Frailty markers comprise blood metabolites involved in antioxidation, cognition, and mobility

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As human society ages globally, age-related disorders are becoming increasingly common. Due to decreasing physiological reserves and increasing organ system dysfunction associated with age, frailty affects many elderly people, compromising their ability to cope with acute stressors. Frail elderly people commonly manifest complex clinical symptoms, including cognitive dysfunction, hypomobility, and impaired daily activity, the metabolic basis of which remains poorly understood. We applied untargeted, comprehensive LC-MS metabolomic analysis to human blood from 19 frail and nonfrail elderly patients who were clinically evaluated using the Edmonton Frail Scale, the MoCA-J for cognition, and the TUG for mobility. Among 131 metabolites assayed, we identified 22 markers for frailty, cognition, and hypomobility, most of which were abundant in blood. Frailty markers included 5 of 6 markers specifically related to cognition and 6 of 12 markers associated with hypomobility. These overlapping sets of markers included metabolites related to antioxidation, muscle or nitrogen metabolism, and amino acids, most of which are decreased in frail elderly people. Five frailty-related metabolites that decreased—1,5-anhydroglucitol, acetyl-carnosine, ophthalmic acid, leucine, and isoleucine—have been previously reported as markers of aging, providing a metabolic link between human aging and frailty. Our findings clearly indicate that metabolite profiles efficiently distinguish frailty from nonfrailty. Importantly, the antioxidant ergothioneine, which decreases in frailty, is neuroprotective. Oxidative stress resulting from diminished antioxidant levels could be a key vulnerability for the pathogenesis of frailty, exacerbating illnesses related to human aging.

frailty | antioxidants | cognitive impairment | metabolomics | aging marker

Human society is aging globally, in developed as well as in developing countries, and people over age 85 now constitute 1.6% of the world population. While life expectancy is increasing, there is also an alarming rise in the number of frail people who are predisposed to be bedridden and to require nursing care. The prevalence of frailty among those aged 65 and over is estimated at 17%, or approximately 120 million individuals worldwide (1). Frail people suffer not only from physical disabilities, but also from psychophysiological and social problems (2), and thus require more social resources than healthy peers. Frailty compromises their ability to cope with acute stressors due to declining physiological reserves and organ system function (2, 3), although it has been suggested that frailty may be reversible (4). Moreover, human aging is a highly complex biological process exhibiting great individual variation, and until now, its metabolic basis has been little understood.

Because all tissues and organs are supplied by the circulatory system, blood should reflect environmental conditions, genetic and epigenetic factors, nutritional status, exposure to exogenous substances, and lifestyle factors (5, 6). Therefore, human blood samples are expected to document not only individual genetic variability, but also differences in physiological responses and homeostatic mechanisms. For example, recent studies suggest that in circulating leukocytes, telomere length (a biomarker of aging) is affected not only by age, but also by disease, psychophysiological condition, and lifestyle (7, 8).

Metabolomics, a tool for evaluating metabolite profiles, employs liquid chromatography–mass spectrometry (LC-MS) to reveal complex but highly integrated biological processes (5). Although noncellular components (serum or plasma) of blood have most often been used for metabolomic assays (5), we developed whole blood and red blood cell metabolomics (9) to comprehensively investigate metabolic foundations of human aging. Metabolomic analyses enable us to detect metabolites related to amino acid metabolism, the tricarboxylic acid (TCA) cycle, nitrogen, sugar, purine/pyrimidine, lipid metabolism, antioxidation, energy supply, and diet.

Based on these quantitative, reproducible analytical methods, we recently reported 14 age-related metabolites relevant to antioxidative defense and nitrogen metabolism (10). Four recent reports drew divergent, nonoverlapping conclusions (11–14), stemming from different experimental designs. For example, the former two reports applied the Fried CHS index as a diagnostic tool, efficiently detecting hypomobility but offering no cognitive assessment (2), while the latter two used a 70-item clinical Frailty Index focusing mainly on activities of daily living (ADL) assessment (15). Here we report the untargeted metabolomic

Significance

Frailty resulting from age-related deterioration of multiple organ systems displays complex features, including cognitive dysfunction, hypomobility, and impaired daily activity. However, metabolic aspects of frailty remain unclear. We performed untargeted, comprehensive metabolomics of whole blood from 19 frail and nonfrail elderly patients. We identified 22 markers, including 15 for frailty, 6 for cognition, and 12 for hypomobility, most of which are abundant in blood. Frailty markers include 5 of 6 for cognition and 6 of 12 for hypomobility. These overlapping markers include decreased levels of metabolites related to antioxidation, nitrogen, and amino acid metabolism. Ergothioneine, an antioxidant involved in neuronal diseases, declines in frailty. Thus, we reveal essential metabolites linked to the pathogenesis of frailty, including vulnerability to oxidative stress.

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The authors declare no competing interest.

Data deposition: Raw LC-MS data in mzML format are available from the MetaboLights repository, http://www.ebi.ac.uk/metabolights/.

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analysis of blood from frail and nonfrail elderly people. For frailty diagnosis, we applied the Edmonton Frail Scale (EFS) and the Japanese version of the Montreal Cognitive Assessment (MoCA-J) to evaluate cognitive aspects of frailty (16, 17). We show that antioxidants, amino acids, and metabolites related to muscle or nitrogen metabolism link frailty to cognitive impairment and hypomobility.

Results and Discussion

Nineteen elderly participants, including 7 males and 12 females with a mean age of 84.2 ± 6.9 y, were examined using the EFS, the MoCA-J for cognitive function (17, 18), and the Timed Up & Go (TUG) test for motor ability (19) (Fig. 1*A*). The EFS is an efficient diagnostic tool, comprising 10 questions to assess cognitive ability (clock-drawing test), mobility (TUG test), and fundamental daily activity, in which a score ≥7 indicates frailty (on a scale of 0 to 17) (16). It also covers domains related to health status, functional independence, social support, medications, nutrition, mood, continence, and illness burden. The MoCA-J evaluates short-term memory, visuospatial ability, various executive functions, attention, concentration, working memory, language, and temporal and spatial orientation. An MoCA-J score below a threshold of 25 to 26 (out of 30) indicates mild cognitive impairment (10). Clinical attributes of the study participants are summarized in *SI Appendix*, Table S1.

First, we clinically evaluated whether the 19 participants were frail, cognitively impaired, or hypomobile, according to EFS, MoCA-J, and TUG scores. Nine individuals (average age, 88.2 ± 6.8 y) were diagnosed as frail (average EFS, 9.0 ± 1.2), while 10 (average age, 80.5 ± 4.7 y) were not (average EFS, 4.7 ± 1.1) (*SI Appendix*, Table S1). According to the MoCA-J assessment, 15 individuals displayed impaired cognition (average score; 19.3 ± 3.8), while 4 were normal (average 27.0 ± 0.8). Regarding mobility, 12 participants exhibited a prolonged TUG test (>10 s), while 7 were normal (*SI Appendix*, Table S1). Both the MoCA-J and TUG results were significantly diminished in frailty (Fig. 1*B* and *SI Appendix*, Table S1). Significant correlations of EFS with MoCA-J, TUG, and functional independence test results (Fig. 1*C* and *SI Appendix*, Fig. S1) confirm that frailty involves simultaneously deteriorating physiological functions and social activities.

In this context, we performed untargeted analysis of 131 compounds in whole blood (Dataset S1). Our comprehensive comparison of these metabolites between frail and nonfrail elderly identified 15 compounds as frailty markers (Fig. 1*D* and *SI Appendix*, Table S2). In addition, we found that 6 metabolites

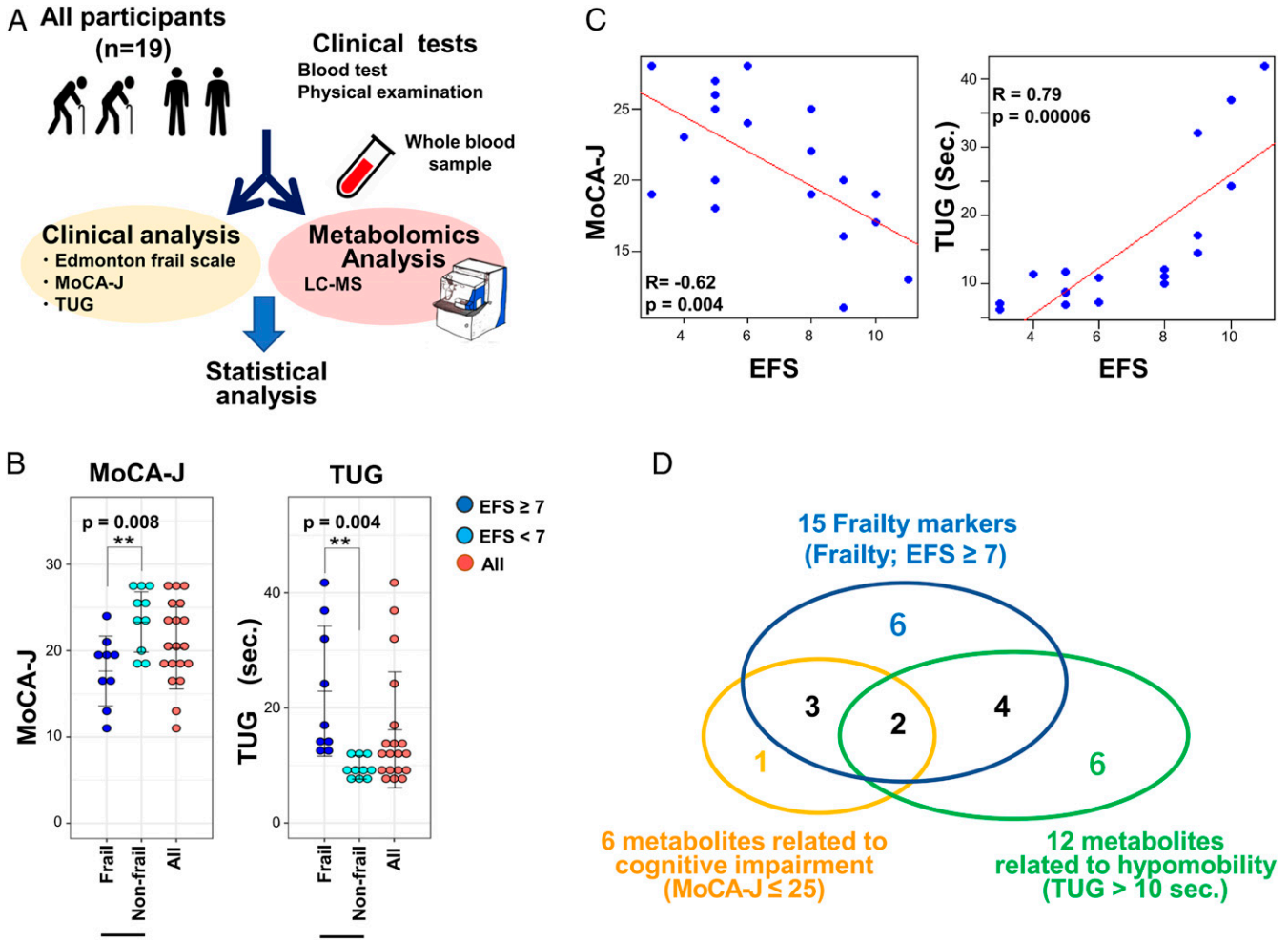

Fig. 1. Metabolomic study of frailty. (*A*) Diagram of the study protocol. All participants were clinically examined, and their blood was analyzed using untargeted comprehensive metabolomics. (*B*) Comparison of MoCA-J and TUG test results between frail and nonfrail subjects. $^{**}P < 0.01$. Error bars represent mean ± SD. (*C*) Pearson's correlation of the linear model between EFS and MoCA-J (*Left*) or TUG (*Right*). (*D*) Overview of identified metabolites related to EFS, MoCA-J, and TUG.

involved in cognitive impairment and 12 metabolites related to low mobility were also significantly changed (Fig. 1*D*).

Among the 15 frailty markers, 13 compounds—acetyl-carnosine, ergothioneine (ET), *S*-methyl-ergothioneine (*S*-methyl-ET), trimethyl-histidine (hercynine), ophthalmic acid (OA), 2-ketobutyrate, urate, 1,5-anhydroglucitol (1,5-AG), proline, isoleucine, leucine, tryptophan, and methionine—decreased in frailty, while two metabolites enriched in red blood cells—creatine and UDP-glucuronate—increased. Ten of 15 frailty markers showed correlations with EFS scores (*SI Appendix*, Table S2). Based on the MoCA-J, a comparison of cognitively impaired subjects and controls detected significant changes in six metabolites—acetyl-carnosine, ET, tryptophan, creatine, UDP-glucuronate, and UDP-glucose—all of which except UDP-glucose were also frailty markers (Fig. 1*D* and *SI Appendix*, Table S3). Among these six metabolites, three compounds—tryptophan, creatine, and UDP-glucuronate—displayed correlations with the MoCA-J results (*SI Appendix*, Table S3). Thus, Pearson's correlation of these marker metabolites also disclosed their congruence with relevant clinical attributes (*SI Appendix*, Fig. S2).

We previously reported 14 aging-related markers (10). In the present study, we noticed that five frailty-related metabolites that were decreased—acetyl-carnosine, OA, isoleucine, leucine, and 1,5-AG—were also among these aging markers (Fig. 2 and *SI Appendix*, Fig. S3*A*), insinuating a metabolic connection between frailty and human aging.

Strikingly, among 15 frailty markers, 7 compounds that were decreased are relevant to antioxidative defense: acetyl-carnosine, ET, *S*-methyl-ET, trimethyl-histidine, OA, 2-ketobutyrate, and urate (Fig. 2*A*). Trimethyl-histidine and *S*-methyl-ET are involved in ET synthesis, mainly in mushrooms and other fungi (20). OA is a tripeptide analog of glutathione (21), the precursor of which is 2-ketobutyrate. Thus, the ergothioneine and OA pathways are greatly affected in frailty. Acetyl-carnosine, formed from β-alanine and histidine, is enriched in muscle (22). Urate is one of the most abundant antioxidants in blood (23). Four of these seven antioxidants are also associated with cognitive impairment or low mobility: acetyl-carnosine, ET, OA, and 2-ketobutyrate (Fig. 2*A*).

We observed significant decreases in five amino acids—methionine, proline, tryptophan, isoleucine, and leucine—in the frail subjects, while tryptophan, methionine, and proline were also reduced in patients manifesting cognitive impairment or low mobility (Fig. 2*B*). Tryptophan is a precursor for the neurotransmitters serotonin and dopamine and is involved in kynurenine metabolism in muscle (24), while leucine and isoleucine are essential for maintaining muscle strength. According to Pearson's correlation analysis, among the 15 metabolites associated with frailty (Fig. 3*A*), five amino acids showed close correlations with several antioxidative metabolites. Correlation coefficients of acetyl-carnosine with methionine and proline were $r = 0.50$ and 0.61, respectively, while those of *S*-methyl-ET with methionine, proline, tryptophan, isoleucine, and leucine were $r = 0.52$ to 0.65 (Fig. 3 *A* and *B*). Interestingly, methionine, proline, and tryptophan, the three amino acids that were decreased in frailty, have been reported as radical scavengers in vitro (25, 26), consistent with recent findings in proteomic analysis (27). Thus, antioxidative defense is greatly impaired in frailty.

Twelve metabolites were identified as hypomobility markers (Fig. 3*C* and *SI Appendix*, Fig. S2*B* and Table S4), some of which are also frailty markers (acetyl-carnosine, OA, 2-ketobutyrate, methionine, proline, and UDP-glucuronate) and cognitive markers (acetyl-carnosine and UDP-glucuronate) (Figs. 2 and 3*D*). Acetyl-carnosine and UDP-glucuronate are linked to increased frailty and to decreased cognition and mobility. The other six hypomobility markers include five decreased metabolites—N3-methyl-histidine, isovaleryl-carnitine, arginine, hippurate, and adenine (Fig. 3*C* and *SI Appendix*, Fig. S3*B*)—and increased *N*-acetyl-aspartate (Fig. 3*D*). Pearson's correlation analysis revealed a close relationship of TUG results with three metabolites: isovaleryl-carnitine, adenine, and UDP-glucuronate) (*SI Appendix*, Table S4). N3-methyl-histidine is an indicator of muscle deterioration (28), while isovaleryl-carnitine, enriched in muscle, supplies acetyl-CoA to mitochondria. Hippurate, synthesized in liver mitochondria from metabolized polyphenol, is involved in nitrogen metabolism (29). Arginine is also utilized in the urea cycle. These four hypomobility metabolites are involved in muscle or nitrogen metabolism, in addition to the involvement of three muscle-related amino acids—tryptophan, isoleucine, and leucine—in frailty (Figs. 2*B* and 3*C*).

Women are at an intrinsic increased risk of frailty by virtue of lower lean mass and strength compared with age-matched men (2). There were significant differences in the skeletal muscle mass index between males and females in our cohort (average, 7.49 vs 5.31; $P = 0.00003$), while metabolites related to muscle mass include compounds involved in nitrogen metabolism (30). Such compounds could be affected in sarcopenic frailty, with an inherent risk in women.

In addition to increased *N*-acetyl-aspartate in hypomobility, three up-regulated metabolites were identified: creatine and UDP-glucuronate for frailty and UDP-glucose for cognition (Fig. 3*D*). Creatine, which is increased in cognitive impairment and frailty, serves as major energy storage in brain and muscle (31). Creatine supplementation is effective for strengthening muscle in athletes (32) and for treating some types of genetic mental retardation associated with cerebral creatine deficiency (33). Increased creatine might compensate for brain and muscle dysfunction in frailty. Moreover, metabolites in UDP-glucuronate biosynthesis are much up-regulated. UDP-glucuronate is involved in the synthesis of ascorbic acid, formation of polysaccharides, and detoxification (34, 35). UDP-glucuronate is increased in frailty, cognitive impairment, and low mobility, and UDP-glucose is up-regulated in cognitive impairment (Fig. 3*D*). It is noteworthy that the antioxidant ET displays significant negative correlations with creatine and UDP glucuronate (Pearson's $r = -0.76$ and -0.56, respectively) (Fig. 3*A* and *SI Appendix*, Fig. S4). It is possible that increased creatine or UDP-glucuronate compensates for decreased antioxidative defense in frailty, which would make frailty reversible. Oxidative damage has been proposed to have a substantial impact both on organismal aging in experimental models (36) and in illnesses of aging, such as Alzheimer's disease (37). As such, declining antioxidative defense could be involved in the pathogenesis of frailty, and oxidative stress may be a key vulnerability for frail elderly people. While a total of 22 metabolites were identified relative to frailty, cognitive impairment, and hypomobility, levels of 16 compounds—acetyl-carnosine, ET, *S*-methyl-ET, OA, urate, methionine, proline, tryptophan, isoluecine, leucine, 1,5-AG, creatine, N3-methyl-histidine, isovaleryl-carnitine, hippurate, and UDP-glucose—are abundant or moderately present in blood (Fig. 4*A*), suggesting a possible involvement in pathogenesis.

Among the 22 metabolites that we identified as relevant to frailty, cognitive /impairment, and hypomobility (Fig. 4*A*), partly overlapping but distinct metabolite profiles support the notion that frailty is an integrated spectrum of age-related disorders. We addressed the question whether these metabolites are useful for diagnosis of frailty. Heatmap comparisons indicated distinct distributions of 15 frailty markers between frail and nonfrail groups (Fig. 4*B*). Similar results were observed among 6 metabolites for cognitive impairment and among 12 metabolites for low mobility (Fig. 4*B*).

Next, we applied principal component analysis (PCA) based on 10 metabolites related to EFS, MoCA-J, and TUG: acetyl-carnosine, ET, OA, methionine, proline, tryptophan, N3-methyl-histidine, creatine, UDP-glucuronate, and UDP-glucose. PCA clearly distinguished frail elderly people from healthy counterparts (Fig. 4*C*). Interestingly, nonfrail people with cognitive impairment or hypomobility were also separated from the frail and

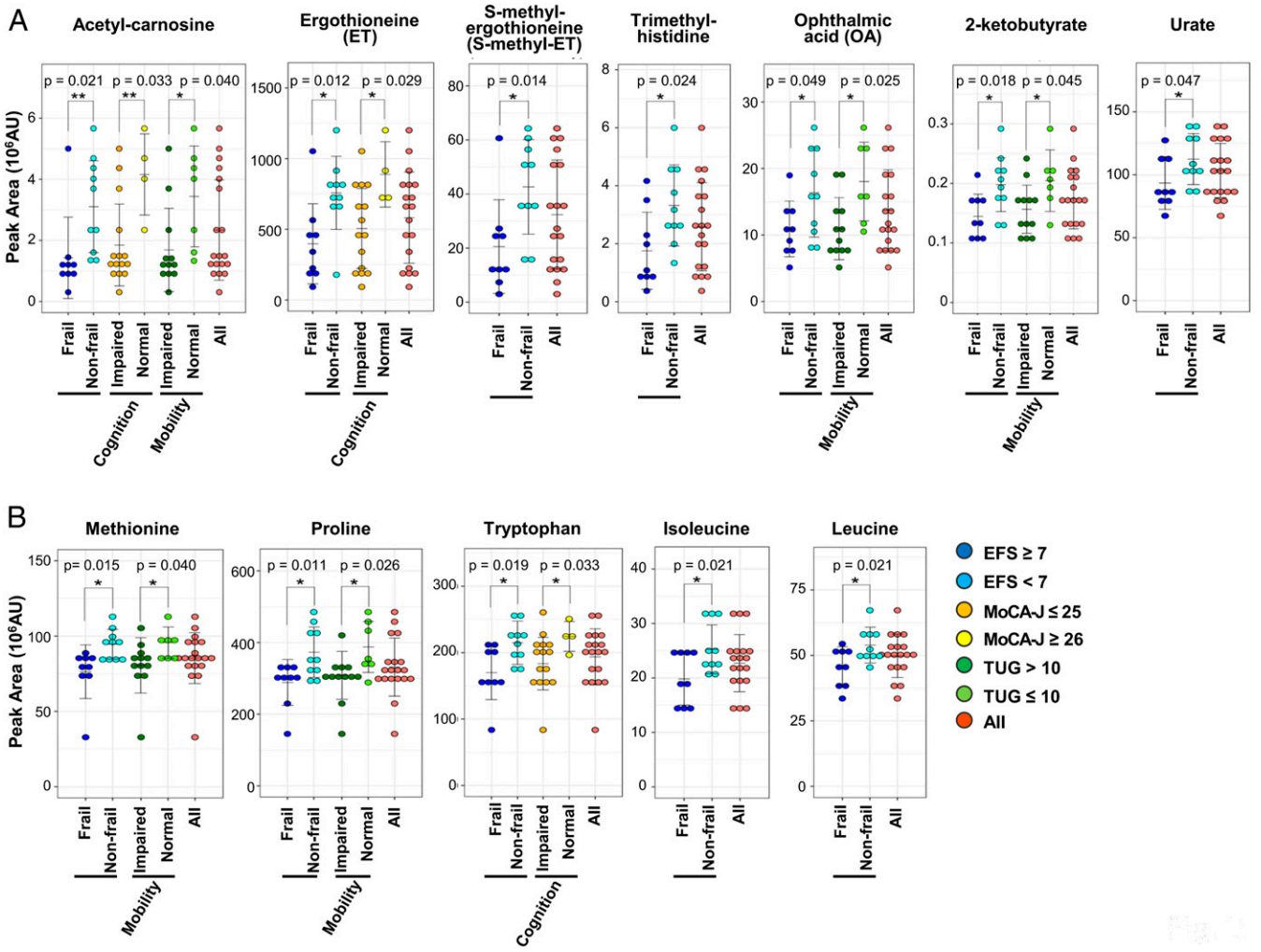

Fig. 2. Antioxidants and amino acids were significantly decreased in frailty. (*A*) Seven antioxidants were identified as frailty markers: acetyl-carnosine, ET, *S*-methyl-ET, trimethyl-histidine, ophthalmic acid, 2-ketobutyrate, and urate. Decreased antioxidants were also observed in cognitive impairment and hypomobility. (*B*) Five amino acids were decreased in frailty: isoleucine, leucine, methionine, tryptophan, and proline. Tryptophan was also decreased in cognitive impairment, while methionine and proline were also decreased in hypomobility. $*P < 0.05$. Error bars represent mean ± SD.

healthy groups. Analysis of a correlation network between EFS and 131 metabolites using Cytoscape 3.7.2 (38, 39) identified 17 metabolites significantly correlated with EFS, including 10 frailty markers (*SI Appendix*, Table S2). Our metabolomic dissection of frailty markers suggests an involvement of antioxidation in cognition and an involvement of nitrogen metabolism in mobility. These findings enhance our understanding of the pathogenesis of frailty and offer hope for interventions to maintain normal physiological levels of these metabolites.

Materials and Methods

Clinical Assessment. All clinical data were collected at Kyoto University Hospital. Patients who were bedridden or who had kidney dysfunction (serum creatinine >2.0 mg/dL) or liver damage (serum aspartate aminotransferase and alanine aminotransferase >50 U/L), were excluded from the study. Clinical interviews, physical examinations, and blood tests were performed for 19 elderly participants. The EFS is an efficient diagnostic tool comprising 10 domains to assess cognitive ability (the clock-drawing test), mobility (TUG test), and fundamental daily activity by questionnaire, in which a score ≥7 indicates frailty (range, 0 to 17) (16). The EFS elicits information on functional independence, including meal preparation, shopping, transportation, telephone use, housekeeping, laundry, money management, and medications. In the TUG test, the time it takes to stand up from a chair, walk normally to a point 3 m away, and return to sitting is measured (19). In the EFS, a TUG test cutoff score of >10 s is classified as hypomobility.

The MoCA-J evaluates short-term memory, visuospatial ability, various executive functions, attention, concentration, working memory, language, and temporal and spatial orientation. An MoCA-J score below a threshold of 25 to 26 (out of 30) is considered to indicate mild cognitive impairment (17, 18).

Blood Sample Preparation for Metabolomic Analysis. Preparation of human blood samples for metabolomic analysis has been described previously (9, 10, 40). In the morning, blood for clinical tests and metabolomic analysis was drawn at the laboratory of Kyoto University Hospital. Until the time of blood sampling, all participants were requested not to have breakfast to ensure overnight fasting at least for 12 h, although they were allowed to spend their time normally and to drink beverages without calories. Since some metabolites are labile, blood samples were quickly quenched at −40 °C in methanol to ensure quick sample processing. Then 10 nmol Hepes and Pipes were added to each sample to serve as internal standards.

LC-MS Conditions. Untargeted, comprehensive analysis by LC-MS was carried out as described previously (9, 10, 40). LC-MS data were obtained using an Ultimate 3000 DGP-3600RS liquid chromatograph and an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). LC separation was done using a ZIC-pHILIC column (Merck SeQuant; 150 mm × 2.1 mm, 5 μm particle size). The mobile phase was composed of ammonium carbonate buffer (10 mM, pH 9.3) and acetonitrile. Gradient elution from 80 to 20% acetonitrile over

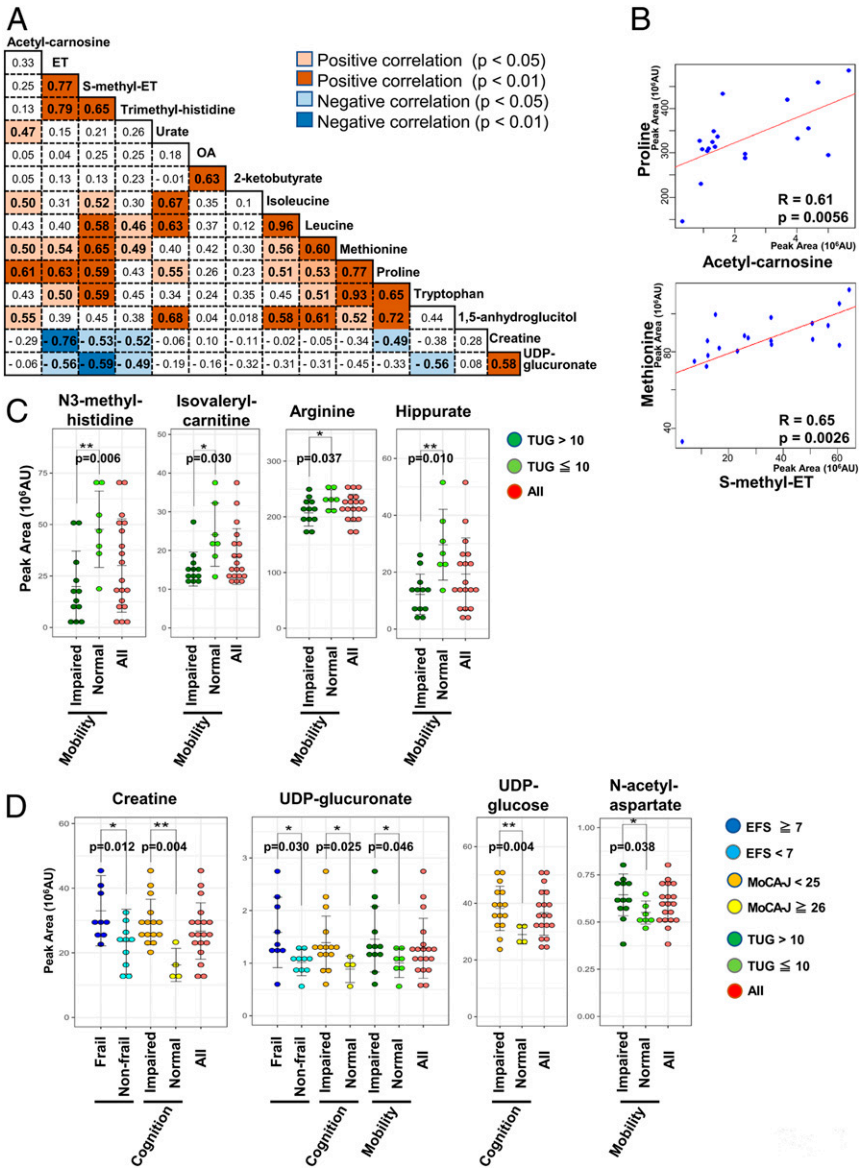

Fig. 3. Compounds relevant to muscle or nitrogen metabolism were decreased in hypomobility, while creatine and UDP-glucuronate were increased in frailty. (*A*) Pearson's correlation analysis for 15 frailty markers. Positive and negative correlations are shown in red and blue, respectively. (*B*) Statistical analysis of the correlation between antioxidants and amino acids, acetyl-carnosine and proline (*Upper*) and *S*-methyl-ET and methionine (*Lower*). (*C*) Compounds involved in muscle or nitrogen metabolism declined in the low mobility group, including N3-methyl-histidine, isovaleryl-carnitine, arginine, and hippurate. (*D*) Four metabolites increased in frailty, cognitive impairment, or hypomobility. $*P < 0.05$; $**P < 0.01$. Error bars represent mean ± SD.

30 min at a flow rate of 100 μL/min was used. An electrospray ionization (ESI) source was used for MS detection. An injection of 1 μL was performed twice for each sample, once with the ESI in positive ionization mode and once with the ESI in negative mode. Spray was set to 4.0 kV for positive ESI and 2.8 kV for negative ESI, while the capillary was adjusted to 350 or 300 °C. Nitrogen gas was used as a carrier. The mass spectrometer was operated in full scanning mode with a 100 to 1,000 *m/z* range and with MS/MS fragmentation scanning in an automatic data-dependent manner.

LC-MS Data Processing and Analysis. MZmine 2 (version 2.29) software (http://mzmine.github.io) was used to measure peak areas for metabolites (41). Isotopic peaks were eliminated. Lists of peaks for individual samples were aligned according to their retention times and corresponding *m/z* values. A total of 131 nonselective metabolites were identified for each sample by comparing retention times and *m/z* values of peaks with those of standards (Dataset S1) (9, 10, 40). If no standard compound was available, metabolites were identified by the analysis of MS/MS spectra. Then all data acquired were transferred into a spreadsheet, followed by analysis with R statistical software (http://www.r-project.org). Statistical analysis included Student's t test to confirm significant differences between groups (with statistical significance set at $P < 0.05$) and 95% confidence intervals, the ordinary least squares method to confirm linear regression, and Pearson's correlation to confirm relationships between metabolites and clinical data (assuming $P < 0.05$). PCA was used to visualize the metabolomic model. A correlation network involving EFS and 131 metabolites was analyzed using Cytoscape 3.7.2 (38) with Metscape (39).

Data Availability. Raw LC-MS data in mzML format are available from the MetaboLights repository, http://www.ebi.ac.uk/metabolights/ (accession no. MTBLS1540).

Ethics Statement. All participants signed informed consent forms prior to examination, in accordance with the Declaration of Helsinki. Experiments were carried out in agreement with relevant rules and official guidelines in Japan. Approval for study protocols was given both by the Human Research Ethics Committee of Kyoto University and by the Review Committee on Human Subjects Research at Okinawa Institute of Science and Technology.

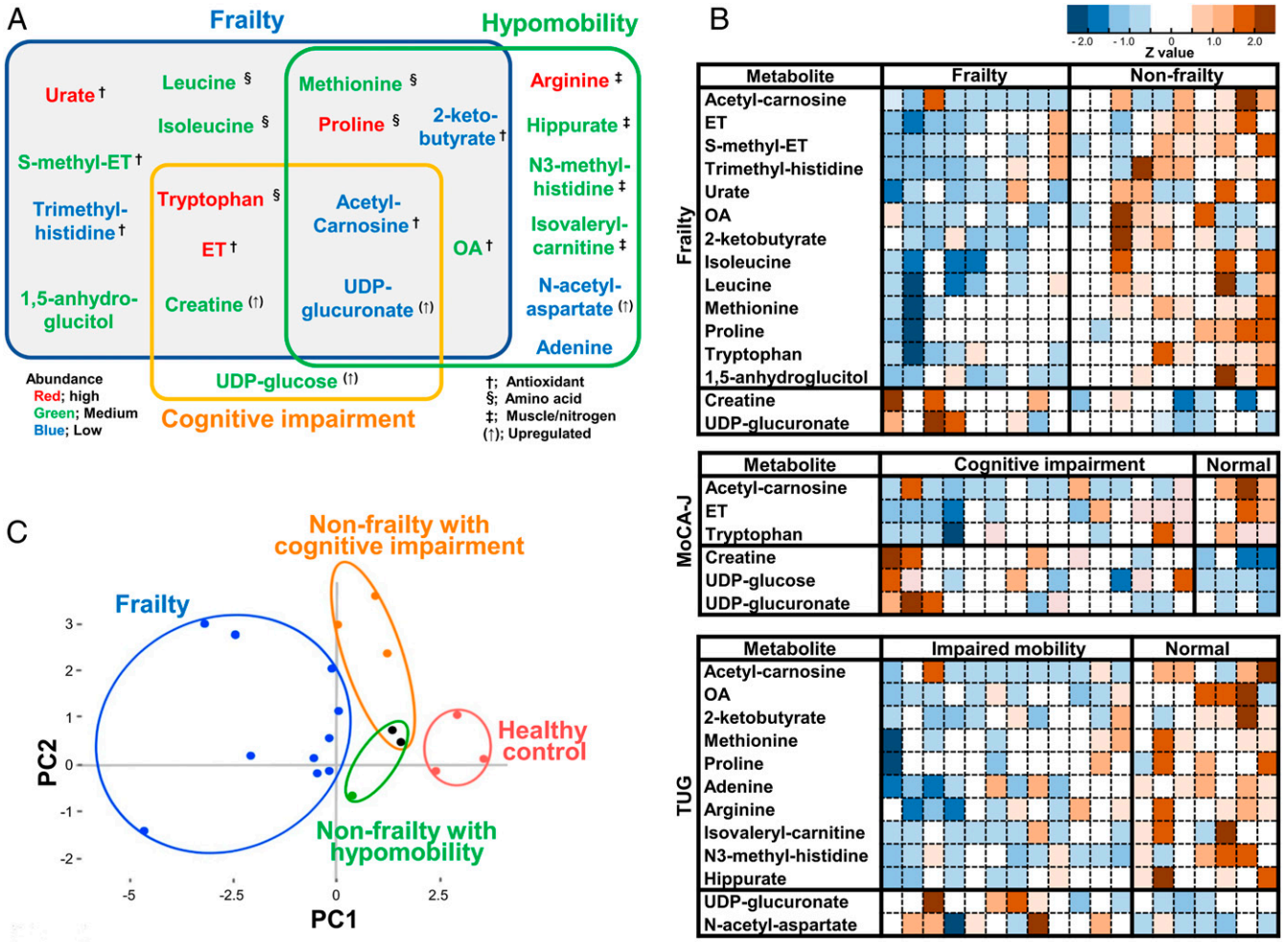

Fig. 4. Heatmap analysis and PCA for frailty. (*A*) Summary of metabolites related to frailty, cognitive impairment, and hypomobility. (*B*) Heatmap analysis of metabolites involved in frailty (*Top*), cognitive impairment (*Middle*), and hypomobility (*Bottom*). The heat map presents *z*-scores of peak areas from LC-MS analysis. (*C*) PCA plot of elderly subjects. Ten metabolites related to EFS, MoCA-J, and TUG were analyzed: acetyl-carnosine, ET, OA, methionine, proline, tryptophan, N3-methyl-histidine, creatine, UDP-glucuronate, and UDP-glucose.

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