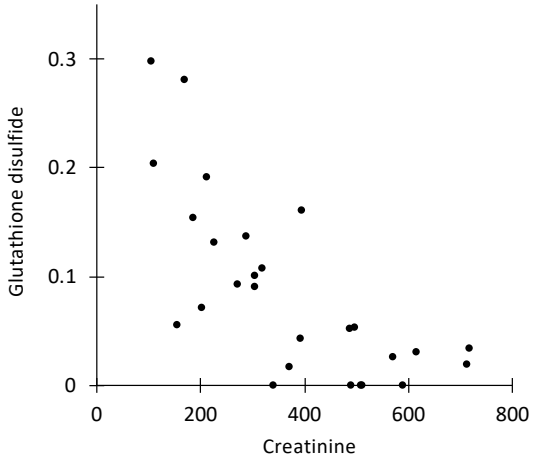
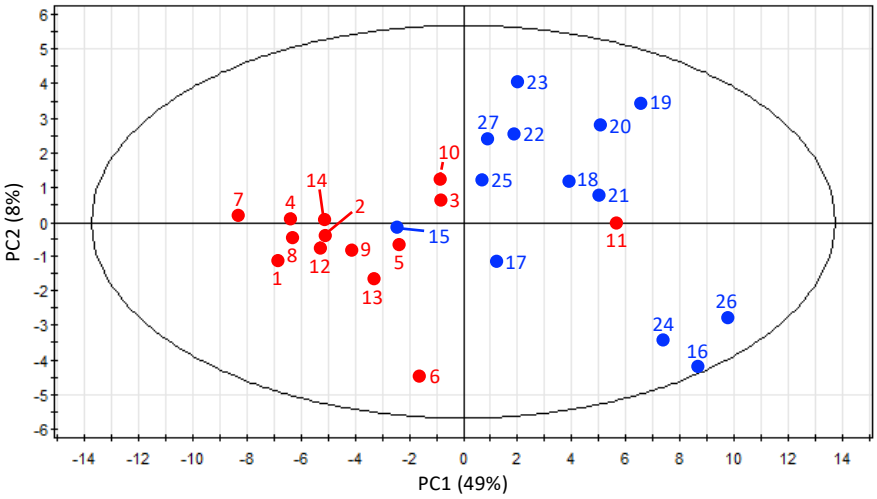


Figure 3.

Category	Suject No. Age/Gender	1 75M	2 79M	3 78M	4 69F	5 78F	6 70F	7 69F	8 77F	9 74F	10 76F	11 81F	12 80F	13 80F	14 75F	15 33M	16 38M	17 29M	18 30M	19 30M	20 34M	21 30M	22 33F	23 29F	24 31F	25 29F	26 25F	27 27F
Antioxidant	Glutathione disulfide ↑	75.1	73.0	52.5	62.4	51.6	56.0	63.8	55.2	57.9	46.3	45.9	48.1	58.7	50.7	50.5	41.9	46.0	42.8	39.6	43.8	43.3	39.6	44.8	41.6	39.6	39.6	39.6
Sugar derivative	myo-Inositol ↑	54.5	42.6	52.8	40.5	49.1	72.6	41.6	75.7	50.0	47.4	51.5	54.2	76.1	46.1	39.4	52.0	40.4	41.3	50.6	44.7	46.9	49.0	44.3	48.6	48.1	50.1	40.0
Nucleosides, nucleobases	Dimethyl-guanosine	37.6	40.6	48.1	42.0	45.7	36.2	29.0	43.5	48.3	51.8	55.0	43.9	43.2	43.5	39.9	63.4	50.1	57.3	73.2	58.4	48.7	59.6	62.4	55.6	56.6	62.9	53.4
	N-Methyl-adenosine	36.4	56.3	39.6	55.8	37.7	41.2	44.8	37.8	35.3	48.1	63.6	38.1	47.5	46.2	55.4	55.1	51.2	56.7	61.8	43.2	68.1	59.2	64.9	48.9	49.9	41.1	66.2
	Pseudouridine	36.2	39.0	49.6	40.7	46.0	45.9	36.6	42.4	40.7	48.9	58.0	41.2	40.9	43.4	42.5	69.8	50.2	55.8	71.5	65.5	60.1	50.1	53.5	60.7	48.6	62.6	49.9
	N-Methyl-guanosine	38.1	39.2	47.6	36.5	45.3	41.5	39.9	38.2	44.8	52.4	52.6	41.6	44.3	36.1	42.2	61.7	50.0	58.3	73.1	57.3	55.5	60.6	58.1	54.8	60.7	66.4	53.3
	Adenosine	38.4	41.7	41.4	41.5	50.4	42.1	39.5	45.2	49.2	56.5	56.8	45.1	28.5	47.8	41.8	67.2	48.1	66.0	51.9	49.0	49.4	63.4	58.5	68.0	44.1	64.9	53.6
	Hypoxanthine	37.1	38.2	61.0	40.9	54.2	35.7	53.1	40.9	45.2	58.1	65.3	37.7	39.0	33.9	43.4	52.6	50.2	53.9	57.3	56.5	52.7	71.4	63.1	47.2	60.5	51.3	49.7
Sugar derivatives	Gluconate	37.7	43.3	50.3	34.2	41.5	48.7	38.3	45.4	40.1	66.4	66.0	43.1	44.9	46.7	44.3	75.5	43.3	48.5	63.5	57.2	54.5	49.3	49.9	49.8	57.6	62.8	47.2
	N-Acetyl-glucosamine	37.9	45.1	43.9	45.3	49.1	32.0	43.4	47.3	45.6	61.7	73.2	45.9	37.0	39.0	42.4	48.9	44.3	57.3	63.4	47.6	70.0	55.9	55.8	52.8	46.6	61.1	57.4
	1,5-Anhydroglucitol	43.4	42.2	44.8	45.2	43.8	44.7	40.4	41.8	42.7	46.8	56.9	45.7	45.5	43.5	52.8	66.3	47.1	54.1	64.1	53.8	52.3	46.0	47.3	54.7	49.2	88.1	46.5
Sugar phosphates	Glycerol-phosphate	42.1	42.7	59.4	42.9	44.6	45.1	42.7	42.4	44.4	44.7	57.4	43.3	41.7	45.9	43.5	61.1	49.6	47.7	51.0	86.9	54.3	47.6	54.9	55.0	43.8	67.1	48.2
	Fructose-1,6-diphosphate	41.1	41.3	43.3	40.8	40.5	51.6	41.4	40.9	40.4	50.6	65.5	40.6	48.3	57.9	48.2	70.2	65.1	46.0	58.3	53.4	42.3	53.1	76.5	44.5	51.6	52.3	44.3
	Fructose-6-phosphate	41.1	41.8	66.4	39.8	41.8	47.7	40.0	39.0	41.1	48.5	83.1	41.6	57.2	40.7	43.3	58.7	51.5	49.9	55.0	44.8	58.6	57.4	59.3	51.3	48.9	52.5	49.2
	Pentose-phosphate	36.6	39.9	51.1	37.1	48.4	41.6	41.1	45.7	46.2	49.2	72.9	46.3	41.4	43.7	43.2	58.9	49.9	51.6	68.8	66.1	52.3	47.4	70.8	48.1	47.7	59.3	44.5
	Phosphoglycerate	43.8	43.6	49.7	44.3	45.5	45.8	44.3	43.6	44.1	46.4	50.9	44.2	52.9	45.8	44.9	80.5	47.1	48.4	46.4	58.2	47.4	47.5	46.4	65.8	46.6	80.2	45.7
Cholines, ethanolamines	Glycerophosphocholine	44.5	51.8	60.4	42.5	44.9	40.6	45.6	37.3	49.6	48.3	32.9	46.1	33.6	44.0	50.1	43.1	49.6	63.3	73.8	54.0	61.6	56.8	68.8	44.1	57.6	48.1	57.0
	Glycerophosphoethanolamine	39.4	41.1	56.6	40.9	44.5	44.6	41.2	46.6	49.7	41.9	64.1	43.9	38.8	45.0	44.2	46.7	45.5	51.8	51.1	61.7	49.3	56.9	81.9	52.5	47.1	69.6	53.5
Carnitines	(iso)Butyryl-carnitine	41.9	47.3	46.4	43.7	46.9	41.8	41.6	45.2	50.2	49.7	41.6	44.0	44.9	37.0	48.7	56.9	50.7	73.1	45.3	51.8	63.1	46.9	39.0	72.6	46.2	68.9	64.4
	(iso)Valeryl-carnitine	49.4	47.0	57.2	37.4	43.4	39.0	45.5	46.7	49.1	42.4	41.8	46.6	51.2	35.8	52.1	47.7	55.3	64.4	57.7	51.7	77.5	41.2	41.0	64.7	47.9	69.7	46.3
	Decanoyl-carnitine	48.8	42.2	49.3	40.2	44.8	39.4	41.5	44.4	40.5	50.8	48.7	46.1	50.0	36.8	40.6	59.0	47.1	63.0	64.0	77.6	71.6	46.6	47.2	53.9	47.5	60.1	48.4
	Octanoyl-carnitine	53.5	43.9	52.1	38.1	38.1	36.3	40.4	40.2	44.4	54.9	42.8	40.8	44.1	36.6	52.6	58.6	45.8	50.0	65.2	72.6	58.4	51.3	61.0	69.8	48.8	56.5	53.2
Organic acids	2-Hydroxybutyrate	40.1	41.4	54.4	40.9	45.5	42.7	40.6	41.3	42.9	48.0	62.4	43.6	46.8	42.3	43.2	70.4	48.3	51.2	81.0	64.9	52.5	47.9	53.0	49.4	48.1	59.4	47.7
	3-Hydroxybutyrate	40.2	40.4	57.7	41.8	45.4	48.9	40.4	40.0	42.2	42.8	56.9	42.7	49.1	41.9	41.3	63.1	47.6	52.6	64.6	83.5	57.6	48.4	48.4	56.6	46.9	58.9	50.1
Standard amino acids	Histidine	48.3	51.5	46.9	39.8	48.7	47.7	33.5	30.3	48.0	36.7	51.5	34.0	48.6	39.1	50.7	42.9	50.2	59.4	60.1	58.7	65.4	53.9	53.9	63.8	62.5	66.3	57.5
	Phenylalanine	39.2	46.1	43.1	38.9	49.2	53.4	37.6	36.9	46.8	49.8	64.5	41.1	43.0	38.5	44.1	74.5	56.2	52.9	53.6	49.5	55.8	50.1	49.8	67.5	51.7	70.6	45.9
	Tryptophan	41.8	44.8	51.3	40.3	49.8	52.5	35.2	35.4	48.7	42.3	60.2	38.9	48.7	38.0	45.9	70.5	49.8	58.8	56.6	61.5	59.1	46.4	52.7	73.8	44.4	59.6	42.8
	Asparagine	44.0	49.0	45.2	37.1	49.3	60.2	31.5	33.1	54.6	46.4	53.4	41.9	46.7	40.9	42.0	66.1	45.6	50.3	58.6	48.7	53.4	53.9	47.1	75.8	61.1	63.9	50.1
	Glutamine	41.8	45.5	40.9	42.2	44.7	50.6	35.8	40.1	42.2	46.0	64.3	42.7	41.3	43.4	43.5	68.7	54.2	57.8	60.0	57.1	63.6	47.5	44.7	55.5	50.7	76.7	48.7
	Leucine	36.8	42.1	46.7	43.0	44.6	54.0	36.8	38.6	43.6	46.0	68.2	39.8	37.7	52.5	46.3	74.4	57.7	48.7	56.7	56.3	63.0	50.3	46.6	63.5	48.1	62.8	45.3
	Lysine	49.2	53.4	47.3	42.0	56.1	47.2	42.3	43.3	43.3	42.5	56.0	44.3	44.5	43.1	46.3	62.1	90.0	45.3	44.7	54.7	48.6	44.5	54.0	59.1	42.7	58.7	44.7
	Methionine	42.7	49.4	44.7	37.5	49.6	66.1	39.1	39.5	49.5	44.3	63.4	44.9	28.4	39.8	44.5	61.7	59.7	56.5	57.2	47.2	50.4	49.2	50.7	67.5	47.6	69.7	49.4
	Serine	48.8	43.9	40.6	39.1	45.1	54.2	33.7	36.2	44.5	53.1	59.6	38.1	30.6	44.9	45.9	61.1	47.8	66.6	57.3	55.0	57.3	54.0	50.8	69.6	57.1	63.2	51.9
	Threonine	46.1	44.8	48.6	42.9	47.1	72.5	33.0	39.2	42.7	48.4	54.5	41.1	42.8	38.9	39.5	58.5	49.5	63.6	62.2	49.7	59.0	45.5	46.4	68.3	49.0	66.9	49.1
	Aspartate	34.5	40.0	43.4	44.7	50.4	42.0	38.5	43.3	46.8	57.1	52.3	41.9	44.9	49.0	49.9	34.9	48.5	71.5	54.6	50.7	50.3	76.2	48.7	53.6	58.4	58.8	65.1
	Isoleucine	37.4	42.0	51.2	42.9	44.7	50.7	38.2	39.2	40.1	49.7	70.3	41.2	38.7	41.4	44.6	70.3	50.5	53.9	67.3	52.2	63.5	51.0	47.4	62.0	51.1	62.0	46.8
	Valine	38.0	41.3	45.5	42.1	43.2	50.2	38.1	40.4	41.5	49.2	67.3	40.5	37.8	41.4	63.2	72.5	48.3	53.1	62.6	56.2	59.4	50.0	48.8	61.3	49.4	64.0	44.9
Methylated amino acids	Dimethyl-arginine	35.0	46.5	54.9	45.0	50.6	43.0	38.6	40.5	51.0	40.1	49.2	44.4	59.4	39.9	53.3	55.7	60.2	40.4	45.0	64.5	60.2	52.7	43.4	72.0	41.0	73.0	50.8
	N-Methyl-histidine	36.4	44.5	48.1	33.7	47.9	51.0	35.0	43.6	42.7	48.0	48.3	41.5	44.6	43.3	38.8	57.4	58.5	68.5	63.0	53.6	55.2	58.1	54.5	66.6	40.4	66.5	60.3
	Trimethyl-lysine	41.8	42.3	49.9	41.8	44.0	49.5	37.5	53.4	46.8	51.4	42.8	50.2	48.7	45.6	46.5	49.1	54.9	54.2	49.4	55.3	39.6	60.1	51.6	91.8	49.2	57.0	45.3
	Dimethyl-lysine	35.1	42.9	47.3	41.2	52.3	39.5	38.0	38.6	40.4	41.7	69.6	53.4	59.2	45.6	49.5	47.9	63.3	41.1	64.6	49.5	52.3	42.7	59.7	68.1	45.9	58.4	62.3
	Butyro-betaine	43.5	48.7	49.1	41.2	54.7	41.5	44.0	49.4	49.9	42.8	42.8	52.3	44.7	40.8	61.8	45.8	46.0	51.6	51.9	45.5	75.4	44.3	44.0	68.4	45.4	78.9	45.6
Acetylated amino acids	N-Acetyl-arginine	39.5	26.2	50.4	36.4	56.3	63.8	32.0	38.8	51.3	47.0	51.4	38.6	53.2	51.6	45.5	53.5	57.4	56.6	42.3	48.2	53.5	65.1	57.7	61.1	65.1	58.0	49.5
	N2-Acetyl-lysine	40.2	33.4	57.0	36.6	55.7	44.6	34.3	37.9	48.8	44.8	51.5	41.3	61.5	41.9	48.7	52.8	76.6	53.7	43.7	50.4	48.7	65.6	54.4	58.5	59.6	56.5	51.2
	N6-Acetyl-lysine	37.0	53.7	42.9	49.7	41.1	57.8	33.0	33.9	37.1	56.0	50.3	38.3	50.5	38.7	65.6	63.1	57.2	43.5	54.8	52.4	45.3	68.2	58.2	63.8	47.6	55.4	54.9
	N-Acetyl-aspartate	34.6	37.5	43.4	42.4	44.3	45.4	35.1	39.9	38.9	50.1	55.0	41.8	49.9	45.8	49.1	56.8	45.2	64.0	65.9	53.2	57.6	55.9	47.4	60.9	59.8	74.9	55.2
Other amino acids	Creatinine	35.1	38.7	47.0	41.0	46.3	45.3	35.3	41.8	39.6	37.9	56.4	40.6	51.2	44.3	46.2	69.0	56.9	61.1	57.6	69.3	63.6	48.2	51.1	49.9	57.7	62.1	56.6
	Indoxyl-sulfate	37.2	39.2	45.7	44.0	56.6	51.6	36.6	44.2	39.3	43.7	70.4	36.9	44.5	44.5	44.8	66.7	50.7	50.2	49.9	52.2	58.5	54.9	45.0	69.0	45.9	65.4	62.3
	4-Guanidinobutanoate	39.7	41.4	40.0	40.6	41.8	78.6	37.9	41.3	47.6	58.3	63.9	45.5	42.7	43.1	49.9	49.4	54.4	49.1	47.1	46.6	61.9	51.8	42.0	61.6	68.0	55.0	50.8
	Taurine	45.2	45.3	47.5	46.2	46.7	47.1	45.3	46.1	46.3	47.7	56.4	45.7	48.5	47.0	47.4	97.8	47.6	49.3	54.7	52.3	50.6	45.4	48.7	50.0	44.9	52.3	47.9
	Carnosine	44.2	44.6	49.2	42.6	45.7	46.1	42.4	44.2	42.3	43.1	45.6	42.4	56.1	42.2	56.1	74.3	48.8	67.0	52.7	46.5	49.7	47.1	49.2	56.9	43.9	81.9	45.4
	Keto(iso)leucine	41.4	43.2	52.2	42.5	43.9	52.5	41.4	42.3	43.9	43.9	66.2	43.5	88.0	43.5	43.7	51.6	48.0	47.3	66.1	52.8	54.6	45.8	52.4	51.9	50.6	50.1	46.7
	Kynurenine	49.1	45.3	57.6	39.8	44.1	46.6	40.9	38.1	42.5	41.2	64.4	42.1	52.4	39.6	40.0	83.5	43.5	55.3	52.7	55.5	59.1	46.4	49.6	63.3	51.4	54.0	52.2
	Quinolinic acid	37.0	39.0	49.7	38.9	50.5	39.5	36.7	44.4	38.2	62.1	57.3	39.7	47.0	42.1	44.3	47.3	45.1	56.4	66.1	70.3	64.0	58.1	54.6	51.7	52.1	66.8	51.1
	S-adenosyl-homocysteine	51.4	58.0	44.5	52.4	44.9	26.5	43.5	54.8	43.8	37.7	51.0	39.6	46.5	52.2	52.4	38.4	45.6	51.6	59.4	78.6	55.3	53.2	58.6	52.5	51.8	39.9	65.6

Figure 4.

A


PC2 (8%)
PC1 (49%)
23
19
27
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12
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16
6

B

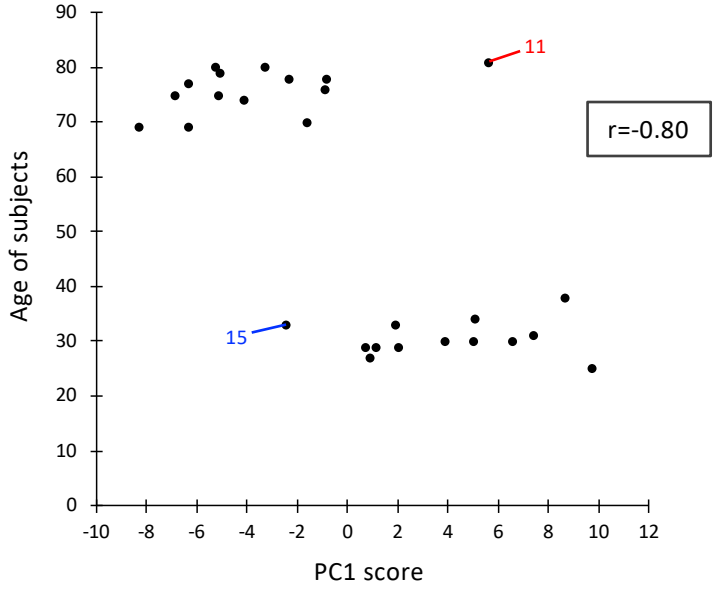
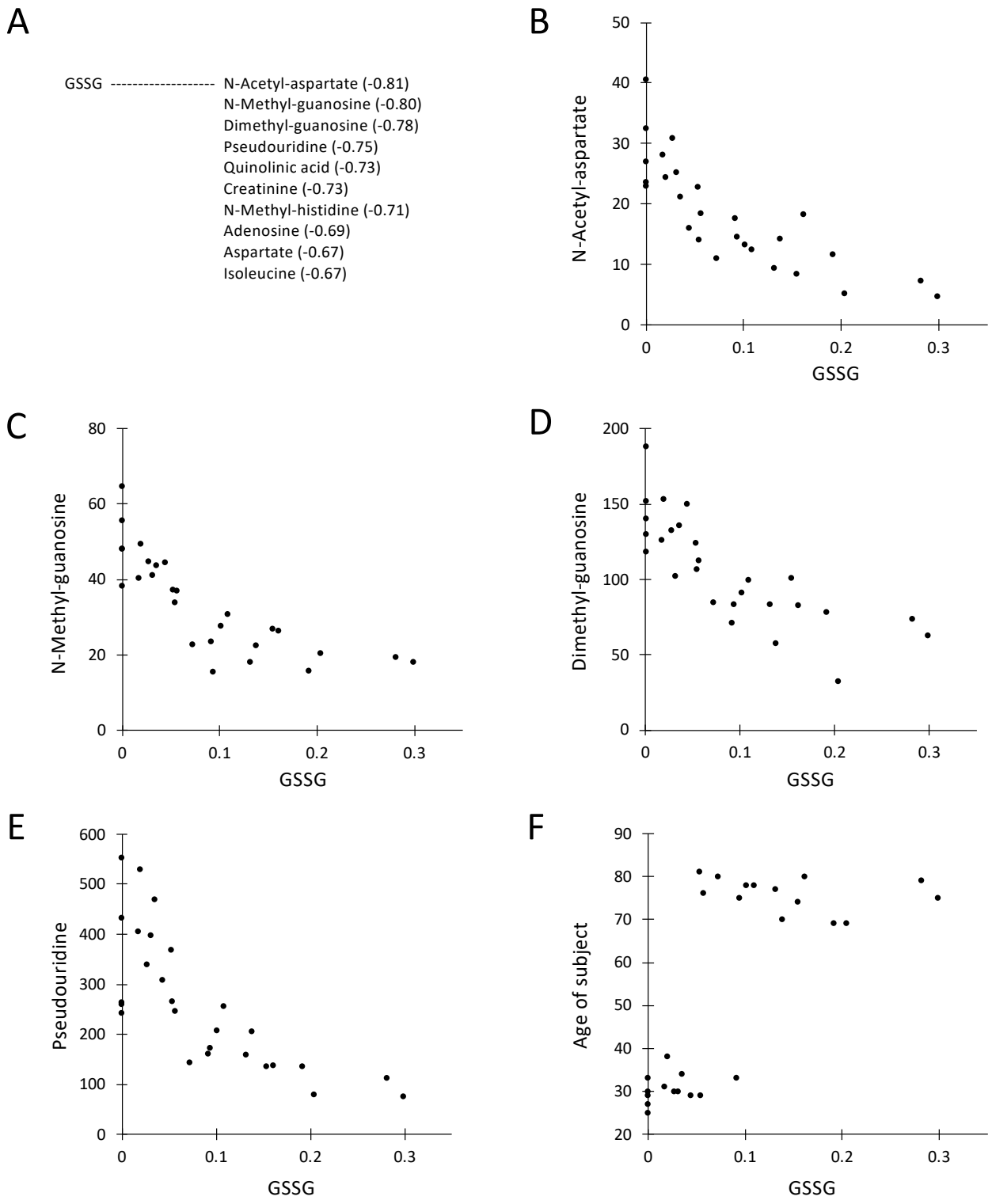

Age of subjects
PC1 score
r=-0.80
11
15

Figure 5.


A
GSSG ---------- N-Acetyl-aspartate (-0.81)
N-Methyl-guanosine (-0.80)
Dimethyl-guanosine (-0.78)
Pseudouridine (-0.75)
Quinolinic acid (-0.73)
Creatinine (-0.73)
N-Methyl-histidine (-0.71)
Adenosine (-0.69)
Aspartate (-0.67)
Isoleucine (-0.67)
B
N-Acetyl-aspartate
GSSG
C
N-Methyl-guanosine
GSSG
D
Dimethyl-guanosine
GSSG
E
Pseudouridine
GSSG
F
Age of subject
GSSG

Supplementary information

Aging markers in human urine: A comprehensive, non-targeted LC-MS study

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Supplementary Table S1. Characteristics of 27 subjects

	Youth(n=13)	Elderly (n=14)	All (n=27)
Age (mean ± standard deviation)	30.6±3.2	75.8±3.9	54.0±22.9
Gender (male/female)	7/6	3/11	10/17
BMI (kg/m^2) (mean ± standard deviation)	22.5±2.7	24.5±3.7	23.5±3.4

Supplemental Table S2. List of 99 urine compounds identified in the present study.

Category /Compound	Peak abundance	p-value elderly/young
Nucleosides, nucleobases, and derivatives (12)		
Dimethyl-guanosine*	H-M	0.00009
N-Methyl-adenosine*	H-M	0.00395
Pseudouridine*	H-M	0.00005
Urate	H-M	0.34988
Dimethyl-xanthine	H-L	0.72031
Xanthine	H-L	0.68485
N-Methyl-guanosine*	M	0.00001
Adenosine*	M-L	0.00228
Caffeine	M-L	0.94295
Cytidine	M-L	0.48795
Hypoxanthine*	M-L	0.04265
Uridine	L	0.07627
Sugar derivatives (4)		
Gluconate*	H-M	0.01448
N-Acetyl-glucosamine*	H-M	0.01675
1,5-Anhydroglucitol*	M-L	0.00003
myo-Inositol*	M-L	0.03766
Sugar phosphates (6)		
Glycerol-phosphate*	H-L	0.00228
Fructose-1,6-diphosphate*	L	0.00780
Fructose-6-phosphate*	L	0.01070
Glucose-6-phosphate	L	0.55018
Pentose-phosphate*	L	0.00662
Phosphoglycerate*	L	0.00662
Vitamins and coenzymes (3)		
Pantothenate	H-M	0.18518
4-Aminobenzoate	H-L	0.48795
Nicotinamide	M-L	1.00000
Choline and ethanolamine derivatives (4)		
Phosphocholine	M	0.32548
Glycerophosphocholine*	M-L	0.00228
Glycerophosphoethanolamine*	L	0.00228
Phosphoethanolamine	L	0.21988
Carnitines (8)		
Carnitine	H-M	0.45827
(iso)Butyryl-carnitine*	H-M	0.00187
(iso)Valeryl-carnitine*	H-L	0.01247
Acetyl-carnitine	H-L	0.65004
Decanoyl-carnitine*	M-L	0.00560
Hexanoyl-carnitine	M-L	0.09448
Octanoyl-carnitine*	M-L	0.00009
Propionyl-carnitine	M-L	0.98097
Organic acids (11)		
Citrate	H-M	0.12786
2-Hydroxybutyrate*	M-L	0.00082
2-Oxoglutarate	M-L	0.42960
3-Hydroxybutyrate*	M-L	0.00395
Arginino-succinate	M-L	0.86738
cis-Aconitate	M-L	0.25884
Citramalate	M-L	0.10476
Glycochenodeoxycholate	M-L	0.42960
Malate	M-L	0.32548
Succinate	M-L	0.11587
Glycerate	L	0.14075
Antioxidant (1)		
Glutathione disulfide*	L	0.00003

Category /Compound	Peak abundance	p-value elderly/young
Standard amino acids (17)		
Arginine	H-M	0.45827
Histidine*	H-M	0.00003
Phenylalanine*	H-M	0.00126
Tryptophan*	H-M	0.00471
Tyrosine	M	0.12786
Asparagine*	M-L	0.00780
Glutamate	M-L	0.40196
Glutamine*	M-L	0.00033
Leucine*	M-L	0.00154
Lysine*	M-L	0.04265
Methionine*	M-L	0.01070
Proline	M-L	0.25884
Serine*	M-L	0.00026
Threonine*	M-L	0.00662
Aspartate*	L	0.00275
Isoleucine*	L	0.00102
Valine*	L	0.00033
Methylated amino acids (12)		
Betaine	H	0.10476
Dimethyl-arginine*	H	0.01675
N-Methyl-histidine*	H	0.00042
Trimethyl-lysine*	H	0.02222
Dimethyl-lysine*	H-M	0.02222
Dimethyl-proline	H-M	0.18518
Trimethyl-tryptophan	H-L	0.75638
Butyro-betaine*	M-L	0.04265
N6-Methyl-lysine	M-L	0.30216
Trimethyl-histidine	M-L	0.61595
Trimethyl-tyrosine	M-L	0.18178
S-Methyl-ergothioneine	L	0.16939
Acetylated amino acids (6)		
N-Acetyl-arginine*	H-M	0.00003
N2-Acetyl-lysine*	M	0.00915
N6-Acetyl-lysine*	M	0.00154
N-Acetyl-aspartate*	M-L	0.01675
N-Acetyl-glutamate	M-L	0.09448
N-Acetyl-(iso)leucine	M-L	0.09448
Other amino acids (15)		
Creatinine*	H	0.00001
Hippurate	H	0.32548
Indoxyl-sulfate*	H	0.00187
4-Guanidinobutanoate*	H-M	0.01448
Taurine*	H-M	0.01448
Acetyl-carnosine	M-L	0.58264
Carnosine*	M-L	0.00053
Citrulline	M-L	0.21988
Creatine	M-L	0.27995
Keto(iso)leucine*	M-L	0.03766
Kynurenine*	M-L	0.01675
Quinolinic acid*	M-L	0.00102
S-Adenosyl-methionine	M-L	0.25884
Ornithine	L	0.06094
S-Adenosyl-homocysteine*	L	0.02547

P-values between age groups were calculated by Mann Whitney U-test. Asterisks indicate 55 compounds that showed significant difference ($p<0.05$). The peak ratio is described in only asterisked compounds. Thirty-one RBC-enriched compounds are underlined (Chaleckis et al., 2016).

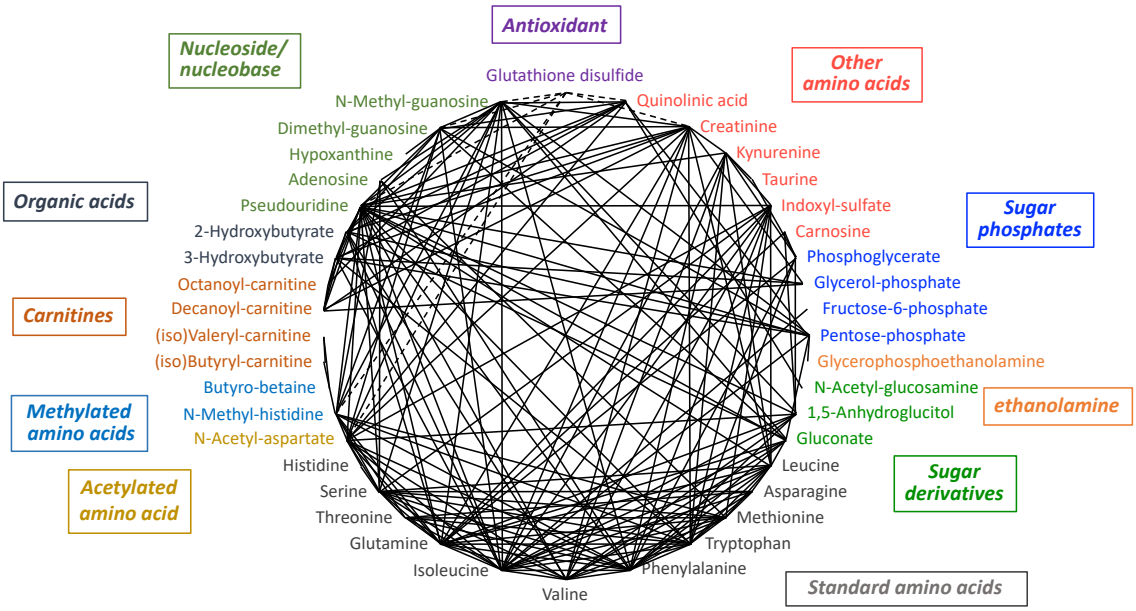
Supplemental Table S3, Chromatogram and mass spectrum data.

Category /Compound	Status	Ionization	Theoretical m/z	Detected m/z	m/z error (ppm)	RT (min)
Nucleosides, nucleobases, and derivatives (12)						
Adenosine	STD	pos	268.1040	268.1036	1.4	6.4
Caffeine	STD	pos	195.0877	195.0872	2.8	3.9
Cytidine	STD	pos	244.0928	244.0927	0.5	10.2
Dimethyl-guanosine	STD	pos	312.1302	312.1298	1.3	6.0
Dimethyl-xanthine	STD	pos	181.0720	181.0713	3.9	4.4
Hypoxanthine	STD	pos	137.0458	137.0453	3.4	7.6
N-Methyl-adenosine	STD	pos	282.1197	282.1192	1.8	12.8
N-Methyl-guanosine	STD	pos	298.1146	298.1141	1.7	7.6
Pseudouridine	STD	neg	243.0623	243.0621	0.9	10.2
Urate	STD	neg	167.0211	167.0210	0.9	12.7
Uridine	STD	neg	243.0623	243.0621	0.7	7.2
Xanthine	STD	neg	151.0261	151.0264	-1.8	8.6
Sugar derivatives (4)						
1,5-Anhydroglucitol	STD	neg	163.0612	163.0614	-1.0	9.9
Gluconate	STD	neg	195.0510	195.0512	-1.3	13.0
myo-Inositol	STD	neg	179.0561	179.0559	0.9	16.8
N-Acetyl-glucosamine	STD	pos	222.0972	222.0968	1.6	10.4
Sugar phosphates (6)						
Fructose-1,6-diphosphate	STD	neg	338.9888	338.9887	0.4	18.7
Fructose-6-phosphate	STD	neg	259.0224	259.0224	-0.1	16.0
Glucose-6-phosphate	STD	neg	259.0224	259.0223	0.3	16.8
Glycerol-phosphate	STD	neg	171.0064	171.0065	-0.6	14.5
Pentose-phosphate	STD	neg	229.0119	229.0118	0.5	15.1
Phosphoglycerate	STD	neg	184.9857	184.9857	-0.1	16.7
Vitamins and coenzymes (3)						
4-Aminobenzoate	STD	pos	138.0550	138.0545	3.8	7.4
Nicotinamide	STD	pos	123.0553	123.0548	3.9	4.9
Pantothenate	STD	pos	220.1179	220.1176	1.2	5.3
Choline and ethanolamine derivatives (4)						
Glycerophosphocholine	STD	pos	258.1101	258.1091	4.0	14.4
Glycerophosphoethanolamine	MS/MS	neg	214.0486	214.0486	0.01	15.4
Phosphocholine	STD	pos	184.0733	184.0729	2.4	15.1
Phosphoethanolamine	STD	neg	140.0118	140.0120	-1.8	16.1
Carnitines (8)						
Acetyl-carnitine	STD	pos	204.1230	204.1227	1.6	9.1
(iso)Butyryl-carnitine	STD	pos	232.1543	232.1540	1.3	6.1
Carnitine	STD	pos	162.1125	162.1120	3.1	12.6
Decanoyl-carnitine	STD	pos	316.2482	316.2476	2.0	3.9
Hexanoyl-carnitine	STD	pos	260.1856	260.1852	1.4	5.0
Octanoyl-carnitine	STD	pos	288.2169	288.2163	1.9	4.0
Propionyl-carnitine	STD	pos	218.1387	218.1383	1.7	7.4
(iso)Valeryl-carnitine	STD	pos	246.1700	246.1696	1.6	5.4
Organic acids (11)						
2-Hydroxybutyrate	STD	neg	103.0401	103.0406	-4.5	4.8
2-Oxoglutarate	STD	neg	145.0142	145.0145	-2.3	14.6
3-Hydroxybutyrate	STD	neg	103.0401	103.0406	-5.0	6.5
Arginino-succinate	STD	pos	291.1299	291.1295	1.4	17.0
cis-Aconitate	STD	pos	175.0237	175.0233	2.0	17.8
Citramalate	STD	neg	147.0299	147.0301	-1.4	13.8
Citrate	STD	neg	191.0197	191.0198	-0.7	17.8
Glycochenodeoxycholate	STD	neg	448.3068	448.3075	-1.5	3.3
Malate	STD	neg	133.0142	133.0146	-3.0	15.2
Glycerate	STD	neg	105.0193	105.0198	-5.2	8.6
Succinate	STD	neg	117.0193	117.0197	-3.4	14.4
Antioxidants (3)						
Glutathione disulfide	STD	pos	613.1592	613.1578	2.3	17.7

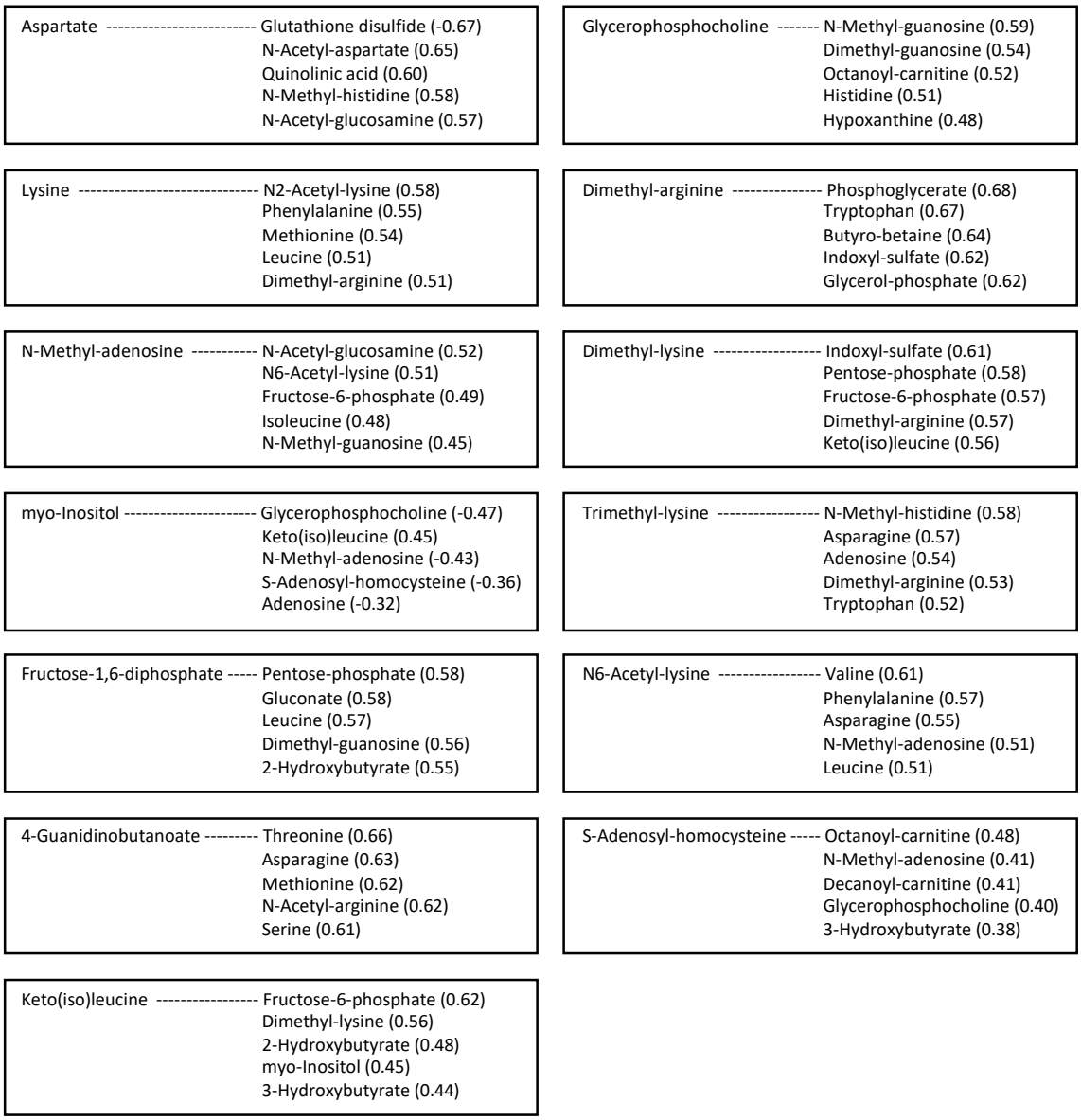
Supplemental Table S3, (continued)

Category /Compound	Status	Ionization	Theoretical m/z	Detected m/z	m/z error (ppm)	RT (min)
Standard amino acids (17)						
Arginine	STD	pos	175.1190	175.1186	2.5	26.2
Asparagine	STD	pos	133.0608	133.0603	4.0	14.5
Aspartate	STD	pos	134.0448	134.0443	3.8	13.9
Glutamate	STD	pos	148.0604	148.0600	2.8	14.0
Glutamine	STD	neg	145.0619	145.0622	-1.9	14.5
Histidine	STD	pos	156.0768	156.0762	3.7	13.9
Isoleucine	STD	neg	130.0874	130.0877	-2.3	9.3
Leucine	STD	neg	130.0874	130.0877	-2.3	8.7
Lysine	STD	neg	145.0983	145.0984	-1.0	25.1
Methionine	STD	pos	150.0583	150.0579	2.9	9.6
Phenylalanine	STD	pos	166.0863	166.0858	3.1	7.6
Proline	STD	pos	116.0706	116.0701	4.3	11.6
Serine	STD	pos	106.0499	106.0493	5.5	15.2
Threonine	STD	pos	120.0655	120.0650	3.9	13.6
Tryptophan	STD	pos	205.0972	205.0968	2.2	10.0
Tyrosine	STD	pos	182.0812	182.0808	2.4	12.2
Valine	STD	neg	116.0717	116.0721	-3.7	11.2
Methylated amino acids (12)						
Betaine	STD	pos	118.0863	118.0857	4.8	9.2
Butyro-betaine	STD	pos	146.1176	146.1171	3.5	12.7
Dimethyl-arginine	STD	pos	203.1503	203.1498	2.4	22.0
Dimethyl-lysine	STD	pos	175.1441	175.1439	1.1	21.5
Dimethyl-proline	STD	pos	144.1019	144.1014	3.7	8.3
N-Methyl-histidine	STD	pos	170.0924	170.0921	1.6	11.5
N6-Methyl-lysine	STD	pos	161.1285	161.1280	3.1	24.0
S-Methyl-ergothioneine	STD	pos	244.1114	244.1112	0.8	8.4
Trimethyl-histidine	STD	pos	198.1237	198.1233	1.8	11.2
Trimethyl-lysine	STD	pos	189.1598	189.1593	2.5	22.7
Trimethyl-tryptophan	STD	pos	247.1441	247.1436	2.0	6.1
Trimethyl-tyrosine	MS/MS	pos	224.1281	224.1277	1.9	7.9
Acetylated amino acids (6)						
N-Acetyl-arginine	STD	pos	217.1295	217.1292	1.4	14.7
N-Acetyl-aspartate	STD	neg	174.0408	174.0409	-0.8	13.8
N-Acetyl-glutamate	STD	pos	190.0710	190.0706	2.0	13.4
N-Acetyl-(iso)leucine	STD	pos	174.1125	174.1120	2.8	3.7
N2-Acetyl-lysine	STD	pos	189.1234	189.1230	2.3	14.9
N6-Acetyl-lysine	STD	pos	189.1234	189.1230	2.3	12.3
Other amino acids (15)						
4-Guanidinobutanoate	STD	pos	146.0924	146.0919	3.3	14.9
Acetyl-carnosine	STD	pos	269.1244	269.1241	0.9	6.9
Carnosine	STD	pos	227.1139	227.1133	2.5	15.6
Citrulline	STD	pos	176.1030	176.1026	2.2	15.7
Creatine	STD	neg	130.0622	130.0625	-2.1	14.2
Creatinine	STD	neg	112.0516	112.0520	-3.8	7.0
Hippurate	STD	pos	180.0655	180.0651	2.1	4.0
Indoxyl-sulfate	STD	neg	212.0023	212.0021	0.8	4.7
Keto(iso)leucine	STD	neg	129.0557	129.0561	-3.0	3.5
Kynurenine	STD	pos	209.0921	209.0917	2.1	8.8
Ornithine	STD	neg	131.0826	131.0829	-2.1	23.0
Quinolinic acid	STD	neg	166.0146	166.0152	-3.4	14.3
S-Adenosyl-homocysteine	STD	pos	385.1289	385.1279	2.6	13.3
S-Adenosyl-methionine	STD	pos	399.1445	399.1439	1.5	17.0
Taurine	STD	neg	124.0074	124.0077	-2.7	13.5

Compounds were identified using either commercially available standards (STD) or by analysis of MS/MS spectra (MS/MS), if no standard was available.



Supplemental Figure S1. Of the 55 aging markers, 13 compounds with correlations <0.7. The 5 most highly correlated compounds for each.



Supplemental Figure S2. Of the 55 aging markers, 13 compounds with correlations <0.7. The 5 most highly correlated compounds for each.