

RESEARCH ARTICLE

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

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Abstract

Metabolites in human biofluids document the physiological status of individuals. We conducted comprehensive, non-targeted, non-invasive metabolomic analysis of urine from 27 healthy human subjects, comprising 13 young adults (30 ± 3 years) and 14 seniors (76 ± 4 years). Quantitative analysis of 99 metabolites revealed 55 that displayed significant differences in abundance between the two groups. Forty-four did not show a statistically significant relationship with age. 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 (glutathione disulfide, myo-inositol) increased in elderly people. The great majority of age-linked markers were highly correlated with creatinine. In contrast, 44 other urinary metabolites, including urate, carnitine, hippurate, and betaine, were not age-linked, neither declining nor increasing in elderly subjects. As metabolite profiles of urine and blood are quite different, age-related information in urine offers additional valuable insights into aging mechanisms of endocrine system. Correlation analysis of urinary metabolites revealed distinctly inter-related groups of compounds.

KEYWORDS

creatinine, glutathione, human endocrine aging, LC-MS, non-targeted metabolomics, pseudouridine, urinary age markers

1 | INTRODUCTION

Elderly people are acutely aware of the progressive aging, 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 multifaceted aging of the human body.¹⁻¹⁶ Age-dependent changes of metabolite abundances may be valuable in identifying molecular causes of impaired organ functions.¹² Technology that enables simple and rapid measurement of urinary metabolites, which can be collected

Abbreviations: AU, arbitrary unit; GSH, reduced glutathione; GSSG, oxidized glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LC-MS, liquid chromatography-mass spectrometry; PCA, principal component analysis; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid).

[Correction added on November 19, 2020, after first online publication: affiliation 2 has been updated correctly.]

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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.^{13,15,17} 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.¹⁸⁻²³ Nonetheless, because urinary metabolites originate in all organ systems, urinary metabolites may be useful to examine human aging in a synthetic way. In this study, we analyzed urinary metabolites in young and elderly subjects, using comprehensive metabolomics to identify metabolites linked to aging. Striking correlations of many urine metabolites with age were found. Previously creatinine, the waste product from muscle and other organs, was identified as an age-related factor.^{1,2,20} Subsequently, dimethyl-guanosine, decanoyl-carnitine, N-acetyl-aspartate, and tryptophan were reported to decline in urine of elderly subjects.^{15,17} In this study, we identified many more age-dependent and age-independent metabolites using comprehensive, non-targeted analysis of urine compounds. Fifty-three compounds were found to decline, while two compounds increase in urine of elderly subjects.

2 | MATERIAL AND METHODS

2.1 | Participants and sample collection

Fourteen elderly (69–81 years) and 13 young (25–38 years) healthy people participated as subjects in this study (Table S1). Serum clinical renal markers were measured at Falco Biosystems Ltd. (Kyoto, Japan). Values of all participants were within normal ranges (urea nitrogen, 8–20 mg/dL; creatinine, female 0.47–0.79 and male 0.61–1.04 mg/dL). 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 similar to those previously reported.²⁴ Midstream urine samples for metabolomic analysis were taken in the morning at home, immediately after awakening. Subjects were asked to ensure at least 8 h of fasting prior to sampling. During fasting, they took water freely. After collection, urine samples were brought to the laboratory within 3 h. 0.2 mL urine were immediately quenched in 1.8 mL of 55% methanol at -40°C . This rapid 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) 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.^{12,25}

2.2 | 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.

2.3 | Chemicals and reagents

Standards for metabolite identification were purchased from commercial sources as described previously.^{12,26-28}

2.4 | LC-MS analysis and data processing

Urinary metabolites were analyzed using an Ultimate 3000 DGP-3600RS HPLC system (Thermo Fisher Scientific) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific), as described.^{12,25} Briefly, LC separation was performed on a ZIC-pHILIC column (Merck SeQuant, Umea, Sweden; 150 mm \times 2.1 mm, 5 μm particle size). Acetonitrile (A) and 10 mM ammonium carbonate buffer, pH 9.3 (B) were used as the mobile phase, with a linear gradient elution from 80% to 20% A over 30 min, at a flow rate of 100 $\mu\text{L}/\text{mL}$. The mass spectrometer was operated in full-scan mode with a 100-1000 m/z scan rate and automatic data-dependent MS/MS fragmentation scans. For each metabolite, we chose a singly charged, $[\text{M} + \text{H}]^{+}$ or $[\text{M} - \text{H}]^{-}$, peak (Table S2). Peak detection and identification of metabolites were performed using MZmine 2 software.²⁹ Detailed data analytical procedures and parameters have been described previously.²⁶ Normalized peak area was calculated from the following formula: Normalized peak area = (Raw peak area of each metabolite/Raw peak area of creatinine) \times 1000.

2.5 | Peak identification and characteristics

We analyzed 99 urine metabolites that were confirmed using standards or MS/MS analysis.^{12,26-28} Metabolite abundances were classified into 3 groups (H, M, and L), according to their peak areas. H denotes compounds with

high peak areas ($>10^8$ AU), M with medium peak areas ($10^7 \sim 10^8$ AU) and L with low peak areas ($<10^7$ AU) (Table S3).

2.6 | Statistical analysis

Peak data processed by MZmine 2 were exported into spreadsheet format and analyzed with R statistical software (<http://www.r-project.org>). Statistical analysis was performed using the Mann Whitney *U*-test. Statistical significance was established at $p < 0.05$. *Q*-values were calculated from *p*-values using the Benjamini-Hochberg method.

2.7 | Data and materials availability

Raw LC-MS data in mzML format are accessible via the MetaboLights repository (URL: <http://www.ebi.ac.uk/metabolights>). Data for the 27 volunteers are available under accession number MTBLS1407.

3 | RESULTS

3.1 | Collection of urine samples

Samples of morning urine, immediately after awaking, were collected from healthy volunteer subjects (elderly, 75.8 ± 3.9 years, and young, 30.6 ± 3.2 years; Table S1 shows gender, BMI, urea nitrogen, and creatinine) in Onna Village, Okinawa, Japan. Precautions taken for sample collection are described in the Material and Methods. Basic data analytical procedures were similar to those previously described.^{12,25,26}

3.2 | 99 urine metabolites identified

Ninety-nine urinary metabolites were identified and quantified using liquid chromatography-mass spectrometry (LC-MS) and the peak identification software MZmine 2^{26,29} (chromatogram and mass spectrum data in Table S2, and *p*-values, *q*-values, and age markers in Table S3). These compounds were subdivided into 12 groups, containing standard amino acids, methylated amino acids, acetylated and other amino acids, nucleosides, nucleobases, and their derivatives, sugar derivatives, sugar phosphates, vitamins

and coenzymes, choline and ethanolamine derivatives, carnitines, organic acids, and an antioxidant. 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.^{12,27} Raw abundance data are shown in the database.

Some compounds varied widely among individuals 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 belonged to the abundant class (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.^{12,27} Below, we show that about half of all urinary metabolites are age-related.

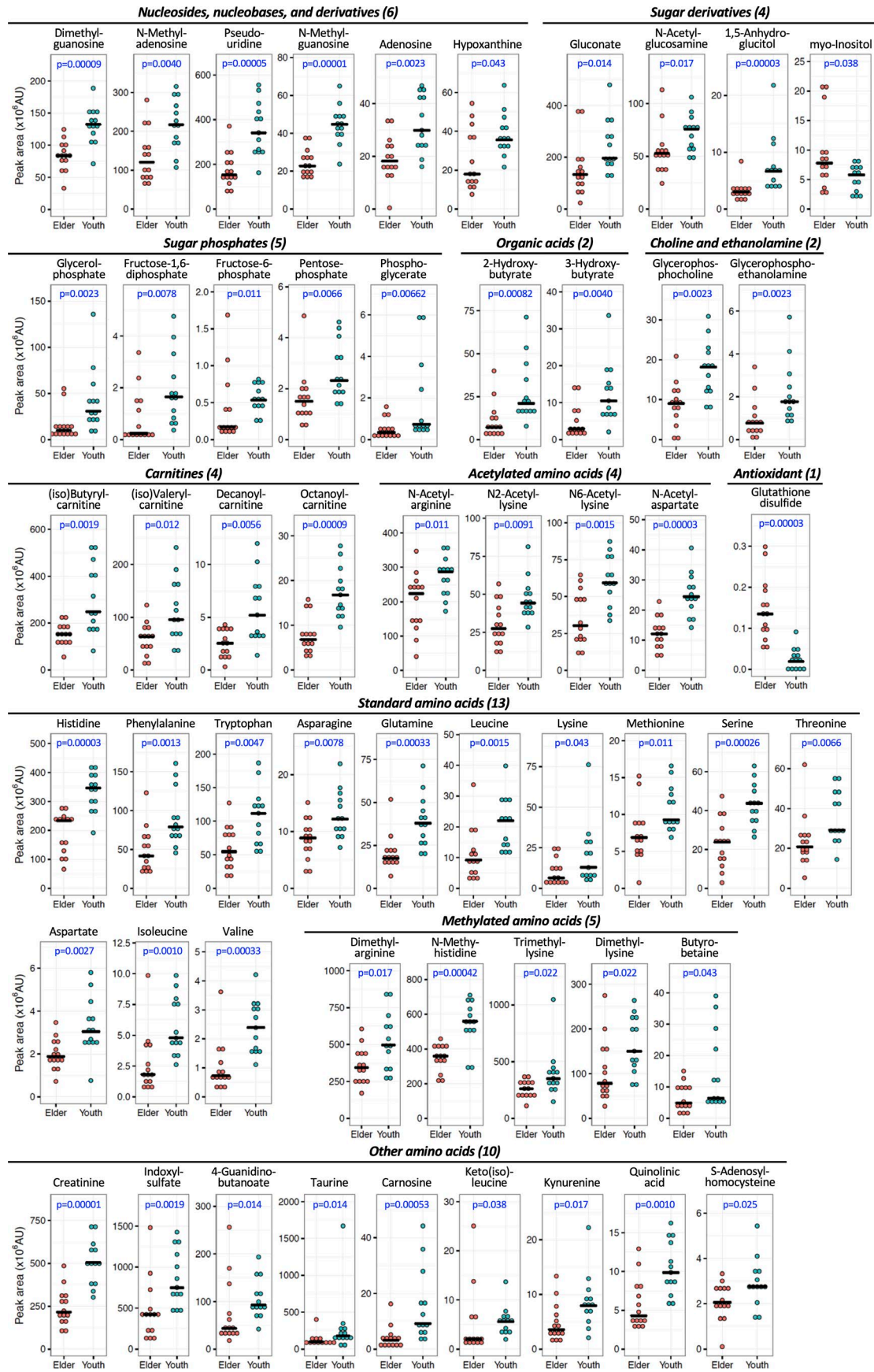
3.3 | 55 urinary compounds are less abundant in elderly samples

Of 99 urinary metabolites assayed in 27 subjects, 55 showed statistically significant differences between young and elderly subjects (*p*-values, $0.00003 < p < 0.05$) (Figure 1; Table 1A). Thus, about half (55/99) of the urinary metabolites are age-linked to aging, with all but glutathione disulfide (GSSG) and myo-inositol becoming less abundant in elderly subjects. To verify statistical significance of metabolite abundances, we also calculated *q*-values, as an estimate of false discovery rates. *Q*-values shown in Table S3 are consistent with *p*-values of 50 age-related compounds. Five compounds (hypoxanthine, myo-inositol, lysine, butyro-betaine, keto(iso) leucine) had *q*-values of ~ 0.08 . In contrast, the remaining 44 compounds (Tables 1B and S3), did not differ significantly between young and elderly (Figure S1).

3.4 | Creatinine is an age marker

In clinical settings, creatinine is often used for normalization, as it is abundant and stable. Creatinine is also known as a urinary age marker.¹ We found that creatinine and many other age markers decline in elderly subjects, possibly due to similar causes. If creatinine is used for normalization of

FIGURE 1 Dot plot profiles of 55 age-linked urine compounds, which comprise 11 groups, in samples from 27 individuals are shown. Pale red and azure dots represent elderly and young subjects, respectively. Bars represent medians in each group. The peak abundance of compounds and the ratio of median value between elderly and young are shown in Table 1A and 1B. *P*-values were obtained using the non-parametric Mann Whitney *U*-test. Fifty-three of 55 compounds were more abundant in urine of young subjects, while two (myo-inositol and GSSG) were more abundant among the elderly



3 age-linked metabolites (dimethyl-guanosine, tryptophan, N-acetyl-aspartate), such age-linked differences vanish for tryptophan and N-acetyl-aspartate (Figure S1A), while dimethyl-guanosine abundance in elderly urine diminished to a little more than half that of young adult samples. If age-independent metabolites such as urate, citrate, caffeine, betaine are normalized against creatinine, their abundances in elderly urine exceeded those in young adult samples, causing false assignments (Figure S1B). Thus, for 44 urine age-independent compounds, creatinine cannot be used for normalization.

Since urine concentration fluctuates daily due to factors such as water intake, it is common to use creatinine concentration for correcting the measured values. If metabolites of interest are age-linked, normalization against creatinine might be appropriate. If not, correction of quantitative data with creatinine levels might miss certain compounds with interesting features. To check the consistency of urinary metabolite concentrations in first morning urine, we examined the diurnal variation of 8 representative metabolites that were less abundant in urine of elderly people. Three young subjects were asked to collect urine every morning for 5 days. Our data indicated that the diurnal variation of these metabolites was negligible in the same person (Figure S2).

3.5 | Glutathione increased in elderly urine while other compounds declined sharply

Two compounds, myo-Inositol and GSSG, were 1.34- and 6.96-fold, more abundant, respectively, in elderly samples (Table 1A). The increase of GSSG was striking, though its abundance in urine was rather low (Figure S3A). GSSG (and glutathione, GSH) was virtually absent in urine of young adults, while it was abundant in elderly samples. myo-Inositol, like GSSG increased in elderly urine (Figure S3H), a phenomenon which cannot be explained at present.

Creatinine was abundant in urine of young subjects (Figure S3B), but together with pseudouridine, isoleucine, leucine, dimethyl-guanosine and N-methyl-guanosine, it declined sharply in urine of elderly subjects (Figure S3C–G). In fact, 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 octanoyl-carnitine (0.40)].

3.6 | 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. The highest correlation, 0.92, was obtained for isoleucine – leucine and N-methyl guanosine – dimethyl guanosine (Figure 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.

Pairwise correlation analyses revealed 19 pairs of compounds with correlation coefficients >0.85 (Figure 2). These show a network consisting of two groups, one centered around pseudouridine and the other around isoleucine (Figure 2). Creatinine is linked to both. This suggests that compounds within each of these groups may be metabolically linked.

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, and of isoleucine to glutamine required further explanation. In the case of a creatinine-related network, 11 metabolites have correlation coefficients from 0.71–0.84 (Figure 2A). Creatinine, a known waste product from muscle,^{30,31} is thus correlated with a broad array of urinary metabolites.

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 from 55 age-linked compounds (Figure S4). 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.

3.7 | Some age-linked metabolites were not highly correlated

Thirteen age-marker compounds showed only weak correlations (<0.7) with the other 42 age-linked urinary metabolites (Figure S5). 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 0.48 (octanoyl-carnitine). Lysine, dimethyl-lysine, trimethyl-lysine, and N6-acetyl-lysine did not show high correlation coefficients. Fructose-1,6-diphosphate showed a maximal correlation value (0.58) with pentose-phosphate and gluconate. Whether these metabolites decline in elderly subjects as a consequence of aging or as a cause of it, remains to be investigated.

TABLE 1A List of 55 aging-linked compounds that showed a significant difference in abundance between young and elderly people. The abundance of compounds (peak area) displayed: H, high peak areas [$>10^8$ AU (arbitrary unit)]; M, medium peak areas ($10^7 \sim 10^8$ AU); L, low peak areas ($<10^7$ AU). The peak ratio was calculated using the median of peak abundance in elderly and young people, respectively

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

(Continues)

TABLE 1A (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

3.8 | Heatmap analysis of urinary metabolites linked to aging

We then employed a heatmap to visualize the quantitative profile of age-linked urinary metabolites in individual subjects (Figure 3). Since most of these metabolites declined in the elderly, matrix color represents metabolite abundance for individual subjects. Explanation of heatmap patterns is given in the legend of Figure 3. Thus, a heatmap of urinary metabolites graphically illustrates the degree of urinary metabolite aging for individual subjects, so that subjects may be compared one with another. Two subjects (elderly 11, 81F and young 15, 33 M) showed patterns remarkably like those of the opposite age group.

3.9 | Principal component analysis of age-linked metabolites

Implications of urinary compounds in aging should also be cross-examined using results of blood compound analyses. However, as these compounds decrease in urine of elderly people, the results are consistent with actual aging. Some urinary metabolites may be implicated in sustaining health and slowing aging. To integrate quantitative metabolite abundance data from individual subjects, abundances of these 55 age-related metabolites were subjected to principal component analysis (PCA).^{25,32} Subjects were separated into 2 groups representing negative and positive values of principal component (PC) 1, respectively (Figure 4A). One elderly subject 11 (81F) was in the middle of the young group and one young subject 15 (33 M) was in the group of elderly subjects in the PCA plot (Figure 4A). These are consistent with results plotted in the heatmap (Figure 3). The PC1 score

and chronological age of the subject were highly correlated ($r = 0.80$) (Figure 4B).

3.10 | 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.) (Figure 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 (Figure 5B-F). GSSG was low to undetectable in 5 young subjects, but accumulated in elderly samples (Figure 5F).

GSSG showed different degrees of abundance in different subjects, and was virtually absent in young subjects, but in elderly subjects, its abundance increased and paralleled the abundance of creatinine in young subjects (Figure S3A,B). Thus GSSG abundance was inversely related to aging. The reason for this increase of GSSG in elderly urine is probably due to the absence or declining effectiveness of a mechanism to metabolize and reprocess GSSG by the elderly. Such a reductive mechanism does exist in young subjects; however, the redox environment regarding glutathione appears to be altered during aging. Inability or declined efficiency to reduce oxidized GSSG at any recycling step may accelerate aging.

TABLE 1B List of 44 metabolites that did not show a significant difference in abundance between young and elderly people

Category	Compound	Peak abundance	Ratio (elderly/young)
Nucleosides, nucleobases, and derivatives (6)	Urate	H-M	0.79
	Dimethyl-xanthine	H-L	1.35
	Xanthine	H-L	0.93
	Caffeine	M-L	1.05
	Cytidine	M-L	0.98
	Uridine	L	0.64
Sugar phosphate (1)	Glucose-6-phosphate	L	0.78
Vitamins and coenzymes (3)	Pantothenate	H-M	0.79
	4-Aminobenzoate	H-L	3.04
	Nicotinamide	M-L	0.85
Choline and ethanolamine derivatives (2)	Phosphocholine	M	0.66
	Phosphoethanolamine	L	0.69
Carnitines (4)	Carnitine	H-M	0.82
	Acetyl-carnitine	H-L	1.05
	Hexanoyl-carnitine	M-L	0.65
	Propionyl-carnitine	M-L	0.99
Organic acids (9)	Citrate	H-M	0.73
	2-Oxoglutarate	M-L	0.83
	Arginino-succinate	M-L	1.05
	cis-Aconitate	M-L	0.67
	Citramalate	M-L	0.56
	Glycochenodeoxycholate	M-L	0.69
	Malate	M-L	0.89
	Succinate	M-L	0.52
	Glycerate	L	0.54
Standard amino acids (4)	Arginine	H-M	0.89
	Tyrosine	M	0.84
	Glutamate	M-L	0.63
	Proline	M-L	0.79
Methylated amino acids (7)	Betaine	H	0.74
	Dimethyl-proline	H-M	0.56
	Trimethyl-tryptophan	H-L	1.57
	N6-Methyl-lysine	M-L	0.63
	Trimethyl-histidine	M-L	0.75
	Trimethyl-tyrosine	M-L	2.66
	S-Methyl-ergothioneine	L	0.83
Acetylated amino acids (2)	N-Acetyl-glutamate	M-L	0.71
	N-Acetyl-(iso)leucine	M-L	0.68
Other amino acids (6)	Hippurate	H	0.65
	Acetyl-carnosine	M-L	1.42
	Citrulline	M-L	0.78
	Creatine	M-L	0.79
	S-Adenosyl-methionine	M-L	1.21
	Ornithine	L	0.79

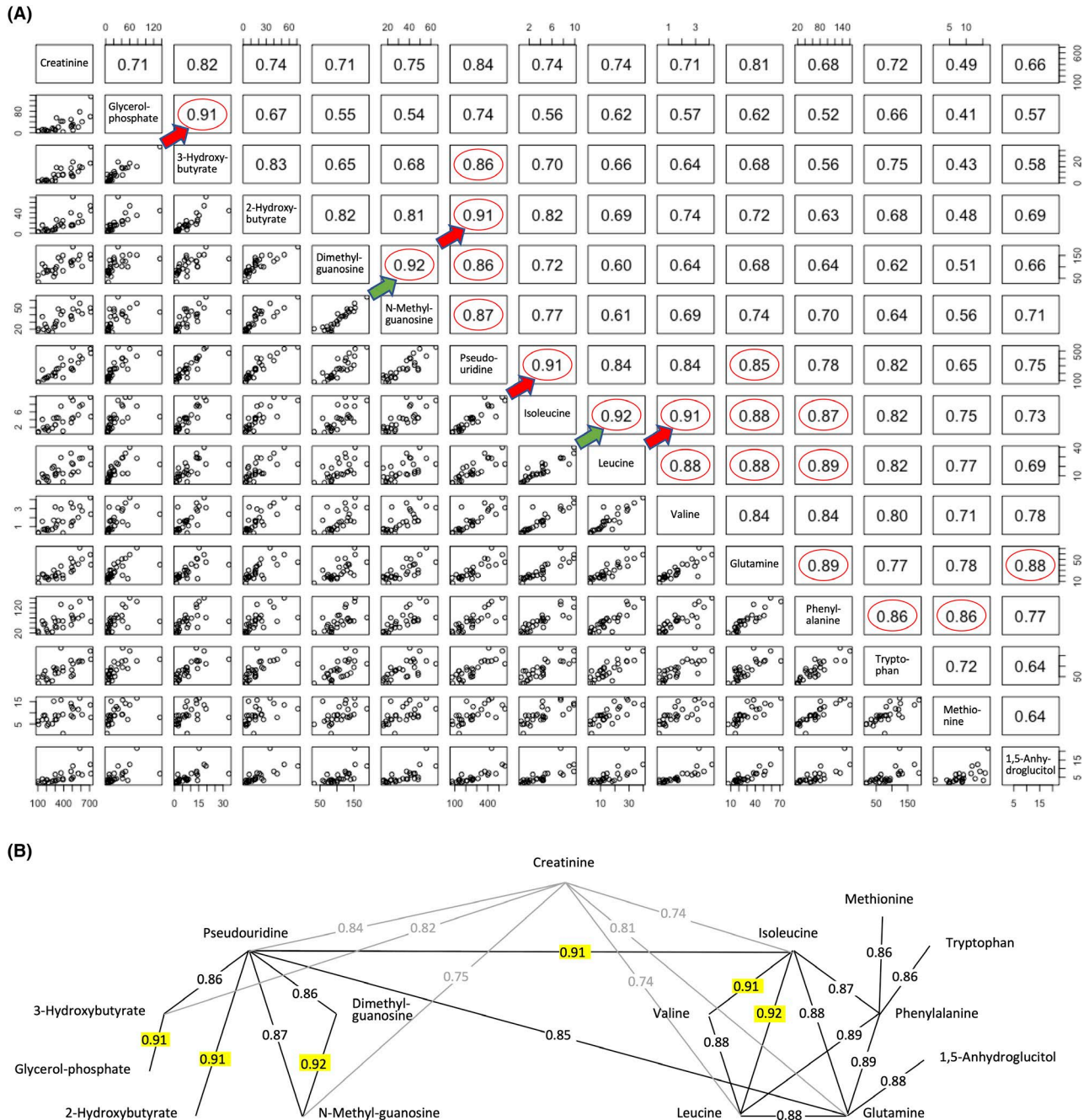


FIGURE 2 Correlation network among age-linked compounds. (A) Values indicate correlation coefficients between paired compounds. Highly correlated pairs of aging markers ($r > 0.85$) are indicated with red circles. The most highly correlated pairs are indicated by green (0.92) and red arrows (0.91). (B) Interrelated compounds form a network. Correlations ($r > 0.90$) are highlighted in yellow. Creatinine was correlated with both groups ($r = 0.74$ – 0.84 , shown in gray)

4 | 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,^{28,33–37} later embarking on human blood metabolomics.^{12,25–27} By adapting the software MZmine 2 for metabolite identification,^{29,38}

we found that metabolomics of fission yeast and humans are surprisingly similar in regard to metabolite composition.²⁷ 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.^{12,25–27} We then undertook human fluids collected non-invasively, such as urine and saliva.

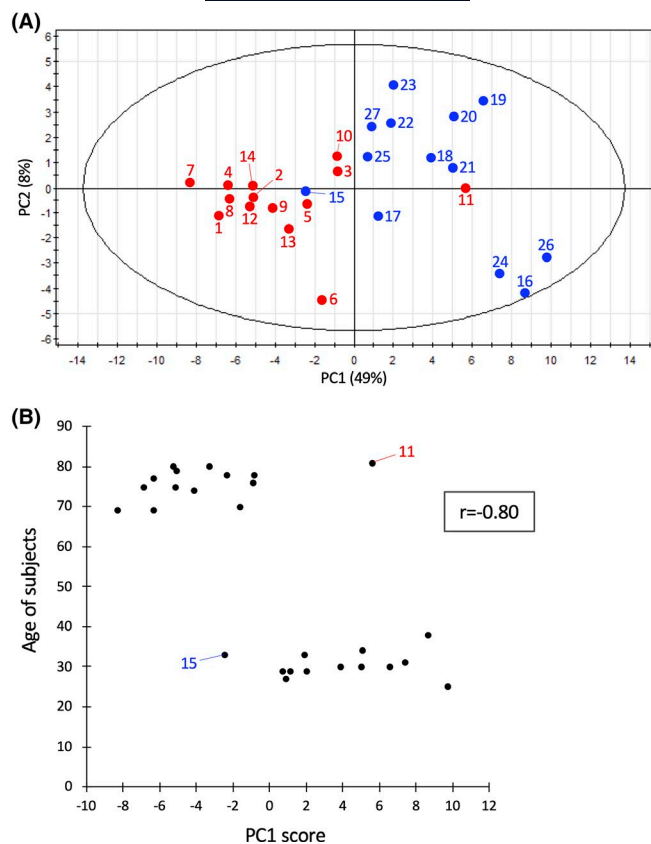


FIGURE 4 PCA of 55 aging markers. (A) Thirteen young and 14 elderly subjects are shown in blue and red, respectively, with their subject numbers (indicated in the heatmap Figure 3). PC1 reflected the abundance of the compounds with strong correlation networks, such as pseudouridine and isoleucine (see Figure 2B and Figure S4). PC2 comprised metabolites that were not strongly correlated (glycerophosphocholine, S-adenosyl-homocysteine, etc.), but that were isolated from a strong correlation network. (B) PC1 score of each subject (*X*-axis) is plotted versus subject age (*Y*-axis). Subject 11 (F, age 81) has a heatmap profile of a young person (Figure 3), while subject 15 (M, age 33) has an exceptionally elderly heatmap profile. The correlation coefficient is shown in the box

S4). Their abundances were reduced, except for the increase of GSSG and myo-inositol. Aging information obtained from urine metabolites may be useful in different ways from blood metabolites. Aging due to declining kidney function, for example, should be a primary research target. 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 age-independent (listed in Table 1B). In aged subjects, levels of protein, nucleic acid and lipid may decrease due to reduced physical activity,³⁹⁻⁴¹ resulting in declining metabolite abundances in urine. 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. However, the role of age-independent metabolites is much less understood. Their correlations are also lower than those of age-linked metabolites. Further study is required.

4.2 | Glutathione disulfide, myo-inositol, and pseudouridine

Two exceptional urinary age metabolites, myo-inositol and GSSG, increased in elderly samples (1.34- and 6.96-fold, respectively). myo-Inositol may be an important second messenger when phosphorylated, causing changes in $[Ca^{2+}]$.^{42,43} In the correlation analysis, myo-inositol was not highly correlated with any other metabolite (Figure S5). Glycerophosphocholine and N-methyl-adenosine showed weak, *negative* correlations with myo-inositol (-0.47 and -0.43 , respectively, Figure S5). Implications of the myo-inositol increase in urine of elderly people remain unclear.

GSSG that we detected was the inactive, oxidized form of GSH. It sharply increased in elderly urine (Figures 1 and S3A). The active SH form of GSH seems to be very unstable and it is difficult to prevent its degradation during preparation of urine (the present study) and blood samples.¹² The active SH form was rapidly oxidized to an inactive disulfide form, which accumulated in elderly subjects, whereas in young subjects the disulfide form GSSG was hardly formed or undetected possibly due to rapid decay in the young body or sample. Consistently, GSSG was undetectable in urine of the five young subjects (Figure S3A). The abundance of GSSG was inversely related to concentrations of pseudouridine, creatinine, and dimethyl-guanosine, which were most abundant in young subjects (Figures 5 and S3). Thus the redox compound GSSG in urine appears to be appropriate as an age marker in urine of elderly subjects. In contrast, the high abundance of the nucleoside, pseudouridine, seems to be emblematic of youth.

4.3 | Highly correlated urine metabolites

Fifteen metabolites, comprising two groups, are highly correlated (correlation coefficients >0.85 , Figure 2A,B). One includes nucleosides pseudouridine, dimethyl-guanosine, N-methyl-guanosine and 2- or 3-hydroxybutyrate and the other group contains branched-chain 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; Figure 2). It remains to be determined what kind of functional connections exist between these two groups of metabolites in health and disease. In addition, a number of compounds showing correlation coefficients >0.70 are only remotely related structurally. Therefore, the high correlations of urinary compounds cannot all be easily

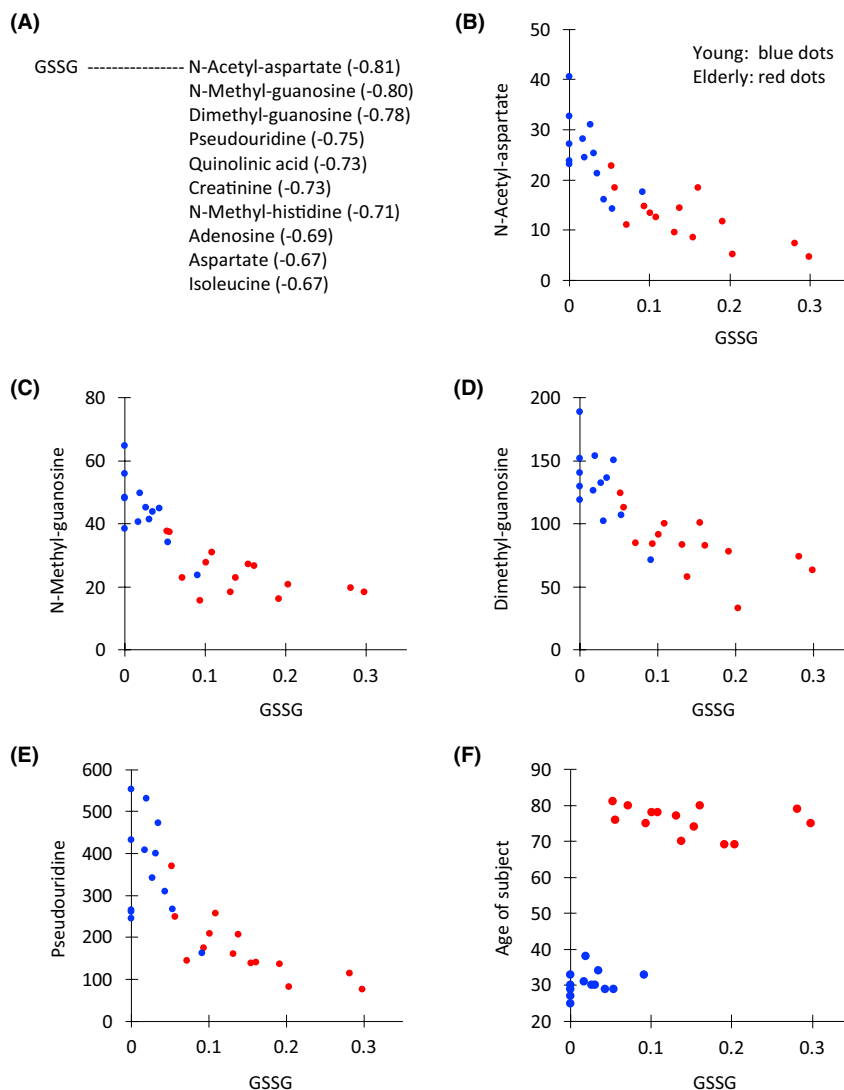


FIGURE 5 Scatter plots between GSSG and correlated compounds or age. (A) Ten metabolites with the relatively high correlations with GSSG are listed. Scatter plot of the peak abundance ($\times 10^6$ AU) between GSSG and N-acetyl aspartate (B), N-methyl guanosine (C), dimethyl guanosine (D), pseudouridine (E), and the age of the subject (F). Dots for young and elderly subjects are blue and red, respectively

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.

4.4 | Thirteen metabolites linked by low correlation

Thirteen age-related metabolites that showed only low correlation coefficients include myo-inositol, two regular amino

acids (aspartate and lysine), methylated or acetylated amino acids, keto(iso)leucine, etc (Figure S5). Creatinine, an abundant aging marker¹ and an amino acid waste product in muscle and other tissues showed higher correlations (0.71–0.84, Figure 2A,B). Its urinary content decreased to 43% in elderly samples (Tables 1A and 1B). 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 urine of elderly persons. Pseudouridine is an oxidized tRNA component,⁴⁴ 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 high correlation coefficients with 10 compounds (Figure 2) and its high abundance in urine may provide a convenient youth marker.

4.5 | An effective overview on aging by heatmap

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 (Figure 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 (subjects 11 and 15) in the present study (Figure 4). Exceptional individuals are of considerable interest, by virtue of their persistent youth or premature aging, if these truly represent metabolic features in endocrine system. Their unusual profiles may be worthy of further investigation. Among 55 age-related urinary markers, 13 compounds did not show correlations >0.7 (Figure S5). 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) (Figure S5). 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.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

M. Yanagida and T. Teruya conceived the research plan and wrote the paper. T. Teruya and H. Goga performed the experiments.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Supplementary Material

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