

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27

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

Takayuki Teruya¹, Haruhisa Goga^{1,2}, and Mitsuhiro Yanagida^{1*}

¹ G0 Cell Unit, Okinawa Institute of Science and Technology Graduate University (OIST), Okinawa, Japan

² Forensic Laboratory, Department of Criminal Investigation, Okinawa Prefectural Police HQ, Okinawa, Japan (on leave of absence)

* To whom correspondence should be addressed

M. Yanagida, G0 Cell Unit, Okinawa Institute of Science and Technology Graduate University (OIST), Onna-son, Okinawa, 904-0495, Japan

Tel +81 98 966 8658

Fax +81 98 966 2890

Email; myanagid@gmail.com

Key words

Age markers in urine, non-targeted metabolomics, human aging, LC-MS, highly correlated metabolites, leucine, pseudouridine,

Short title: Aging markers in human urine

28 **Summary**

29 Metabolites in human biofluids document individual physiological status. We
30 conducted comprehensive, non-targeted, non-invasive metabolomic analysis of urine
31 from 27 healthy human subjects, comprising 13 youths (30 ± 3 yr) and 14 seniors
32 (76 ± 4 yr). Quantitative analysis of 99 metabolites revealed 55 that were linked to
33 aging, displaying significant differences in abundance between the two groups.
34 These include 13 standard amino acids, 5 methylated, 4 acetylated, and 9 other
35 amino acids, 6 nucleosides, nucleobases, and derivatives, 4 sugar derivatives, 5
36 sugar phosphates, 4 carnitines, 2 hydroxybutyrates, 1 choline, and 1 ethanolamine
37 derivative, and glutathione disulfide. Abundances of 53 compounds decreased, while
38 2 increased in elderly people. Many age-linked markers were highly correlated; 42 of
39 55 compounds, showed Pearson's correlation coefficients larger than 0.70. As
40 metabolite profiles of urine and blood are quite different, age-related information
41 in urine components offer yet more valuable insights into aging mechanisms of
42 endocrine system and related organ systems.
43

44 **Introduction**

45 Elderly people are acutely aware of the progressive aging of different body
46 parts, but quantifying and characterizing physiological aging is less intuitive.
47 Nonetheless, assessment of aging and determination of aging type by analyzing
48 metabolites in biofluids, such as blood and urine, may help us to understand broadly-
49 featured aging of the human body (Kampmann et al., 1974) (Ames, 1989) (Short et
50 al., 2005) (Slupsky et al., 2007) (Lawton et al., 2008) (Mishur and Rea, 2012) (Yu et
51 al., 2012) (Menni et al., 2013) (Gonzalez-Covarrubias et al., 2013) (Auro et al., 2014)
52 (Chaleckis et al., 2016) (Hertel et al., 2016) (Jove et al., 2016) (Rist et al., 2017)
53 (Chak et al., 2019). Age-dependent changes of metabolite abundances may be
54 valuable to determine the molecular causes of impaired organ functions. Technology
55 that enables simple and rapid measurement of urinary metabolites, which can be
56 collected non-invasively, has certain advantages over methods using blood. Urinary
57 metabolites are promising biological samples for monitoring health parameters if the
58 metabolic processes resulting in production of those metabolites can be fully
59 understood. To date, few comprehensive approaches to investigate human urinary
60 metabolites in aging have been reported (Thevenot et al., 2015) (Hertel et al., 2016)
61 (Rist et al., 2017). Urination is a primary route by which the body eliminates water-
62 soluble waste products. Accordingly, urine has been broadly utilized for diagnosis of
63 renal dysfunction in diverse kidney diseases (Han et al., 2002) (Eknoyan et al., 2003)
64 (Mishra et al., 2005) (Parikh et al., 2006) (Pisitkun et al., 2006) (Vaidya et al., 2008).
65 Nonetheless, because urinary metabolites originate in all organ systems, they may
66 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

71 **Results**

72 **Collection of urine samples**

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

79

80 **99 urine metabolites identified**

81 Ninety-nine urinary metabolites, about half of which are amino acids and
82 their derivatives, were identified and quantified using liquid chromatography – mass
83 spectrometry (LC – MS) and MZmine 2 (Pluskal et al., 2010a) (Teruya et al., 2019)
84 (**Supplemental Table s2**). These compounds were subdivided into 12 groups,
85 containing 17 standard amino acids, 12 methylated amino acids, 6 acetylated amino
86 acids and 15 other amino acids, 12 nucleosides, nucleobases, and derivatives, 4 sugar
87 derivatives, 6 sugar phosphates, 3 vitamins and coenzymes (pantothenate, 4-
88 aminobenzoate, nicotinamide), 4 choline and ethanolamine derivatives, 8 carnitines,
89 11 organic acids, and 1 antioxidant (oxidized form of glutathione, GSSG). Small amino
90 acids, such as glycine and alanine, were not detected in our analysis due to the
91 mass cutoff (100 m/z) used. Levels of individual compounds, categorized by
92 abundance as H (high, >10⁸), M (medium, 10⁷~10⁸) or L (low, <10⁷), were estimated
93 based upon mass spectroscopic peak area (Chaleckis et al., 2014) (Chaleckis et al.,

94 2016). Some compounds varied widely from one individual to the next and are
95 denoted as H-L, H-M or M-L. According to abundance, there were 7 H, 19 H-M, 7 H-
96 L, 5 M, 45 M-L and 16 L compounds. Twenty-six urinary metabolites were abundant
97 (H or H-M), the great majority of which were amino acids and their derivatives, such
98 as the methylated amino acids, betaine and dimethyl-arginine, but they also
99 included nucleosides, such as pseudouridine and N-methyl-guanosine. In blood,
100 sugar phosphates and methylated amino acids were enriched in red blood cells
101 (Chaleckis et al., 2014) (Chaleckis et al., 2016). In urine, sugar phosphates are age-
102 related, except for glucose-6-phosphate. We show below that about half of all
103 urinary metabolites are age-related.

104

105 **Most urinary age markers decreased with age**

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

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

120

121 **Highly correlated age-linked urinary metabolites**

122 To understand relationships among urine metabolites, Pearson's correlation
123 coefficients were calculated from abundance data for all 55 age-linked urinary
124 metabolites of all 27 subjects (Methods section). The highest correlation, 0.92, was
125 obtained for isoleucine – leucine and N-methyl guanosine – dimethyl guanosine (**Fig.**
126 **2A**). This is probably due to their similar chemical structures and proximity in
127 biochemical pathways (see below and KEGG, <https://www.genome.jp/kegg/>).
128 However, correlation values 0.91 obtained for glycerol-phosphate – 3-
129 hydroxybutyrate, pseudouridine – 2-hydroxybutyrate, and pseudouridine – isoleucine
130 clearly have a different explanation. Fifteen urinary metabolites having correlation
131 values >0.85 formed a network (**Fig. 2B**). The high correlation between
132 pseudouridine and isoleucine seems to be a key connection between two groups of
133 metabolites. Additionally, the connections of creatinine to pseudouridine and 3-
134 hydroxybutyrate to glutamine appeared to be required for further correlation (see
135 below). In the case of a creatinine-related network, 15 metabolites have correlation
136 coefficients from 0.71~0.84 (**Fig. 2C**). Creatinine, a known waste product from
137 muscle (Heymsfield et al., 1983) (Baxmann et al., 2008), is correlated with many
138 metabolites. It even shows a negative correlation with GSSG (**Fig. 2D**).

139 Pairwise correlation analyses revealed 21 pairs of compounds with

140 correlation coefficients >0.85 (**Fig. 2A**). These show a network consisting of two
141 groups, one centered around pseudouridine and the other around isoleucine (**Fig.**
142 **2B**). This suggests that compounds within each of these groups may be
143 metabolically linked.

144 When urinary compounds with correlation coefficients larger than 0.7 were
145 selected, the majority of age-linked metabolites formed a large network consisting of
146 42 metabolites (**Supplemental Fig. S1**). Since all age-linked metabolites displayed
147 p-values < 0.05 in dot plot profiling, the fact that the great majority ($42/55 = 76\%$)
148 form such a large age-linked correlation network is quite impressive.

149

150 **Some age-linked metabolites were not highly correlated**

151 On the other hand, 13 compounds, though they were age-related, showed
152 only weak correlations (<0.7) with the other 42 age-linked urinary metabolites
153 (**Supplemental Fig. S1**). Aspartate, a standard amino acid having an acidic side
154 chain, showed maximal, but inverse correlation with GSSG (-0.67). Aspartate has a
155 correlation coefficient of 0.65 with N-acetyl aspartate, indicating that structural
156 similarity might partly explain the weak correlation. S-adenosyl-homocysteine did not
157 show correlation values higher than 0.41 (N-methyl-adenosine). Although the
158 correlation is rather low, the two compounds exhibit structural similarity. Similarly,
159 lysine, dimethyl-lysine, trimethyl-lysine, and N6-acetyl-lysine did not show the
160 correlation coefficients higher than 0.5 among them. These metabolites diminish
161 significantly in urine of elderly people. Fructose-1,6-diphosphate showed a maximal
162 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

186 these 55 age-related metabolites were subjected to principal component analysis
187 (PCA) (Nakamura et al., 1988) (Kameda et al., 2020). Subjects were separated into
188 2 groups (red for elderly and blue for young subjects) represented by negative and
189 positive values of principal component (PC) 1, respectively (**Fig. 4A**). One elderly
190 subject 11 (81F) was in the middle of the young group and one young subject 15
191 (33M) was in the group of elderly subjects in the PCA plot (**Fig. 4A**). These are
192 consistent with results plotted in the heatmap (**Fig. 3**). The PC1 score and
193 chronological age of the subject showed a high correlation with $r=0.80$. (**Fig. 4B**).

194

195 **Negative correlation between GSSG and age-linked metabolites**

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

207 GSSG showed different degrees of abundance in different subjects, and
208 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

217 **Discussion**

218 We initially investigated metabolomics of a simple model eukaryote, fission
219 yeast (*Schizosaccharomyces pombe*) using yeast genetic technology to identify and
220 determine metabolite profiles in wild type and mutant cell extracts (Pluskal et al.,
221 2010b) (Pluskal et al., 2016) (Pluskal and Yanagida, 2016a) (Pluskal and Yanagida,
222 2016b). By adapting the software MZmine 2 for metabolite identification (Pluskal et
223 al., 2010a) (Pluskal et al., 2012), we found that metabolomics of fission yeast and
224 humans are surprisingly similar in regard to metabolite composition (Chaleckis et al.,
225 2014). Metabolites detected in fission yeast and human whole blood were 75%
226 identical. Given this unexpected similarity, we adapted our techniques to human
227 blood metabolites to better understand health, disease, and longevity (Chaleckis et
228 al., 2014) (Chaleckis et al., 2016) (Teruya et al., 2019) (Kameda et al., 2020).

229

230 **Distinction of aging information between blood and urine.**

231 Urinary metabolites offer a non-invasive means of obtaining aging
232 information. The present results indicate that urinary aging information may be
233 distinct from that obtainable from blood. Blood metabolites derived from plasma and
234 red blood cells of elderly subjects reflect a decrease in antioxidant production and
235 muscle activity or increasing inefficiency of nitrogen metabolism (Chaleckis et al.,
236 2016). Aging information obtained from urine will be useful, as human aging is
237 exceedingly complex. In the present study, we identified 55 human urinary aging
238 markers using non-targeted, comprehensive LC-MS. Since urine contains 99
239 compounds, many urinary metabolites such as vitamins (pantothenate and

240 nicotinamide) and organic acids (citrate, malate) neither significantly decreased nor
241 increased so that about a half of urinary metabolites are not age markers. In aged
242 subjects, levels of protein, nucleic acid and lipid synthesis, modification, and turnover
243 may decrease due to reduced physical activity (Hughes et al., 2002) (Pollack et al.,
244 2002) (Maynard et al., 2015), resulting in declining metabolite abundances.
245 Metabolite data may be considered as an overview of the physiological state of all
246 tissues and organs. Hence these 55 metabolites constitute a panorama of human
247 aging as seen through urine composition. Alternatively, these may not represent
248 actual human aging, but may represent voluntary lifestyle changes that may be
249 reversible regardless of actual age.

250

251 **Myoinositol, GSSG and pseudouridine.**

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

263 2016, the present study). The active SH form was rapidly oxidized to an inactive
264 disulfide form, which accumulated in elderly subjects, whereas in young subjects the
265 disulfide form was hardly formed or undetected possibly due to rapid decay in the
266 young body or sample. Consistently, reduced GSSG was undetectable in urine of the
267 five young subjects **Fig. 2D** and **Fig. 5B-F**). The abundance of GSSG was inversely
268 related to those of pseudouridine, creatinine, and 2-hydroxybutyrate, which were
269 most abundant in young subjects. Thus GSSG in urine appears to be appropriate as
270 an age marker in urine of elderly subjects. In contrast, the high abundance of
271 pseudouridine seems to be emblematic of youth.

272

273 **Highly correlated urine metabolites.**

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

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

291

292 **Thirteen metabolites linked by low correlation.**

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

309

310 **An effective overview on aging by heatmap.**

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

328

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

330

<i>Category</i> /Compound	Peak abundance	Ratio (elderly/young)
<i>Nucleosides, nucleobases, and derivatives (6)</i>		
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
<i>Sugar derivatives (4)</i>		
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
<i>Sugar phosphates (5)</i>		
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
<i>Choline and ethanolamine derivatives (2)</i>		
Glycerophosphocholine	M-L	0.50
Glycerophosphoethanolamine	L	0.44
<i>Carnitines (4)</i>		
(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
<i>Organic acids (2)</i>		
2-Hydroxybutyrate	M-L	0.33
3-Hydroxybutyrate	M-L	0.27
<i>Antioxidant (1)</i>		
Glutathione disulfide	L	6.96
<i>Standard amino acids (13)</i>		
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
<i>Methylated amino acids (5)</i>		
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)**

<i>Category /Compound</i>	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 ($10^8\sim 10^7$ 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

339 **Materials and Methods**

340 **Participants and sample collection**

341 14 elderly (69~81 yr) and 13 young (25~38 yr) healthy people participated as
342 subjects in this study (**Supplemental Table S1**). Measurements of metabolites in
343 first morning urine are more consistent than random daytime sampling to monitor
344 metabolites. Intra-individual coefficients of variation in first morning urine and 24-
345 h collected urine are reportedly similar (Witte et al., 2009).

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

355

356 **Ethics statement**

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

362

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 μ m
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 μ L 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 ($10^7 \sim 10^8$ 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-](http://www.r-project.org)
396 [project.org](http://www.r-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

407 **Acknowledgments**

408 We thank Ms. Junko Takada for providing excellent technical assistance. We

409 gratefully acknowledge the editorial help of Dr. Steven D. Aird. We are greatly
410 indebted to the generous support of Okinawa Institute of Science and Technology
411 Graduate University and its Innovative Technology Research (ITR) fund.

412

413

414 **Declaration of Interests**

415 The authors declare that they have no competing interests.

416

417 **References**

- 418 Ames, B.N. (1989). Endogenous DNA damage as related to cancer and aging.
419 Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 214,
420 41-46.
- 421 Auro, K., Joensuu, A., Fischer, K., Kettunen, J., Salo, P., Mattsson, H., Niironen, M.,
422 Kaprio, J., Eriksson, J.G., Lehtimäki, T., et al. (2014). A metabolic view on
423 menopause and ageing. Nat Commun 5, 4708.
- 424 Baxmann, A.C., Ahmed, M.S., Marques, N.C., Menon, V.B., Pereira, A.B., Kirsztajn,
425 G.M., and Heilberg, I.P. (2008). Influence of muscle mass and physical activity on
426 serum and urinary creatinine and serum cystatin C. Clin J Am Soc Nephrol 3, 348-
427 354.
- 428 Berridge, M.J. (1993). Inositol trisphosphate and calcium signalling. Nature 361, 315-
429 325.
- 430 Berridge, M.J. (2016). The Inositol Trisphosphate/Calcium Signaling Pathway in
431 Health and Disease. Physiol Rev 96, 1261-1296.
- 432 Chak, C.M., Lacruz, M.E., Adam, J., Brandmaier, S., Covic, M., Huang, J., Meisinger,
433 C., Tiller, D., Prehn, C., Adamski, J., et al. (2019). Ageing Investigation Using Two-
434 Time-Point Metabolomics Data from KORA and CARLA Studies. Metabolites 9.
- 435 Chaleckis, R., Ebe, M., Pluskal, T., Murakami, I., Kondoh, H., and Yanagida, M.
436 (2014). Unexpected similarities between the Schizosaccharomyces and human
437 blood metabolomes, and novel human metabolites. Mol Biosyst 10, 2538-2551.
- 438 Chaleckis, R., Murakami, I., Takada, J., Kondoh, H., and Yanagida, M. (2016).
439 Individual variability in human blood metabolites identifies age-related differences.
440 Proc Natl Acad Sci U S A 113, 4252-4259.
- 441 Charette, M., and Gray, M.W. (2000). Pseudouridine in RNA: what, where, how, and
442 why. IUBMB Life 49, 341-351.
- 443 Eknoyan, G., Hostetter, T., Bakris, G.L., Hebert, L., Levey, A.S., Parving, H.-H.,
444 Steffes, M.W., and Toto, R. (2003). Proteinuria and other markers of chronic kidney
445 disease: a position statement of the national kidney foundation (NKF) and the

- 446 national institute of diabetes and digestive and kidney diseases (NIDDK). American
447 Journal of Kidney Diseases 42, 617-622.
- 448 Gonzalez-Covarrubias, V., Beekman, M., Uh, H.W., Dane, A., Troost, J.,
449 Paliukhovich, I., van der Kloet, F.M., Houwing-Duistermaat, J., Vreeken, R.J.,
450 Hankemeier, T., et al. (2013). Lipidomics of familial longevity. *Aging Cell* 12, 426-434.
- 451 Han, W.K., Bailly, V., Abichandani, R., Thadhani, R., and Bonventre, J.V. (2002).
452 Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule
453 injury. *Kidney Int* 62, 237-244.
- 454 Hertel, J., Friedrich, N., Wittfeld, K., Pietzner, M., Budde, K., Van der Auwera, S.,
455 Lohmann, T., Teumer, A., Volzke, H., Nauck, M., et al. (2016). Measuring Biological
456 Age via Metabonomics: The Metabolic Age Score. *J Proteome Res* 15, 400-410.
- 457 Heymsfield, S.B., Arteaga, C., McManus, C., Smith, J., and Moffitt, S. (1983).
458 Measurement of muscle mass in humans: validity of the 24-hour urinary creatinine
459 method. *Am J Clin Nutr* 37, 478-494.
- 460 Hughes, V.A., Frontera, W.R., Roubenoff, R., Evans, W.J., and Singh, M.A. (2002).
461 Longitudinal changes in body composition in older men and women: role of body
462 weight change and physical activity. *Am J Clin Nutr* 76, 473-481.
- 463 Jove, M., Mate, I., Naudi, A., Mota-Martorell, N., Portero-Otin, M., De la Fuente, M.,
464 and Pamplona, R. (2016). Human Aging Is a Metabolome-related Matter of Gender.
465 *J Gerontol A Biol Sci Med Sci* 71, 578-585.
- 466 Kameda, M., Teruya, T., Yanagida, M., and Kondoh, H. (2020). Frailty markers
467 comprise blood metabolites involved in antioxidation, cognition, and mobility. *Proc*
468 *Natl Acad Sci U S A*.
- 469 Kampmann, J., Siersbaek-Nielsen, K., Kristensen, M., and Hansen, J.M. (1974).
470 Rapid evaluation of creatinine clearance. *Acta Med Scand* 196, 517-520.
- 471 Lawton, K.A., Berger, A., Mitchell, M., Milgram, K.E., Evans, A.M., Guo, L., Hanson,
472 R.W., Kalhan, S.C., Ryals, J.A., and Milburn, M.V. (2008). Analysis of the adult
473 human plasma metabolome. *Pharmacogenomics* 9, 383-397.
- 474 Maynard, S., Fang, E.F., Scheibye-Knudsen, M., Croteau, D.L., and Bohr, V.A.

- 475 (2015). DNA Damage, DNA Repair, Aging, and Neurodegeneration. Cold Spring
476 Harb Perspect Med 5.
- 477 Menni, C., Kastenmuller, G., Petersen, A.K., Bell, J.T., Psatha, M., Tsai, P.C., Gieger,
478 C., Schulz, H., Erte, I., John, S., et al. (2013). Metabolomic markers reveal novel
479 pathways of ageing and early development in human populations. *Int J Epidemiol* 42,
480 1111-1119.
- 481 Mishra, J., Dent, C., Tarabishi, R., Mitsnefes, M.M., Ma, Q., Kelly, C., Ruff, S.M.,
482 Zahedi, K., Shao, M., Bean, J., et al. (2005). Neutrophil gelatinase-associated
483 lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *The*
484 *Lancet* 365, 1231-1238.
- 485 Mishur, R.J., and Rea, S.L. (2012). Applications of mass spectrometry to
486 metabolomics and metabonomics: detection of biomarkers of aging and of age-
487 related diseases. *Mass Spectrom Rev* 31, 70-95.
- 488 Nakamura, E., Miyao, K., and Ozeki, T. (1988). Assessment of biological age by
489 principal component analysis. *Mechanisms of Ageing and Development* 46, 1-18.
- 490 Parikh, C.R., Mishra, J., Thiessen-Philbrook, H., Dursun, B., Ma, Q., Kelly, C., Dent,
491 C., Devarajan, P., and Edelstein, C.L. (2006). Urinary IL-18 is an early predictive
492 biomarker of acute kidney injury after cardiac surgery. *Kidney Int* 70, 199-203.
- 493 Pisitkun, T., Johnstone, R., and Knepper, M.A. (2006). Discovery of urinary
494 biomarkers. *Mol Cell Proteomics* 5, 1760-1771.
- 495 Pluskal, T., Castillo, S., Villar-Briones, A., and Oresic, M. (2010a). MZmine 2:
496 modular framework for processing, visualizing, and analyzing mass spectrometry-
497 based molecular profile data. *BMC Bioinformatics* 11, 395.
- 498 Pluskal, T., Nakamura, T., Villar-Briones, A., and Yanagida, M. (2010b). Metabolic
499 profiling of the fission yeast *S. pombe*: quantification of compounds under different
500 temperatures and genetic perturbation. *Mol Biosyst* 6, 182-198.
- 501 Pluskal, T., Nakamura, T., and Yanagida, M. (2016). Preparation of Intracellular
502 Metabolite Extracts from Liquid *Schizosaccharomyces pombe* Cultures. *Cold Spring*
503 *Harb Protoc* 2016.

- 504 Pluskal, T., Uehara, T., and Yanagida, M. (2012). Highly accurate chemical formula
505 prediction tool utilizing high-resolution mass spectra, MS/MS fragmentation, heuristic
506 rules, and isotope pattern matching. *Anal Chem* *84*, 4396-4403.
- 507 Pluskal, T., and Yanagida, M. (2016a). Measurement of Metabolome Samples Using
508 Liquid Chromatography-Mass Spectrometry, Data Acquisition, and Processing. *Cold*
509 *Spring Harb Protoc* *2016*.
- 510 Pluskal, T., and Yanagida, M. (2016b). Metabolomic Analysis of
511 *Schizosaccharomyces pombe*: Sample Preparation, Detection, and Data
512 Interpretation. *Cold Spring Harb Protoc* *2016*.
- 513 Pollack, M., Phaneuf, S., Dirks, A., and Leeuwenburgh, C. (2002). The role of
514 apoptosis in the normal aging brain, skeletal muscle, and heart. *Ann N Y Acad Sci*
515 *959*, 93-107.
- 516 Rist, M.J., Roth, A., Frommherz, L., Weinert, C.H., Kruger, R., Merz, B., Bunzel, D.,
517 Mack, C., Egert, B., Bub, A., et al. (2017). Metabolite patterns predicting sex and age
518 in participants of the Karlsruhe Metabolomics and Nutrition (KarMeN) study. *PLoS*
519 *One* *12*, e0183228.
- 520 Short, K.R., Bigelow, M.L., Kahl, J., Singh, R., Coenen-Schimke, J., Raghavakaimal,
521 S., and Nair, K.S. (2005). Decline in skeletal muscle mitochondrial function with
522 aging in humans. *Proc Natl Acad Sci U S A* *102*, 5618-5623.
- 523 Slupsky, C.M., Rankin, K.N., Wagner, J., Fu, H., Chang, D., Weljie, A.M., Saude,
524 E.J., Lix, B., Adamko, D.J., Shah, S., et al. (2007). Investigations of the effects of
525 gender, diurnal variation, and age in human urinary metabolomic profiles. *Anal Chem*
526 *79*, 6995-7004.
- 527 Teruya, T., Chaleckis, R., Takada, J., Yanagida, M., and Kondoh, H. (2019). Diverse
528 metabolic reactions activated during 58-hr fasting are revealed by non-targeted
529 metabolomic analysis of human blood. *Sci Rep* *9*, 854.
- 530 Thevenot, E.A., Roux, A., Xu, Y., Ezan, E., and Junot, C. (2015). Analysis of the
531 Human Adult Urinary Metabolome Variations with Age, Body Mass Index, and
532 Gender by Implementing a Comprehensive Workflow for Univariate and OPLS
533 Statistical Analyses. *J Proteome Res* *14*, 3322-3335.

534 Vaidya, V.S., Waikar, S.S., Ferguson, M.A., Collings, F.B., Sunderland, K., Gioules,
535 C., Bradwin, G., Matsouaka, R., Betensky, R.A., Curhan, G.C., et al. (2008). Urinary
536 biomarkers for sensitive and specific detection of acute kidney injury in humans. *Clin*
537 *Transl Sci* 1, 200-208.

538 Witte, E.C., Lambers Heerspink, H.J., de Zeeuw, D., Bakker, S.J., de Jong, P.E., and
539 Gansevoort, R. (2009). First morning voids are more reliable than spot urine samples
540 to assess microalbuminuria. *J Am Soc Nephrol* 20, 436-443.

541 Yu, Z., Zhai, G., Singmann, P., He, Y., Xu, T., Prehn, C., Romisch-Margl, W., Lattka,
542 E., Gieger, C., Soranzo, N., et al. (2012). Human serum metabolic profiles are age
543 dependent. *Aging Cell* 11, 960-967.

544

545

546 **Figure Legends**

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

555

556 **Figure 2.** Correlation network among age-linked compounds. (A) Values indicate
557 correlation coefficients between paired compounds. Highly correlated pairs of aging
558 markers ($r > 0.85$) are indicated with red circles. The most highly correlated pairs are
559 indicated by green (0.92) and red arrows (0.91). (B) Interrelated compounds form a
560 network. Correlations ($r > 0.90$) are highlighted in yellow. (C) Fifteen metabolites with
561 the highest correlation with creatinine are listed. (D) Scatter plot of the peak
562 abundance ($\times 10^6$ AU) between creatinine and GSSG. GSSG was negatively
563 correlated with creatinine.

564

565 **Figure 3.** Heatmap representing urinary metabolic profiles of elderly and young
566 subjects. Standardized data for each metabolite are shown for 27 subjects using a
567 color matrix representing relative abundance data of 55 urinary aging markers.
568 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 ($\times 10^6$ 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.

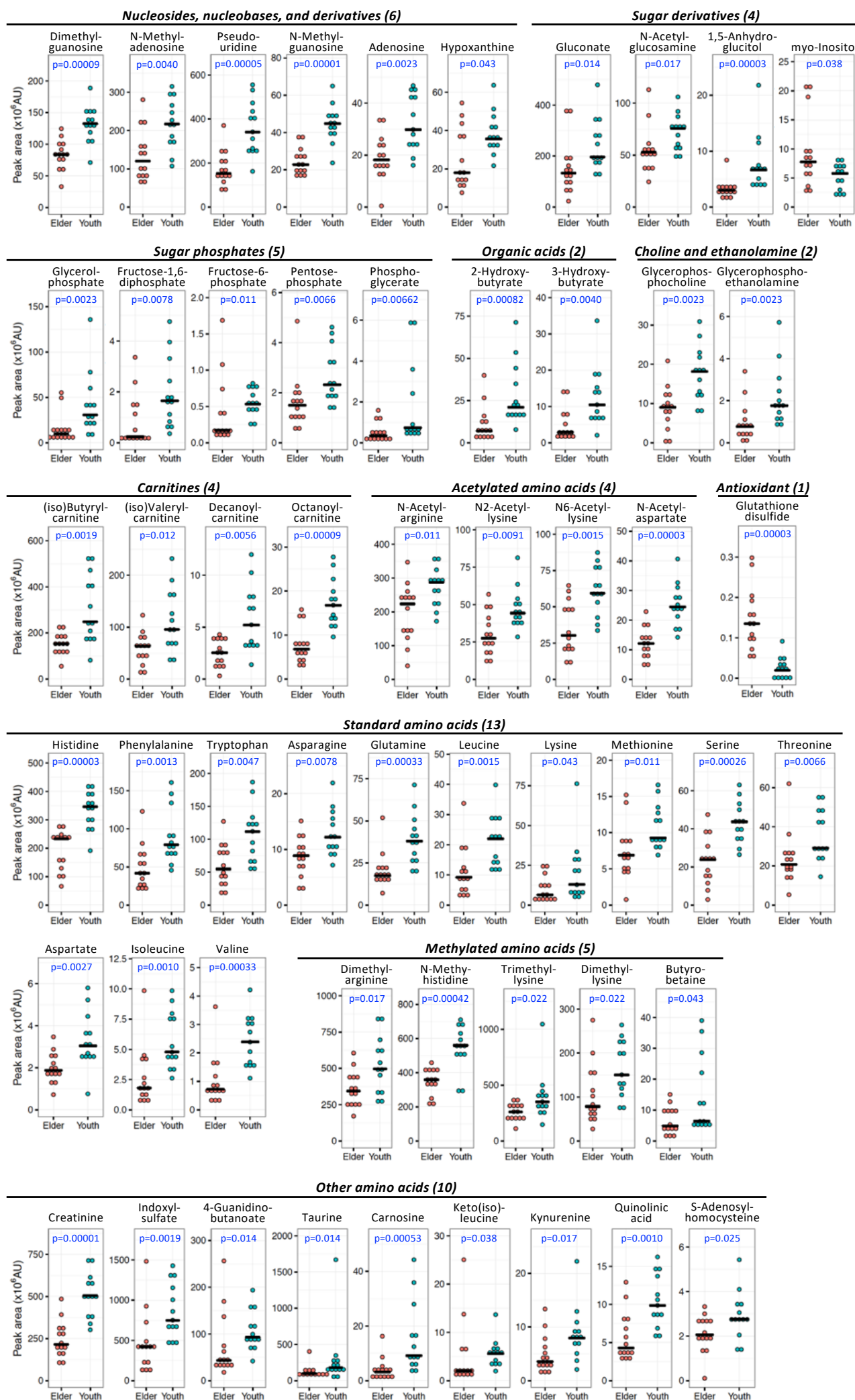


Figure 2.

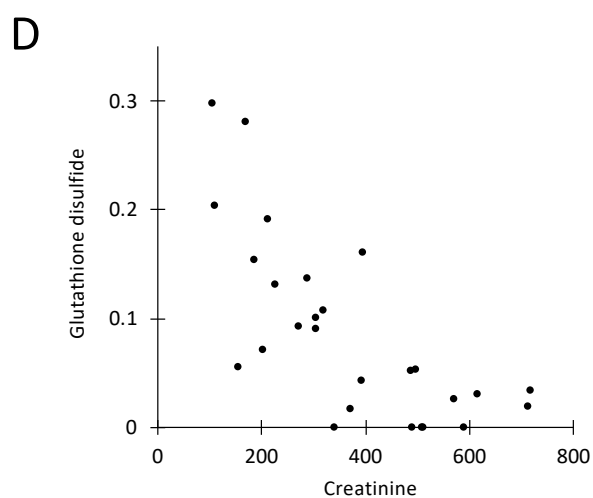
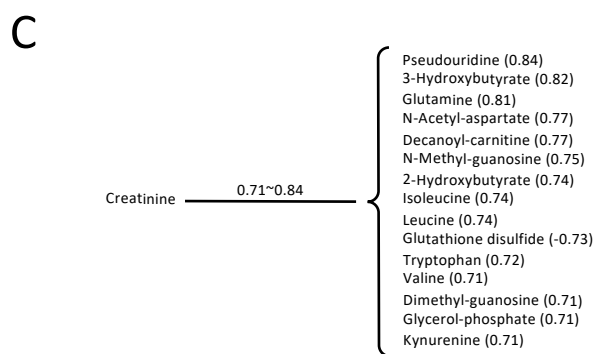
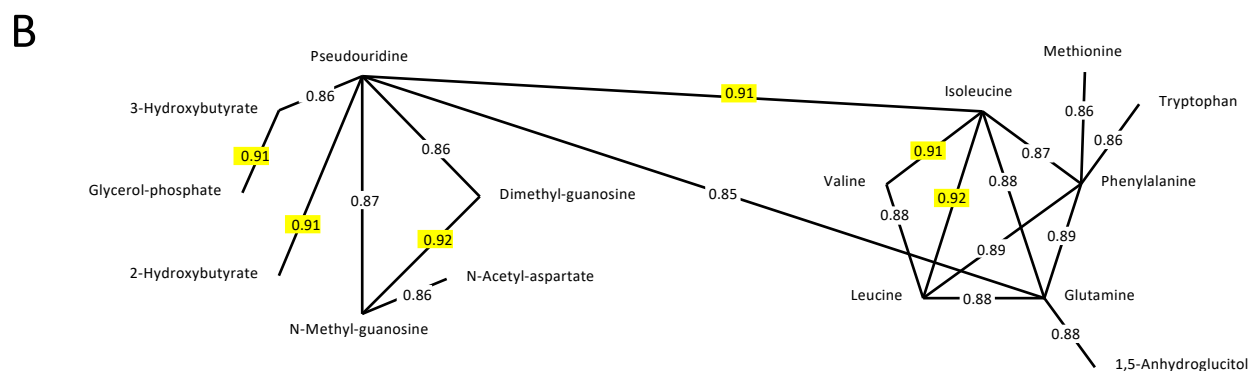
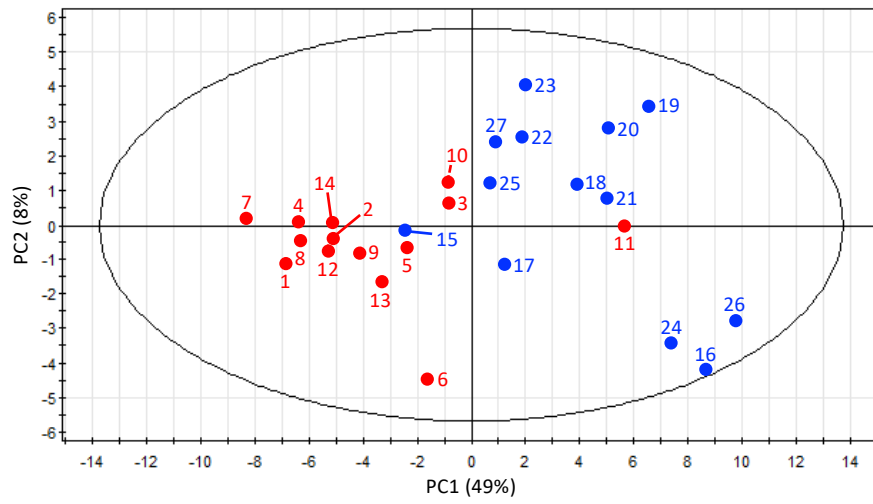


Figure 3.

Category	Subject No. Age/Gender	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
		75M	79M	78M	69F	78F	70F	69F	77F	74F	76F	81F	80F	80F	75F	33M	38M	29M	30M	30M	34M	30M	33F	29F	31F	29F	25F	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	
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
Hydroxanthine	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
	Inositol-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	68.1	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	68.1	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	68.5	5		

Figure 4.

A



B

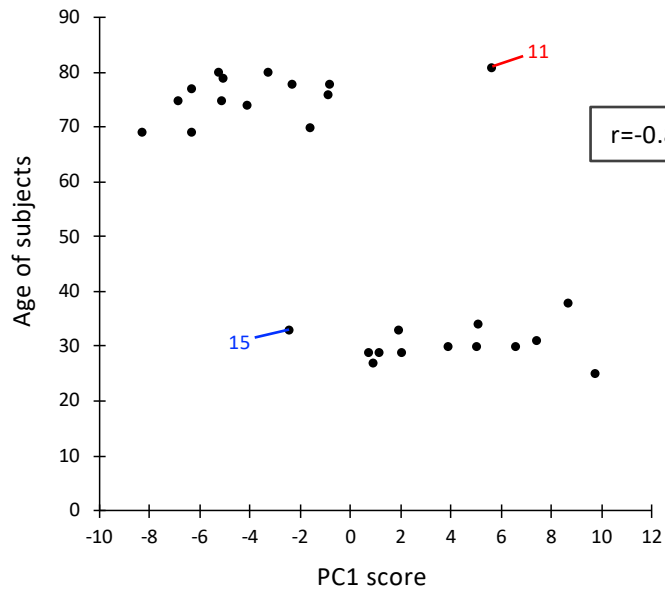
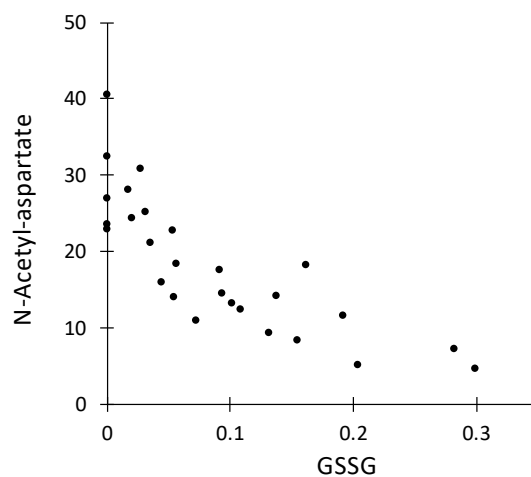


Figure 5.

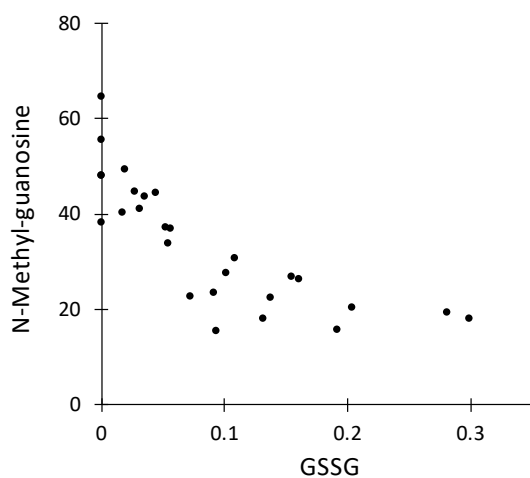
A

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)

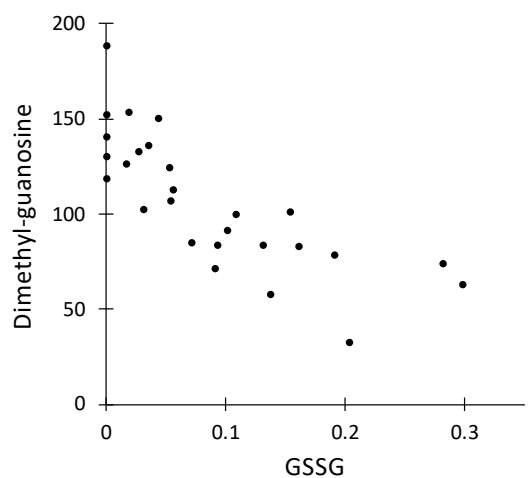
B



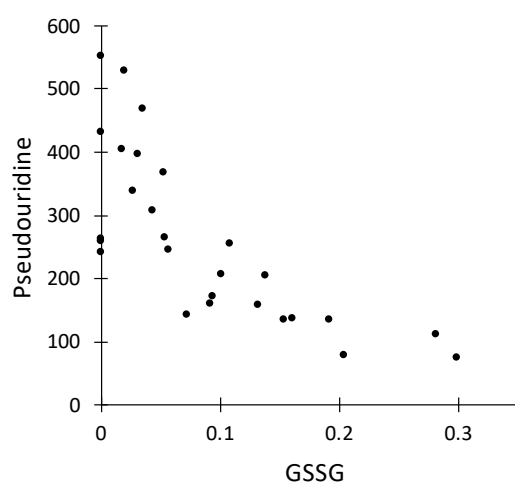
C



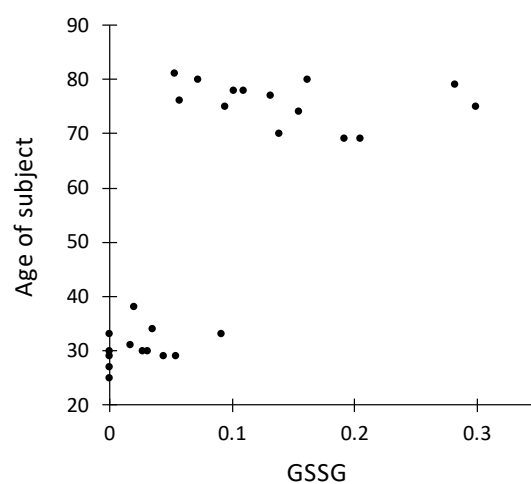
D



E



F



Supplementary information

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

Takayuki Teruya¹, Haruhisa Goga^{1,2}, and Mitsuhiro Yanagida^{1*}

¹ G0 Cell Unit, Okinawa Institute of Science and Technology Graduate University (OIST),
Okinawa, Japan

² Forensic Laboratory, Department of Criminal Investigation, Okinawa Prefectural Police HQ,
Okinawa, Japan (on leave of absence)

* To whom correspondence should be addressed

M. Yanagida, G0 Cell Unit, Okinawa Institute of Science and Technology Graduate University
(OIST), Onna-son, Okinawa, 904-0495, Japan

Tel +81 98 966 8658

Fax +81 98 966 2890

Email; myanagid@gmail.com

Supplementary Table S1. Characteristics of 27 subjects

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

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

<i>Category</i> /Compound	Peak abundance	p-value elderly/young	<i>Category</i> /Compound	Peak abundance	p-value elderly/young
<i>Nucleosides, nucleobases, and derivatives (12)</i>			<i>Standard amino acids (17)</i>		
Dimethyl-guanosine*	H-M	0.00009	Arginine	H-M	0.45827
N-Methyl-adenosine*	H-M	0.00395	Histidine*	H-M	0.00003
Pseudouridine*	H-M	0.00005	Phenylalanine*	H-M	0.00126
<u>Urate</u>	H-M	0.34988	Tryptophan*	H-M	0.00471
Dimethyl-xanthine	H-L	0.72031	Tyrosine	M	0.12786
Xanthine	H-L	0.68485	Asparagine*	M-L	0.00780
N-Methyl-guanosine*	M	0.00001	<u>Glutamate</u>	M-L	0.40196
Adenosine*	M-L	0.00228	Glutamine*	M-L	0.00033
Caffeine	M-L	0.94295	Leucine*	M-L	0.00154
Cytidine	M-L	0.48795	Lysine*	M-L	0.04265
Hypoxanthine*	M-L	0.04265	Methionine*	M-L	0.01070
Uridine	L	0.07627	Proline	M-L	0.25884
<i>Sugar derivatives (4)</i>			Serine*	M-L	0.00026
<u>Gluconate*</u>	H-M	0.01448	Threonine*	M-L	0.00662
<u>N-Acetyl-glucosamine*</u>	H-M	0.01675	<u>Aspartate*</u>	L	0.00275
1,5-Anhydroglucitol*	M-L	0.00003	Isoleucine*	L	0.00102
myo-Inositol*	M-L	0.03766	Valine*	L	0.00033
<i>Sugar phosphates (6)</i>			<i>Methylated amino acids (12)</i>		
<u>Glycerol-phosphate*</u>	H-L	0.00228	Betaine	H	0.10476
<u>Fructose-1,6-diphosphate*</u>	L	0.00780	Dimethyl-arginine*	H	0.01675
<u>Fructose-6-phosphate*</u>	L	0.01070	N-Methyl-histidine*	H	0.00042
<u>Glucose-6-phosphate</u>	L	0.55018	<u>Trimethyl-lysine*</u>	H	0.02222
<u>Pentose-phosphate*</u>	L	0.00662	Dimethyl-lysine*	H-M	0.02222
<u>Phosphoglycerate*</u>	L	0.00662	<u>Dimethyl-proline</u>	H-M	0.18518
<i>Vitamins and coenzymes (3)</i>			<u>Trimethyl-tryptophan</u>	H-L	0.75638
<u>Pantothenate</u>	H-M	0.18518	<u>Butyro-betaine*</u>	M-L	0.04265
4-Aminobenzoate	H-L	0.48795	N6-Methyl-lysine	M-L	0.30216
<u>Nicotinamide</u>	M-L	1.00000	<u>Trimethyl-histidine</u>	M-L	0.61595
<i>Choline and ethanolamine derivatives (4)</i>			<u>Trimethyl-tyrosine</u>	M-L	0.18178
Phosphocholine	M	0.32548	<u>S-Methyl-ergothioneine</u>	L	0.16939
Glycerophosphocholine*	M-L	0.00228	<i>Acetylated amino acids (6)</i>		
Glycerophosphoethanolamine*	L	0.00228	N-Acetyl-arginine*	H-M	0.00003
Phosphoethanolamine	L	0.21988	N2-Acetyl-lysine*	M	0.00915
<i>Carnitines (8)</i>			N6-Acetyl-lysine*	M	0.00154
Carnitine	H-M	0.45827	N-Acetyl-aspartate*	M-L	0.01675
(iso)Butyryl-carnitine*	H-M	0.00187	<u>N-Acetyl-glutamate</u>	M-L	0.09448
(iso)Valeryl-carnitine*	H-L	0.01247	N-Acetyl-(iso)leucine	M-L	0.09448
<u>Acetyl-carnitine</u>	H-L	0.65004	<i>Other amino acids (15)</i>		
Decanoyl-carnitine*	M-L	0.00560	Creatinine*	H	0.00001
Hexanoyl-carnitine	M-L	0.09448	Hippurate	H	0.32548
Octanoyl-carnitine*	M-L	0.00009	Indoxyl-sulfate*	H	0.00187
<u>Propionyl-carnitine</u>	M-L	0.98097	4-Guanidinobutanoate*	H-M	0.01448
<i>Organic acids (11)</i>			Taurine*	H-M	0.01448
Citrate	H-M	0.12786	Acetyl-carnosine	M-L	0.58264
2-Hydroxybutyrate*	M-L	0.00082	<u>Carnosine*</u>	M-L	0.00053
2-Oxoglutarate	M-L	0.42960	Citrulline	M-L	0.21988
3-Hydroxybutyrate*	M-L	0.00395	<u>Creatine</u>	M-L	0.27995
Arginino-succinate	M-L	0.86738	Keto(iso)leucine*	M-L	0.03766
cis-Aconitate	M-L	0.25884	Kynurenine*	M-L	0.01675
<u>Citramalate</u>	M-L	0.10476	Quinolinic acid*	M-L	0.00102
Glycochenodeoxycholate	M-L	0.42960	<u>S-Adenosyl-methionine</u>	M-L	0.25884
<u>Malate</u>	M-L	0.32548	Ornithine	L	0.06094
<u>Succinate</u>	M-L	0.11587	<u>S-Adenosyl-homocysteine*</u>	L	0.02547
Glycerate	L	0.14075	P-values between age groups were calculated by Mann Whitney U-test.		
<i>Antioxidant (1)</i>			Asterisks indicate 55 compounds that showed significant difference (p<0.05). The peak ratio is described in only asterisked compounds.		
<u>Glutathione disulfide*</u>	L	0.00003	Thirty-one RBC-enriched compounds are underlined (Chaleckis et al., 2016).		

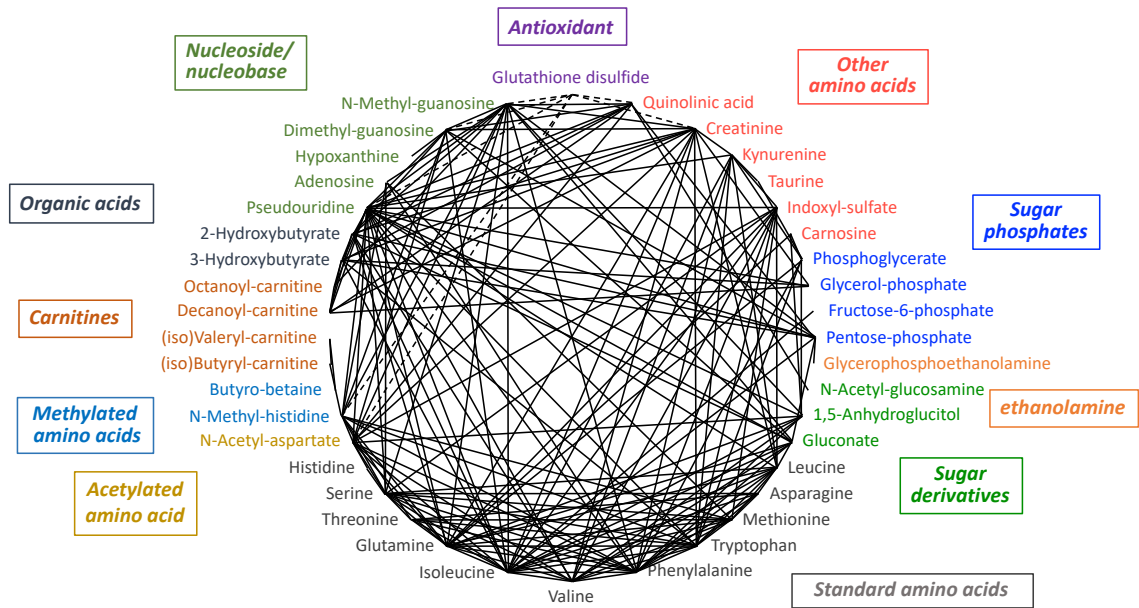
Supplemental Table S3, Chromatogram and mass spectrum data.

<i>Category /Compound</i>	Status	Ionization	Theoretical m/z	Detected m/z	m/z error (ppm)	RT (min)
<i>Nucleosides, nucleobases, and derivatives (12)</i>						
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
<i>Sugar derivatives (4)</i>						
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
<i>Sugar phosphates (6)</i>						
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
<i>Vitamins and coenzymes (3)</i>						
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
<i>Choline and ethanolamine derivatives (4)</i>						
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
<i>Carnitines (8)</i>						
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
<i>Organic acids (11)</i>						
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
<i>Antioxidants (3)</i>						
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.

Aspartate -----	Glutathione disulfide (-0.67) N-Acetyl-aspartate (0.65) Quinolinic acid (0.60) N-Methyl-histidine (0.58) N-Acetyl-glucosamine (0.57)	Glycerophosphocholine -----	N-Methyl-guanosine (0.59) Dimethyl-guanosine (0.54) Octanoyl-carnitine (0.52) Histidine (0.51) Hypoxanthine (0.48)
Lysine -----	N2-Acetyl-lysine (0.58) Phenylalanine (0.55) Methionine (0.54) Leucine (0.51) Dimethyl-arginine (0.51)	Dimethyl-arginine -----	Phosphoglycerate (0.68) Tryptophan (0.67) Butyro-betaine (0.64) Indoxyl-sulfate (0.62) Glycerol-phosphate (0.62)
N-Methyl-adenosine -----	N-Acetyl-glucosamine (0.52) N6-Acetyl-lysine (0.51) Fructose-6-phosphate (0.49) Isoleucine (0.48) N-Methyl-guanosine (0.45)	Dimethyl-lysine -----	Indoxyl-sulfate (0.61) Pentose-phosphate (0.58) Fructose-6-phosphate (0.57) Dimethyl-arginine (0.57) Keto(iso)leucine (0.56)
myo-Inositol -----	Glycerophosphocholine (-0.47) Keto(iso)leucine (0.45) N-Methyl-adenosine (-0.43) S-Adenosyl-homocysteine (-0.36) Adenosine (-0.32)	Trimethyl-lysine -----	N-Methyl-histidine (0.58) Asparagine (0.57) Adenosine (0.54) Dimethyl-arginine (0.53) Tryptophan (0.52)
Fructose-1,6-diphosphate -----	Pentose-phosphate (0.58) Gluconate (0.58) Leucine (0.57) Dimethyl-guanosine (0.56) 2-Hydroxybutyrate (0.55)	N6-Acetyl-lysine -----	Valine (0.61) Phenylalanine (0.57) Asparagine (0.55) N-Methyl-adenosine (0.51) Leucine (0.51)
4-Guanidinobutanoate -----	Threonine (0.66) Asparagine (0.63) Methionine (0.62) N-Acetyl-arginine (0.62) Serine (0.61)	S-Adenosyl-homocysteine -----	Octanoyl-carnitine (0.48) N-Methyl-adenosine (0.41) Decanoyl-carnitine (0.41) Glycerophosphocholine (0.40) 3-Hydroxybutyrate (0.38)
Keto(iso)leucine -----	Fructose-6-phosphate (0.62) Dimethyl-lysine (0.56) 2-Hydroxybutyrate (0.48) myo-Inositol (0.45) 3-Hydroxybutyrate (0.44)		

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