



# Whole-blood metabolomics of dementia patients reveal classes of disease-linked metabolites

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**Dementia is caused by factors that damage neurons. We quantified small molecular markers in whole blood of dementia patients, using nontargeted liquid chromatography–mass spectroscopy (LC-MS). Thirty-three metabolites, classified into five groups (A to E), differed significantly in dementia patients, compared with healthy elderly subjects. Seven A metabolites present in plasma, including quinolinic acid, kynurenine, and indoxyl-sulfate, increased. Possibly they act as neurotoxins in the central nervous system (CNS). The remaining 26 compounds (B to E) decreased, possibly causing a loss of support or protection of the brain in dementia. Six B metabolites, normally enriched in red blood cells (RBCs), all contain trimethylated ammonium moieties. These metabolites include ergothioneine and structurally related compounds that have scarcely been investigated as dementia markers, validating the examination of RBC metabolites. Ergothioneine, a potent antioxidant, is significantly decreased in various cognition-related disorders, such as mild cognitive impairment and frailty. C compounds also include some oxidoreductants and are normally abundant in RBCs (NADP<sup>+</sup>, glutathione, adenosine triphosphate, pantothenate, S-adenosyl-methionine, and gluconate). Their decreased levels in dementia patients may also contribute to depressed brain function. Twelve D metabolites contains plasma compounds, such as amino acids, glycerophosphocholine, dodecanoyl-carnitine, and 2-hydroxybutyrate, which normally protect the brain, but their diminution in dementia may reduce that protection. Seven D compounds have been identified previously as dementia markers. B to E compounds may be critical to maintain the CNS by acting directly or indirectly. How RBC metabolites act in the CNS and why they diminish significantly in dementia remain to be determined.**

dementia | Alzheimer's disease | antioxidants | ergothioneine | trimethyl-ammonium compounds

“Dementia” is a collective term to describe various symptoms of cognitive impairment in a condition in which intelligence is irreversibly diminished due to acquired organic disorders of the brain, characterized by deterioration of memory, thinking, behavior, and the ability to perform daily activities (1, 2). Though a common cause is Alzheimer's disease (AD), a neurodegenerative disease in which memory is rapidly impaired due to hippocampal atrophy, multiple types of dementia, known as “mixed dementia,” can coexist (3, 4). Mental and physical exercise and avoidance of obesity may reduce the risk of dementia (5–7). No medications or supplements have been definitively shown to decrease risk (8, 9). Dementia most often begins in people over 65 y of age, and about 6% of seniors are afflicted with it. It is one of the most costly diseases in developed countries (10).

In this study, we conducted nontargeted, comprehensive analysis of blood metabolites in dementia patients. Thorough metabolomic evaluation can supply complete information about metabolite abundance in each subject. While nontargeted analysis is far more laborious than targeted analysis, the effort expended in this “no assumptions” approach is often recompensed by identification of diagnostic compounds overlooked by targeted analysis. A wealth of metabolite information may provide clues

to understanding the profound metabolic changes occurring in dementia. Liquid chromatography–mass spectroscopy (LC-MS) was employed for whole-blood metabolite profiling of dementia patients, and we found metabolic compounds not previously known to be related to dementia. Metabolomics of blood cells have scarcely been investigated, particularly in relation to diseases, despite the fact that red blood cells (RBCs) account for about 40% of all blood metabolites (11–13). Thus, metabolomic information from RBCs also provides crucial information on health and disease (14–16). Here we identified 33 dementia-linked markers (12 of which are RBC-enriched) and validated them by principal component analysis (PCA), correlation, and heatmap analyses, confirming that these markers actually are involved in development of dementia. Our results suggest that detailed molecular diagnosis of dementia is now possible. Somewhat unexpectedly, markers deduced from dementia only partially overlap with amino acid markers obtained from frailty patients with cognitive defects (16), so that frailty and dementia partly share the diminished cognitive markers. We also show that an antioxidant, ergothioneine (ET), an RBC component involved in human cognitive ability (16, 17), and two related compounds are reduced in dementia.

## Significance

**Dementia is a slowly progressing, chronic, and usually irreversible decline in cognitive function. Mechanistic causes and definitive treatments remain elusive. Using comprehensive metabolomics, we identified five groups of 33 metabolites (A to E), 13 of them previously reported, possibly useful for diagnosis and therapy of forms of dementia, such as Alzheimer's disease. Seven A compounds may act as neurotoxins, whereas B to E compounds may protect the nervous system against oxidative stress, maintain energy reserves, supply nutrients and neuroprotective factors. Five metabolites, ergothioneine, S-methyl-ergothioneine, trimethyl-histidine, methionine, and tryptophan, overlap with those reported for frailty. Interventions for cognitive diseases involving these dementia metabolomic markers may be accomplished either by inhibiting A compounds or by supplementing B to E compounds in patients.**

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## Results

**Collection of blood samples from dementia subjects.** To identify dementia-related blood metabolites, quantitative comparisons were conducted of blood samples of dementia patients and healthy elderly (HE) and healthy young (HY) subjects. Blood samples of dementia patients (age 75 to 88 y) diagnosed and hospitalized at the National Hospital Organization Ryukyu Hospital, Kin-town, Okinawa were obtained from each patient after informed consent (*Materials and Methods*). The same numbers of HE (67 to 80 y) and HY (28 to 34 y) volunteers from Onna Clinic, Onna-village, Okinawa were also recruited (*SI Appendix, Fig. S1 and Table S1*). Twenty-four subjects comprising eight dementia patients, eight HE subjects, and eight HY subjects participated in this study. All blood samples were drawn at each hospital as described (14). Venous blood samples were taken into tubes with heparin as an anticoagulant.

**Thirty-three dementia-linked blood metabolites comprise five subgroups, A to E.** In all whole-blood samples collected, 124 metabolites were identified and quantified by nontargeted LC-MS (*SI Appendix, Table S2*). They consisted of 14 subgroups. Fifty-one compounds, comprising 5 subgroups (nucleotides, vitamins and coenzymes, nucleotides/sugar derivatives, sugar phosphates, and antioxidants) are RBC-enriched (14). Of these 124 compounds, 33 metabolites differed significantly between dementia patients and HE subjects (range of  $P$  values,  $0.00016 < P < 0.05$ ) (Fig. 1 and Table 1). Five compounds, adenosine triphosphate (ATP), glutathione disulfide (GSSG), glutamine, phenylalanine, and betaine, are highly abundant (ranked H). Five other compounds, glycerophosphocholine, ET, methionine, tryptophan, and tyrosine, are of high to medium (H-M) abundance. Three additional compounds vary widely in abundance among healthy subjects (high to low; H-L): caffeine, dimethyl-xanthine, and trimethyl-tryptophan. The remaining 20 compounds are of medium to low abundance (M-L, M, L) (Table 1). Twelve of the 33 compounds are RBC-enriched, which has been scarcely reported. Characteristically, 9 dementia-related compounds contain trimethyl-ammonium moieties (Table 1).

Peak ratios (dementia/HE in Table 1) were calculated using the median of peak abundance in each group. Seven compounds (group A) that increased in dementia showed peak ratios  $>1.0$ . Twenty-six others had ratios  $<1.0$  that declined in dementia. Four compounds exhibiting the greatest decreases are caffeine-related: dimethyl-xanthine (E; 0.04), trimethyl-tyrosine (B; 0.08), caffeine (E; 0.09), and trimethyl-tryptophan (B; 0.10). Curiously, all are aromatics. Their variances are also broad in healthy subjects.

To quantify individual variability of the 124 metabolites, coefficients of variation (CVs) for all experimental populations of the 24 subjects were calculated (*SI Appendix, Table S2*). In the 33 dementia-linked compounds, CVs of ATP (0.20), GSSG (0.14), and  $\text{NADP}^+$  (0.33), which have vital functions, were relatively low, while trimethyl-tryptophan (1.09), trimethyl-tyrosine (2.21), trimethyl-phenylalanine (3.17), and caffeine (1.49) were high. These values were substantially in agreement with those in our previous study, an independent dataset obtained from 30 HE and HY subjects (14). Thus, the great variability of data in Fig. 1 reflects genuine individual variation in metabolites, which were accurately detected by our metabolomic analysis. These data demonstrate that compounds having small to large individual variability are implicated in dementia.

**Increased group A compounds may be toxic.** Seven group A compounds were identified by their increases in the dementia patients compared to HE (Fig. 14). They consisted of indoxyl-sulfate (peak ratio dementia/HE, 1.93), quinolinic acid (1.79), adenosine (1.51), dimethyl-guanosine (1.45),  $\text{N}^6$ -acetyl-lysine (1.47), pseudouridine (1.19), and kynurenine (1.12). Two of the seven metabolites were previously reported as AD-related markers (18, 19). Some of them are reportedly toxic (20), suggesting that they may be inhibitory in

the brain (see below). To determine the statistically difference, the non-parametric Mann–Whitney  $U$  test was used. This test examines the significant difference between groups based on ranks and not values. Since ranks represent the relative position of an individual in comparison to others, it is not affected by outlier value.

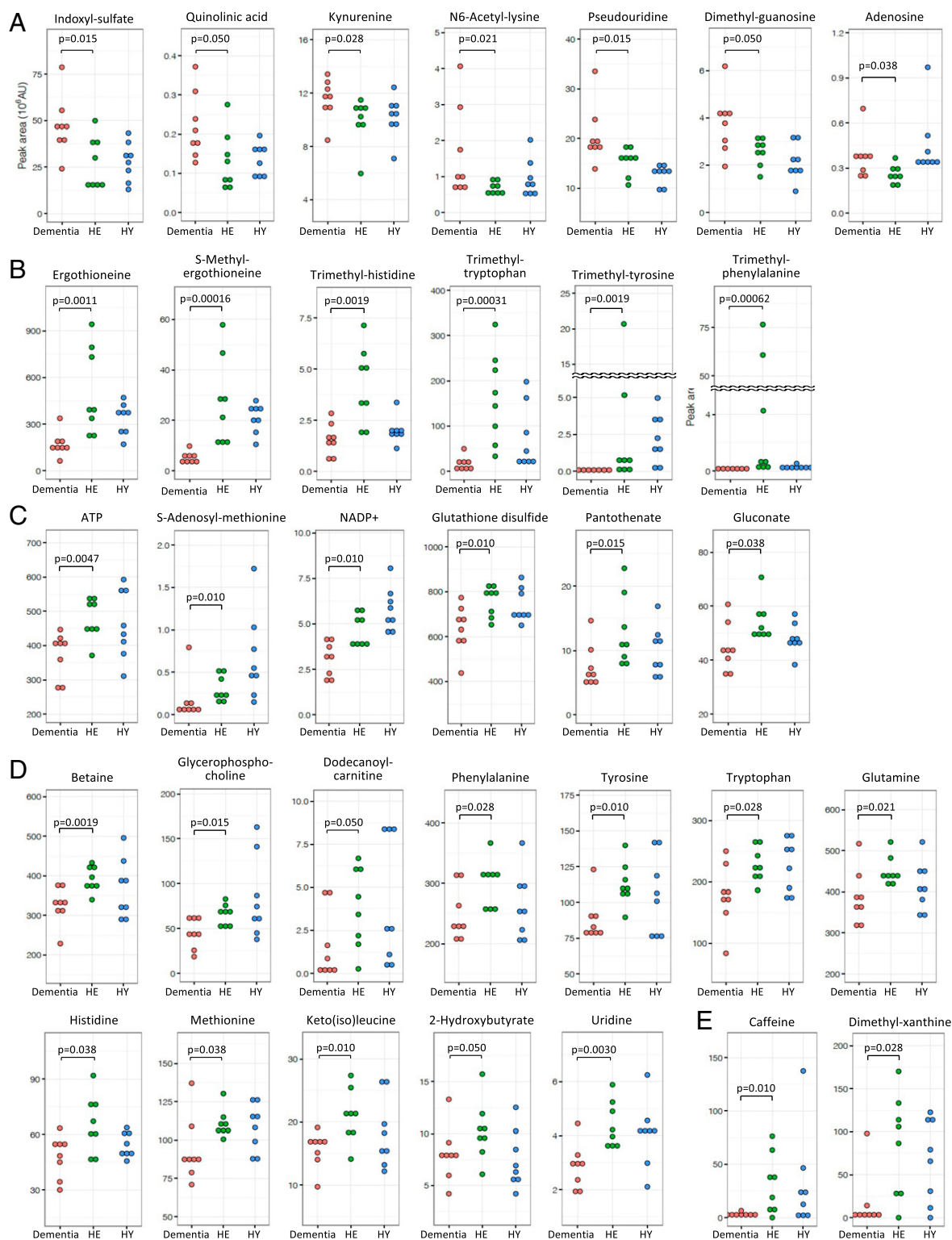
**Twenty-six blood dementia compounds consist of four groups, B to E.** The 26 remaining compounds decreased in dementia patients ( $P < 0.05$ ) (Fig. 1 B–E). They consisted of four subgroups (B to E), having distinct characteristics. Group B compounds include ET and five other trimethyl-ammonium compounds. To our knowledge, except for ET (17), these are all not previously reported as dementia markers, probably because they are enriched in RBCs and scarcely studied in connection with dementia. ET is an antioxidant, a thiourea derivative of trimethyl-histidine. Two other ET-related, but less abundant, compounds, *S*-methyl-ET and trimethyl-histidine (hercynine), also declined strikingly in blood of dementia patients.

Group C compounds also decreased in dementia patients. These included ATP,  $\text{NADP}^+$  (oxidoreductive coenzyme), GSSG (redox compound), pantothenate (vitamin B5), *S*-adenosyl-methionine (SAM; methyl donor) (21), and gluconate (zinc carrier) (22). They are related to energy, redox reactions, methylation, and metal ions. Group C compounds were all enriched in RBCs, and four of the six are not previously reported as dementia markers. Two of them (SAM and GSSG) were previously shown to be AD-related (21, 23).

Trimethyl-tryptophan (hypaphorine), trimethyl-phenylalanine, glycerophosphocholine, dodecanoyl-carnitine (24), and trimethyl-tyrosine, all of which contain trimethyl-ammonium ions, also declined. The extent of reduction for trimethyl-tryptophan (0.10) and trimethyl-tyrosine (0.08) was striking. These reductions may be due to instability or reduced synthesis, or to reduced import in dementia patients. Of the nine compounds that contain a trimethyl-ammonium moiety, six of them that contain ET are enriched in RBCs and classified as group B compounds (Table 1).

Twelve group D metabolites (Table 1) are enriched in blood plasma and seven of them were previously reported to be dementia or AD markers. They include standard amino acids, glutamine (19, 25), phenylalanine (19, 26), tyrosine (19), histidine (19, 25), methionine, and tryptophan (regular amino acids) (18, 19), a pyrimidine nucleoside, uridine (27), and organic acids, 2-hydroxybutyrate (lipid-degradation product) and keto(iso)leucine (keto acid). Caffeine is a known dementia marker (28). Dimethyl-xanthine is a metabolite of caffeine. These greatly declined in dementia and are highly correlated with and isolated from other metabolites (see below) so they are designated as group E. Consistency of group D plasma metabolites as dementia markers but not group B and C RBC metabolites validated the method of searching dementia markers that we employed in the present study. The great majority of metabolites enriched in RBCs were not identified in the previous studies.

**Nine trimethylated ammonium compounds were diminished in dementia patients.** Of nine trimethylated compounds that decreased in dementia, six are enriched in RBCs (*SI Appendix, Fig. S2*). Three of them (betaine, glycerophosphocholine, and dodecanoyl-carnitine) are present in plasma and are synthesized in the human body, whereas the other six, containing an aromatic moiety, are derived from food (29, 30). Most strikingly, six of these nine compounds are highly abundant (H, H-M, or H-L) in plasma and RBCs in healthy subjects, and are highly correlated so that their behavior may be highly coordinated. Hence, the sharp declines of these amphipathic compounds (possessing both hydrophilic and lipophilic properties and forming the basis of lipid polymorphism) in blood of dementia patients may strongly affect the physicochemical properties of neuronal systems.



**Fig. 1.** Dot plot profiles of 33 dementia-related metabolites. Dementia metabolites were selected using  $P$  values ( $<0.05$ ) obtained by comparison of peak abundance between dementia and HE subjects. Compounds were grouped into A to E (see text).

**Seven metabolites increased in dementia.** Interestingly, the seven metabolites of group A, comprising three nucleosides and four amino acid derivatives, increased in dementia (Fig. 1A). None was highly abundant and none was enriched in RBCs, and their increase in dementia occurred in plasma. Indoxyl-sulfate, kynurenine, and quinolinic acid (18) are involved in tryptophan

metabolism and possibly act as excitatory toxins in the brain (31, 32), while *N6*-acetyl-lysine is implicated in histone and nonhistone protein modification (33). Pseudouridine, adenosine (19), and dimethyl-guanosine are degradation products of RNAs present in urine and are thought to be oxidized (34, 35). Increases of these metabolites in dementia are of great interest, as

**Table 1. List of 33 dementia markers**

Group	Compound	Peak abundance	Ratio dementia/HE	RBC-enriched	Trimethylated compound	Refs.
A	Indoxyl-sulfate	M	1.93 ↑			(18)
	Quinolinic acid	L	1.79 ↑			
	Kynurenine	M-L	1.12 ↑			
	N6-acetyl-lysine	L	1.47 ↑			
	Pseudouridine	M-L	1.19 ↑			
	Dimethyl-guanosine	L	1.45 ↑			
B	Adenosine	L	1.51 ↑			(19)
	ET	H-M	0.41 ↓	•	○	
	S-methyl-ET	M-L	0.20 ↓	•	○	
	Trimethyl-histidine	L	0.37 ↓	•	○	
	Trimethyl-tryptophan	H-L	0.10 ↓	•	○	
	Trimethyl-tyrosine	M-L	0.08 ↓	•	○	
	Trimethyl-phenylalanine	M-L	0.23 ↓	•	○	
	ATP	H	0.83 ↓	•		
C	SAM	L	0.35 ↓	•		(21)
	NADP <sup>+</sup>	L	0.70 ↓	•		
	GSSG	H	0.82 ↓	•		
C	Pantothenate	M-L	0.57 ↓	•		(23)
	Gluconate	M	0.85 ↓	•		
D	Betaine	H	0.85 ↓		○	(19, 26)
	Glycerophosphocholine	H-M	0.66 ↓		○	
	Dodecanoyl-carnitine	L	0.15 ↓		○	
	Phenylalanine	H	0.73 ↓			
	Tyrosine	H-M	0.75 ↓			
	Tryptophan	H-M	0.80 ↓			
	Glutamine	H	0.84 ↓			
	Histidine	M	0.80 ↓			
	Methionine	H-M	0.80 ↓			
	Keto(iso)leucine	M-L	0.77 ↓			
	2-Hydroxybutyrate	M-L	0.79 ↓			
	Uridine	L	0.72 ↓			
	E	Caffeine	H-L	0.09 ↓		
Dimethyl-xanthine		H-L	0.04 ↓			

Thirty-three compounds showed significant differences in abundance between dementia patients and HE subjects. Abundances of compounds (peak areas) are displayed. The peak ratio was calculated using the median of peak abundance in dementia and HE subjects, respectively. Twelve RBC-enriched compounds and nine trimethylated compounds are shown with closed and open circles, respectively. The references for dementia markers previously reported are listed.

some are reportedly toxic in the central nervous system (CNS) and may lead to impairment of the brain (36–38).

**PCA separates dementia patients from nondementia subjects.** To distinguish between dementia and HE subjects, we then applied PCA. We calculated PC (PC1, PC2) values using abundance data of the 33 dementia-related metabolites in the 16 subjects. Dementia and HE subjects were clearly separated (Fig. 2A). We then attempted to achieve the same degree of resolution using fewer metabolites and found that six metabolites (dimethyl-guanosine, pseudouridine, NADP<sup>+</sup>, trimethyl-histidine, ET, and S-methyl-ET) were still able to separate dementia from nondementia subjects almost perfectly (Fig. 2B).

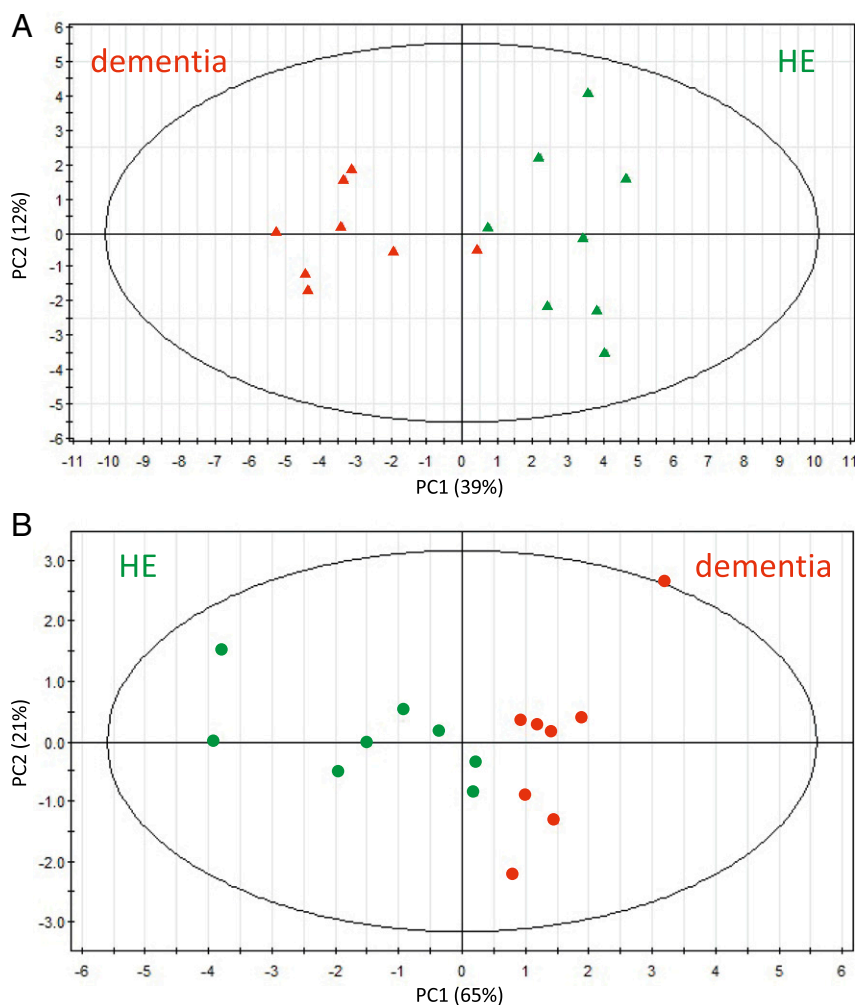
**Correlation analysis corroborates five metabolite groups.** Levels of some blood metabolites linked in a biochemical pathway and/or function show correlations (14). We first confirmed correlations within ET derivatives (ET, S-methyl-ET, and trimethyl-histidine) and between caffeine and dimethyl-xanthine using Pearson's correlation coefficient  $r$ . Abundance of these compounds indicated high correlations ( $0.92 > r > 0.76$ ,  $P < 0.0001$ ) (SI Appendix, Fig. S3) as previously reported (14). Thus, the dataset of the present study is reproducible and statistically valid.

Second, to gain insight into how these 33 dementia metabolites are related, we searched for relationships among them. Significant positive and negative correlations ( $0.89 > r > 0.50$  or  $-0.68 <$

$r < -0.50$ ) in groups A to E support the classification of the subgroups shown in Fig. 3.

Five of the seven group A markers were correlated (0.89 to 0.57) within the subgroup (quinolinic acid, dimethyl-guanosine, pseudouridine, indoxyl-sulfate, and kynurenine). This striking correlation strongly supported their classification within group A and opposition to groups B to E. How group A compounds molecularly oppose groups B to D (Fig. 3, green color) remains to be determined. It is unknown whether the mode of interaction is indirect or direct and remains to be studied.

Negative correlations exist not only between group A and B compounds but also between group A and C compounds. Group B and C metabolites are correlated within the groups, again validating their subgroup designations. Group B metabolites are structurally related, as they commonly contain a trimethylated ammonium group, indicating that they are antioxidative. Group C metabolites are not structurally related, but three of them (NADP<sup>+</sup>, GSSG, and ATP), implicated in redox and energy metabolism, are highly correlated and enriched in RBCs. These distinct metabolites may coordinate to support directly or indirectly their metabolite pathways. Finally, two metabolites, caffeine and dimethyl-xanthine, in group E were highly correlated; the latter is the precursor of caffeine. There are reports on caffeine showing that these purines are beneficial to relieve dementia (Discussion).



**Fig. 2.** PCA of 33 AD or 6 selected dementia-related compounds showed significant differences between patients with dementia and HE subjects. Blood data of dementia and HE subjects were subjected to PCA. (A) A PCA plot using abundances of 33 (7 increased and 26 decreased) dementia-related compounds. Dementia and HE subjects were separated into two domains (see text). Red, dementia; green, HE. (B) PCA was also performed using only 6 selected dementia markers (dimethyl-guanosine, pseudouridine, 5-methyl-ET, ET, trimethyl-histidine, and NADP<sup>+</sup>).

**Heatmap comparison of dementia and HE subjects.** Using abundance data of 33 dementia compounds in eight dementia patients and eight HE subjects, a heatmap was constructed (Fig. 4 and *SI Appendix, Fig. S4*). In dementia patients, the 7 group A compounds mostly displayed red color cells in dementia patients due to the increased abundances, whereas HE subjects mostly showed blue color cells due to the decreased abundances. In the remaining 26 compounds, dementia subjects displayed blue cells, due to their decrease, whereas HE subjects showed red cells, due to their increase, more than the average. Strikingly, the seven group A compounds increased nearly uniformly in dementia patients, whereas in HE and also HY (*SI Appendix, Fig. S4*) subjects, the levels were mostly below 50, indicating that these may be appropriate as diagnostic markers for dementia. Thus, the heatmap profiles showed individual variations of dementia-linked metabolites, and further study will provide more evidence for their use as a diagnostic tool.

## Discussion

A principal conclusion of this study is that plasma-enriched dementia factors, group A compounds, increased in dementia patients and might have a negative toxic impact on CNS functions by themselves or their degradation products (*SI Appendix, Fig. S5*) (20, 32, 39–42). Other group B to E metabolites may be

beneficial for the CNS, as their quantity all declined in dementia patients. RBC-enriched group B metabolites all containing the trimethyl-ammonium ion may protect the CNS through their antioxidative and other unknown activity. Group C compounds, also RBC-enriched, have cellular functions implicated in energy, redox, and so forth, and may be important for maintaining CNS brain functions. Two compounds of group C, SAM and GSSG, had been reported to be AD- and dementia-related markers (21, 23). Group D contains 12 plasma compounds, half of which had been reported as AD-related markers. We thus speculate the 7 group A compounds pathologically enhance or lead to severe dementia such as AD. This presumed dementia deterioration by group A factors is opposed if group B to E metabolites are sufficiently supplied.

Correlation analysis allowed us to categorize 33 dementia markers into five subgroups (A to E) (Fig. 3). Seven group A compounds seem to be opposed by 26 group B to E compounds. Group A compounds were oxidized, and 5 of them are highly correlated, perhaps due to common relation to tryptophan degradation and/or nucleoside metabolism. Group A compounds may act as inhibitors, poisons, or wastes (20, 39). Kynurenine, quinolinic acid (40), and indoxyl-sulfate, known as toxins, may target the brain and worsen dementia (32). *N*6-acetyl-lysine may be related to the accumulation of acetylated tau and alpha-tubulin in the AD

Groups	33 dementia markers	Correlation values (r)																																
		N6-Acetyl-lysine	Adenosine	Dimethyl-guanosine	Pseudouridine	Indoxyl-sulfate	Quinolinic acid	Kynurenine	S-Methyl-ergothioneine	Ergothioneine	Trimethyl-histidine	Trimethyl-tryptophan	Trimethyl-phenylalanine	Trimethyl-tyrosine	Pantothenate	Glucuronate	S-Adenosyl-methionine	NADP+	Glutathione disulfide	ATP	Methionine	Tryptophan	Glutamine	Betaine	Phenylalanine	Tyrosine	Histidine	Uridine	Keto(iso)leucine	Cyctrophosphocholine	2-Hydroxybutyrate	Dodecanoyl-carnitine	Caffeine	Dimethyl-xanthine
A Toxic (waste) compounds	N6-Acetyl-lysine ↑	1.00	0.33	0.07	0.23	0.16	0.32	0.35	-0.35	-0.38	-0.38	-0.17	-0.12	-0.12	-0.11	-0.16	0.10	-0.25	-0.38	-0.25	-0.64	-0.68	-0.37	-0.43	-0.52	-0.41	-0.41	-0.43	-0.41	-0.09	-0.02	-0.11	-0.22	0.00
	Adenosine ↑	0.33	1.00	0.15	-0.07	0.24	0.04	0.44	-0.19	-0.10	-0.35	-0.14	-0.17	-0.04	-0.09	-0.20	0.07	0.17	-0.06	0.20	0.16	0.20	0.07	0.32	0.18	0.14	-0.07	-0.06	-0.19	0.64	-0.27	-0.37	-0.10	-0.05
	Dimethyl-guanosine ↑	0.07	0.15	1.00	0.89	0.69	0.60	0.57	-0.45	-0.30	-0.23	-0.26	-0.11	-0.03	-0.14	-0.13	-0.45	-0.29	-0.13	0.05	-0.01	-0.10	0.16	0.00	0.27	-0.06	-0.10	-0.30	0.05	0.02	0.06	-0.14	-0.29	-0.38
	Pseudouridine ↑	0.23	-0.07	0.89	1.00	0.75	0.69	0.43	-0.49	-0.39	-0.25	-0.23	-0.09	-0.07	-0.18	-0.13	-0.38	-0.46	-0.29	-0.11	-0.36	-0.43	-0.02	-0.23	0.01	-0.31	-0.19	-0.35	0.07	-0.19	0.14	-0.01	-0.31	-0.29
	Indoxyl-sulfate ↑	0.16	0.24	0.69	0.75	1.00	0.60	0.37	-0.64	-0.51	-0.42	-0.19	-0.12	-0.08	-0.12	-0.30	-0.50	-0.60	-0.37	-0.28	-0.24	-0.27	-0.11	-0.17	0.11	-0.19	-0.07	-0.35	-0.04	-0.11	-0.06	-0.24	-0.10	-0.08
	Quinolinic acid ↑	0.32	0.04	0.60	0.69	0.60	1.00	0.48	-0.66	-0.64	-0.52	-0.24	-0.22	-0.16	-0.01	0.04	-0.40	-0.42	-0.54	-0.30	-0.40	-0.46	-0.16	-0.41	-0.04	-0.20	-0.35	-0.52	-0.12	-0.13	0.01	0.02	-0.15	-0.32
	Kynurenine ↑	0.35	0.44	0.57	0.43	0.37	0.48	1.00	-0.54	-0.45	-0.48	-0.21	-0.46	-0.19	0.06	0.04	-0.29	-0.23	-0.33	-0.16	-0.02	-0.01	-0.06	-0.07	0.16	0.07	-0.01	-0.13	0.02	0.12	-0.08	-0.14	-0.02	-0.03
B Trimethylated amino acids	S-Methyl-ergothioneine ↓	-0.35	-0.19	-0.45	-0.49	-0.64	-0.66	-0.54	1.00	0.92	0.72	0.29	0.21	0.13	0.08	0.19	0.24	0.58	0.52	0.55	0.30	0.45	0.21	0.37	0.21	0.27	0.17	0.49	0.33	0.16	0.26	0.23	0.13	0.24
	Ergothioneine ↓	-0.38	-0.10	-0.30	-0.39	-0.51	-0.64	-0.45	0.92	1.00	0.77	0.37	0.30	0.20	0.07	0.18	0.14	0.45	0.51	0.54	0.34	0.52	0.35	0.45	0.31	0.31	0.30	0.55	0.32	0.24	0.21	0.05	0.12	0.26
	Trimethyl-histidine ↓	-0.38	-0.35	-0.23	-0.25	-0.42	-0.52	-0.48	0.72	0.77	1.00	0.57	0.51	0.39	0.30	0.39	0.04	0.22	0.39	0.33	0.21	0.32	0.21	0.48	0.17	0.15	0.36	0.46	0.21	0.09	0.21	0.06	0.08	0.17
	Trimethyl-tryptophan ↓	-0.17	-0.14	-0.26	-0.23	-0.19	-0.24	-0.21	0.29	0.37	0.57	1.00	0.45	0.36	0.63	0.47	0.15	0.23	0.33	0.23	0.17	0.23	0.14	0.31	0.18	0.49	0.49	0.25	0.16	0.16	0.08	0.02	0.54	0.53
	Trimethyl-phenylalanine ↓	-0.12	-0.11	-0.09	-0.12	-0.22	-0.46	0.21	0.30	0.51	0.45	1.00	0.70	0.15	0.48	-0.07	0.06	0.07	0.29	0.11	-0.09	0.26	0.29	-0.06	0.11	0.37	0.17	-0.09	0.05	0.27	-0.24	0.14	0.12	
	Trimethyl-tyrosine ↓	-0.12	-0.04	-0.03	-0.07	-0.08	-0.16	-0.19	0.13	0.20	0.39	0.36	0.70	1.00	0.15	0.57	-0.07	0.15	0.07	0.38	0.13	0.08	0.31	0.41	0.04	0.19	0.59	0.30	0.08	0.31	0.05	-0.21	0.29	0.33
C Other RBC-enriched compounds	Pantothenate ↓	-0.11	-0.09	-0.14	-0.18	-0.12	-0.01	0.06	0.08	0.07	0.30	0.63	0.15	0.15	1.00	0.35	0.05	0.28	0.32	0.09	0.26	0.16	0.12	0.18	0.19	0.49	0.20	0.11	0.17	0.14	0.00	0.15	0.29	0.28
	Glucuronate ↓	-0.16	-0.20	-0.13	-0.13	-0.30	0.04	0.04	0.19	0.18	0.39	0.47	0.48	0.57	0.35	1.00	-0.08	-0.17	-0.04	0.32	0.18	0.14	0.24	0.30	0.21	0.29	0.56	0.45	0.39	0.24	0.43	0.03	0.23	0.21
	S-Adenosyl-methionine ↓	0.10	-0.07	-0.45	-0.38	-0.50	-0.40	-0.29	0.24	0.14	0.04	0.15	-0.07	-0.07	-0.05	-0.08	1.00	0.50	0.36	0.09	-0.11	-0.03	-0.06	-0.25	-0.37	-0.15	-0.14	0.17	-0.09	0.18	-0.05	0.47	0.07	0.20
	NADP+ ↓	-0.25	0.17	-0.29	-0.46	-0.60	-0.42	-0.23	0.58	0.45	0.22	0.23	0.06	0.15	0.28	0.17	0.50	1.00	0.68	0.66	0.50	0.45	0.30	0.33	0.21	0.44	0.01	0.27	0.26	0.53	0.09	0.31	0.18	0.07
	Glutathione disulfide ↓	-0.38	-0.06	-0.13	-0.29	-0.37	-0.54	-0.33	0.52	0.51	0.39	0.33	0.07	0.07	0.32	-0.04	0.36	0.68	1.00	0.64	0.64	0.60	0.58	0.45	0.44	0.49	0.27	0.43	0.46	0.33	0.14	0.34	0.04	0.10
ATP ↓	-0.25	0.20	0.05	-0.11	-0.28	-0.30	-0.16	0.55	0.54	0.33	0.23	0.29	0.38	0.09	0.32	0.09	0.66	0.64	1.00	0.57	0.47	0.63	0.67	0.56	0.46	0.40	0.49	0.46	0.54	0.31	0.10	-0.07	0.00	
D Plasma compounds	Methionine ↓	-0.64	0.16	-0.01	-0.36	-0.24	-0.40	-0.02	0.30	0.34	0.21	0.17	0.11	0.13	0.26	0.18	-0.11	0.50	0.64	0.57	1.00	0.84	0.68	0.67	0.76	0.73	0.48	0.46	0.37	0.39	0.16	0.01	0.16	0.00
	Tryptophan ↓	-0.68	0.20	-0.10	-0.43	-0.27	-0.46	-0.01	0.45	0.52	0.32	0.23	-0.09	0.08	0.16	0.14	-0.03	0.45	0.60	0.47	0.84	1.00	0.55	0.66	0.72	0.72	0.52	0.57	0.46	0.45	0.04	0.03	0.28	0.17
	Glutamine ↓	-0.37	0.07	0.16	-0.02	-0.11	-0.16	-0.06	0.21	0.35	0.21	0.14	0.26	0.31	0.12	-0.24	-0.06	0.30	0.58	0.63	0.68	0.55	1.00	0.57	0.61	0.52	0.53	0.50	0.45	0.51	0.18	0.11	0.10	0.01
	Betaine ↓	-0.43	0.32	0.00	-0.23	-0.17	-0.41	-0.07	0.37	0.45	0.48	0.31	0.29	0.41	0.18	0.30	-0.25	0.33	0.45	0.67	0.67	0.66	0.57	1.00	0.62	0.48	0.63	0.52	0.33	0.62	-0.02	-0.26	-0.10	-0.02
	Phenylalanine ↓	-0.52	0.18	0.27	0.01	0.11	-0.04	0.16	0.21	0.31	0.17	0.18	-0.06	0.04	0.19	0.21	-0.37	0.21	0.44	0.56	0.76	0.72	0.61	0.62	1.00	0.62	0.52	0.39	0.55	0.39	0.33	0.09	0.07	0.06
	Tyrosine ↓	-0.41	0.14	-0.06	-0.31	-0.19	-0.20	0.07	0.27	0.31	0.15	0.49	0.11	0.19	0.49	0.29	-0.15	0.44	0.49	0.46	0.73	0.72	0.52	0.48	0.62	1.00	0.49	0.24	0.33	0.29	-0.02	-0.02	0.50	0.29
	Histidine ↓	-0.41	-0.07	-0.10	-0.19	-0.07	-0.35	-0.01	0.17	0.30	0.36	0.49	0.37	0.59	0.20	0.56	-0.14	0.01	0.27	0.40	0.48	0.52	0.53	0.63	0.52	0.49	1.00	0.67	0.48	0.29	0.15	-0.15	0.29	0.48
	Uridine ↓	-0.43	-0.06	-0.30	-0.35	-0.35	-0.52	-0.13	0.49	0.55	0.46	0.25	0.17	0.30	0.11	0.45	0.17	0.27	0.43	0.49	0.46	0.57	0.50	0.52	0.39	0.24	0.67	1.00	0.65	0.32	0.32	0.24	0.05	0.29
	Keto(iso)leucine ↓	-0.41	-0.19	0.05	0.07	-0.04	-0.12	0.02	0.33	0.32	0.21	0.16	-0.09	0.08	0.17	0.39	-0.09	0.26	0.46	0.46	0.37	0.46	0.45	0.33	0.55	0.33	0.48	0.65	1.00	0.24	0.48	0.34	0.05	0.18
	Glycerophosphocholine ↓	-0.09	0.64	0.02	-0.19	-0.11	-0.13	0.12	0.16	0.24	0.09	0.16	0.05	0.31	0.14	0.24	0.18	0.53	0.33	0.54	0.39	0.45	0.51	0.62	0.39	0.29	0.29	0.32	0.24	1.00	0.13	0.07	-0.11	0.05
	2-Hydroxybutyrate ↓	-0.02	-0.27	0.06	0.14	-0.06	0.01	-0.08	0.26	0.21	0.21	0.08	0.27	0.05	0.00	0.43	-0.05	0.09	0.14	0.31	0.16	-0.04	0.18	-0.02	0.33	0.02	0.15	0.32	0.48	-0.13	1.00	0.44	-0.04	0.03
	Dodecanoyl-carnitine ↓	-0.11	-0.37	-0.14	-0.01	-0.24	0.02	-0.14	0.23	0.05	0.06	0.02	-0.24	-0.21	0.15	0.03	0.47	0.31	0.34	0.10	0.01	0.03	0.11	-0.26	0.09	-0.02	-0.15	0.24	0.34	-0.07	0.44	1.00	0.04	0.13
	E Caffeine metabolites	Caffeine ↓	-0.22	-0.10	-0.29	-0.31	-0.10	-0.15	-0.02	0.13	0.12	0.08	0.54	0.14	0.29	0.29	0.23	0.07	0.18	0.04	-0.07	0.16	0.28	-0.10	-0.10	0.07	0.50	0.29	0.05	0.05	-0.11	-0.04	0.04	1.00
Dimethyl-xanthine ↓		0.00	-0.05	-0.38	-0.29	-0.08	-0.32	-0.03	0.24	0.26	0.17	0.53	0.12	0.33	0.28	0.21	0.20	0.07	0.10	0.00	0.00	0.17	0.01	-0.02	0.06	0.29	0.48	0.29	0.18	0.05	0.03	0.13	0.76	1.00

Fig. 3. Correlation analysis of 33 dementia markers. Correlation values (r) larger than +0.5 are marked with red, whereas values below -0.5 are in green. Compounds that increased or decreased in dementia patients are indicated by arrows. Five subgroups of 33 compounds are shown with frames in yellow.

brain (41, 42). Note, however, that the seven group A markers increased in dementia were not found in frail subjects (16). If the change in group A is the causal reason for dementia, then a cognitive cause in frailty may be distinct from that of dementia. There seemed to be only a small overlap between dementia- and frailty-related cognition markers in the outcome of patients' metabolite analysis. Five markers are common: They are ET, S-methyl-ET, trimethyl-histidine, methionine, and tryptophan. While aging is thought to be a major risk factor for dementia, we have not found clear age markers among dementia factors except these five.

Six group B compounds contained trimethyl-ammonium moieties, presumably acting as antioxidants, so group B may act in

33 dementia markers		Dementia								HE									
		#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16		
Higher in dementia (7)	A	Quinolinic acid	52.4	59.9	56.2	48.0	51.6	68.9	76.9	45.6	38.4	53.8	39.4	36.8	48.2	46.0	40.6	64.6	
		Dimethyl-guanosine	49.2	63.0	51.8	42.3	60.9	79.7	58.3	62.2	42.7	49.8	52.7	38.4	52.8	47.9	50.8	47.0	
		Pseudouridine	53.3	56.8	54.1	45.3	55.7	84.4	65.2	54.8	41.5	54.3	50.0	38.8	53.9	49.2	49.2	49.4	
		Indoxyl-sulfate	57.1	63.4	53.2	53.6	43.7	78.1	58.8	57.2	37.3	59.9	47.4	38.1	52.8	52.4	39.1	38.7	
		Kynurenine	67.0	50.9	56.7	37.6	63.3	53.5	57.2	60.1	47.9	52.6	50.9	22.8	44.4	55.3	44.4	51.5	
		N6-Acetyl-lysine	85.1	46.3	45.4	49.7	57.8	48.4	71.7	44.9	45.8	48.2	42.9	44.5	47.8	44.8	43.8	46.0	
		Adenosine	69.8	51.9	43.1	44.0	50.3	50.9	45.6	51.5	43.0	45.8	43.4	46.3	43.1	50.4	40.2	39.5	
Lower in dementia (26)	B	S-Methyl-ergothioneine	40.7	40.2	44.2	40.5	41.2	38.9	40.2	42.0	52.5	45.2	57.6	70.9	45.2	57.7	79.0	45.5	
		Ergothioneine	40.5	42.8	41.8	43.4	42.0	41.5	37.3	49.9	52.0	44.6	53.1	71.0	49.9	68.0	77.8	45.3	
		Trimethyl-histidine	38.9	48.4	51.5	44.1	42.2	43.6	37.4	44.6	65.5	54.0	46.3	69.2	64.4	55.1	77.5	45.6	
		Trimethyl-tryptophan	41.1	43.0	42.0	43.0	42.0	41.2	46.3	43.2	56.6	76.3	44.5	59.8	67.6	65.3	51.8	47.2	
		Trimethyl-phenylalanine	46.9	46.9	46.9	46.9	46.9	46.9	46.9	47.0	47.2	46.9	47.2	78.2	86.2	49.0	47.1	46.9	
		Trimethyl-tyrosine	45.5	45.7	45.7	45.8	45.7	45.5	45.5	45.5	45.6	45.9	47.4	46.9	93.6	47.4	57.5	45.9	
		Pantothenate	42.0	41.6	40.1	39.1	43.9	38.6	59.7	50.1	69.2	77.2	45.5	51.3	57.7	47.7	52.5	45.6	
	C	Glucuronate	43.8	44.4	64.1	33.6	42.5	32.2	56.1	39.8	50.9	53.4	49.5	51.5	76.4	59.1	52.1	60.5	
		S-Adenosyl-methionine	42.5	41.3	41.2	41.3	60.6	42.1	43.9	43.6	53.7	46.4	45.8	51.0	44.7	53.1	43.9	45.9	
		NADP+	35.1	40.4	33.6	31.6	48.0	42.2	47.2	44.8	55.5	45.2	57.7	58.5	46.8	45.4	53.7	45.3	
		Glutathione disulfide	37.3	51.5	22.0	42.0	46.0	46.5	36.2	56.3	62.3	58.7	61.2	58.5	47.2	50.1	58.4	44.0	
		ATP	40.8	45.4	30.9	31.9	48.0	46.3	47.1	51.0	42.3	51.5	60.2	60.7	58.5	50.8	61.9	51.0	
	D	Methionine	35.3	52.9	40.2	39.8	41.2	39.5	30.7	69.3	54.1	52.1	65.4	56.4	51.4	48.0	50.5	53.5	
		Tryptophan	36.9	53.9	43.6	44.7	42.1	40.7	22.5	58.4	52.5	52.9	56.8	49.3	44.7	61.4	61.9	49.8	
		Glutamine	44.6	45.2	33.1	40.6	41.2	53.6	33.6	66.9	49.3	51.3	54.8	54.9	60.9	54.9	52.6	67.6	
		Betaine	45.4	52.5	44.4	45.8	40.7	42.6	27.3	52.4	52.8	59.5	53.1	56.0	62.3	46.3	60.8	51.3	
		Phenylalanine	36.9	59.2	41.6	41.4	37.8	48.5	41.7	58.3	47.2	59.3	69.5	47.6	47.5	59.4	58.8	58.5	
		Tyrosine	41.3	38.6	39.6	44.9	44.7	40.5	39.3	59.7	44.5	67.3	60.5	53.5	52.9	56.4	53.7	51.2	
		Histidine	44.4	49.4	48.4	49.0	41.8	33.9	30.6	55.6	43.5	64.7	58.4	42.7	76.6	65.2	53.9	52.5	
		Uridine	42.9	43.0	45.7	42.9	37.6	33.8	34.2	55.9	53.9	48.2	49.1	49.1	59.8	68.3	62.7	51.5	
		Keto(iso)leucine	40.8	46.1	46.1	43.2	31.7	51.8	47.8	46.0	56.2	56.3	65.3	41.1	50.3	69.4	56.8	50.0	
		Glycerophosphocholine	48.8	48.8	43.4	35.5	43.6	49.5	37.8	43.3	52.0	50.5	45.6	46.2	55.7	53.5	47.0	50.6	
		2-Hydroxybutyrate	48.3	46.9	51.9	40.9	34.7	47.0	66.3	48.6	48.8	41.3	74.7	61.5	56.0	57.0	53.2	53.6	
		Dodecanoyl-carnitine	39.5	40.0	39.5	42.0	44.7	55.5	55.1	39.7	54.5	51.0	59.6	44.9	40.0	46.6	60.3	62.2	
		E	Caffeine	44.8	43.5	43.6	44.4	43.4	43.7	44.7	45.2	45.7	54.3	55.5	43.3	62.7	66.8	49.2	45.6
			Dimethyl-xanthine	57.7	40.4	40.7	40.7	40.1	40.4	40.3	42.3	45.5	60.7	59.2	39.8	64.2	71.1	55.6	44.3

Fig. 4. Construction of a heatmap using abundance data of 33 compounds for each subject. Standardized scores (T scores) are represented by colors. Average value (50), white; values above average, pale red to deep red; values below average, pale blue to deep blue. Seven compounds that increased in dementia patients tended to be red in dementia whereas they tended to be white to blue in HE subjects. For the 26 remaining compounds, colors are reversed in dementia and HE subjects.

concentrations were positively correlated with 10 compounds in subgroups B to D (Fig. 3) so that the level of ATP may affect or be affected by concentrations of many metabolites, including oxidoreductive compounds, such as GSH, betaine, ET, and S-methyl-ET, and amino acids, such as glutamine and tryptophan. Thus, it is quite possible that ATP might assist antioxidant in the brain via many metabolites present in plasma and RBCs so that it may contribute to brain activities against dementia. NADP<sup>+</sup> and GSSG may be synergistic in maintaining the level of ATP and ET (54). Hence, oxidoreductive NADP<sup>+</sup>, antioxidative GSH, and presumably neuroprotective trimethylated ammonium compounds may all function together to sustain brain mitochondrial ATP production level against dementia. Note that glycerophosphocholine and dodecanoyl-carnitine, which also contain a trimethyl-ammonium moiety, belong to group D and may also enhance mitochondrial function.

Nine compounds possessing trimethylated ammonium ions (Table 1 and *SI Appendix, Fig. S2*) are amphipathic compounds (possessing both hydrophilic and lipophilic properties) and form the basis of lipid polymorphism. All of them showed a sharp

decline in abundance in dementia subjects. Distribution of ET in the brain has been demonstrated in various mammalian species (55, 56) and humans (57). The cause of its decline might be due to the rise of ROS (reactive oxygen species) in dementia patient brains. These amphipathic compounds may have similar roles, such as forming a higher-ordered, assembled structure. In addition, these compounds are abundant. They might act as major neuroprotectants or antioxidants in the brain, and their levels are sensitive to both antioxidants and ROS. In addition, membrane defects have been observed in dementia patient brains that degrade glycerophosphocholine (58, 59). Alternatively, amphipathic moieties might deliver pertinent compounds of nanomolar concentrations to the CNS (60). An advantage of metabolomic analysis over proteomic and genomic studies exists in the search for drugs. As these metabolites exist in human body fluids, they may be employed for studies of drug therapy. In the present study, 33 metabolites are obvious targets for future study, the majority of which have not been studied at all relative to their clinical potential.

## Materials and Methods

**Participants.** Eight dementia patients, hospitalized at the National Hospital Organization Ryukyu Hospital, in Kin-town, Okinawa, participated as subjects in this study (SI Appendix, Table S1). They were judged carefully by doctors at Ryukyu Hospital to understand the study objectives and contents, privacy protection, and free choice of participation, and were selected. In addition, eight young and eight elderly healthy volunteers, who live in Onna-village, Okinawa, participated as volunteers. Blood samples for LC-MS measurements were collected between 2017 and 2018.

**Diagnosis of subjects.** Patients were diagnosed based on the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-4) (61). Careful interviews on impairment of activities of daily living were performed to rule out delirium or other psychiatric diseases. Blood tests and interviews of patients ruled out the possibility of other systemic diseases. To assess cognitive ability and pathological changes, cognitive tests (HDS-R, MMSE, COGNISTAT), MRI for hippocampal atrophy for all patients, and voxel-based specific regional analysis system for Alzheimer's disease (VSRAD) for some patients were collected (SI Appendix, Table S1). Both the revised Hasegawa's Dementia Scale (HDS-R) (62) and the Mini-Mental State Examination (MMSE) (63, 64) have a maximum of 30 points, and cutoff values are 20/21 and 23/34, respectively. Dementia patients were below the cutoff values for each test, while HE people who agreed to the test were almost perfect (28 to 30). The results of the Japanese version of the neurobehavioral cognitive status examination, COGNISTAT (65), and the voxel-based specific regional analysis system for Alzheimer's disease, VSRAD (66), were done supplementally. The Z score of VSRAD indicates the degree of atrophy of the middle temporal area, including the hippocampus (67). These clinical data were collected before January 2018 at Ryukyu Hospital. Information about patient medications is shown in SI Appendix, Table S4.

**Ethics statement.** Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Consent of patient spouses or guardians was also obtained, in addition to that of the patients themselves. All experiments were performed in compliance with relevant Japanese laws and institutional guidelines. All protocols were approved by the Ethics Committee on Human Research of Ryukyu Hospital and by the Human Subjects Research Review Committee of the Okinawa Institute of Science and Technology Graduate University.

**Chemicals and reagents.** Standards for metabolite identification were purchased from commercial sources, as described previously (14, 15, 53, 68). LC-MS-grade acetonitrile, methanol, and ultrapure water were obtained from FUJIFILM Wako Pure Chemical.

**Blood sample preparation.** Metabolomic samples were prepared as described previously (14). Briefly, venous blood samples were collected in heparinized tubes before breakfast. Subjects were asked to ensure at least 8 h of fasting prior to sampling. During fasting, subjects took water freely. Immediately, 0.2 mL of blood was quenched in 1.8 mL of 55% methanol at  $-40^{\circ}\text{C}$ . Ten nanomoles each of Hepes and Pipes was added to each sample to serve as

standards. After brief vortexing, samples were transferred to Amicon Ultra 10-kDa cutoff filters (Millipore) to remove proteins and cellular debris. After sample concentration by vacuum evaporation, each sample was resuspended in 40  $\mu\text{L}$  of 50% acetonitrile, and 1  $\mu\text{L}$  was used for each injection into the LC-MS system, as described.

**LC-MS analysis.** Nontargeted LC-MS conditions were as described previously (14, 16). Briefly, LC-MS data were obtained using an UltiMate 3000 DGP-3600RS high-performance liquid chromatography system (Thermo Fisher Scientific) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). LC separation was performed on a ZIC-pHILIC column (Merck SeQuant;  $150 \times 2.1$  mm, 5- $\mu\text{m}$  particle size). Acetonitrile (A) and 10 mM ammonium carbonate buffer (pH 9.3) (B) were used as the mobile phase, with a linear gradient from 80 to 20% A over 30 min, at a flow rate of 100  $\mu\text{L}/\text{min}$ . The mass spectrometer was operated in full-scan mode with a 100 to 1,000  $m/z$  scan rate and automatic data-dependent MS/MS fragmentation scans.

**LC-MS data processing and peak characteristics.** Peak areas of metabolites of interest were measured using MZmine 2 software (69). Data analytical procedures and parameters have been described previously (15). We analyzed 124 blood metabolites that were confirmed using standards or MS/MS analysis (Dataset S1). According to their peak areas, metabolite abundances were classified into three groups (H, M, and L). H denotes compounds with high peak areas ( $>10^8$  AU; arbitrary unit), M denotes compounds with medium peak areas ( $10^7$  to  $10^8$  AU), and L denotes compounds with low peak areas ( $<10^7$  AU) (SI Appendix, Table S2).

**Statistical analysis.** Peak data processed with 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 using the Benjamini-Hochberg method. The plot of PCs was generated with SIMCA-P+ software (Umetrics). Heatmaps represent standardized abundance data for each metabolite. T scores were calculated from the following formula:  $T \text{ score} = [(\text{sample peak area} - \text{average of population peak area}) \times 10/\text{SD of population peak area}] + 50$ . Therefore, the mean and SD are 50 and 10, respectively.

**Data Availability.** Raw LC-MS data in mzML format reported in this article are accessible via the MetaboLights repository (<https://www.ebi.ac.uk/metabolights>). Data for the 24 subjects are available under accession no. MTBLS2109. All data are included in the manuscript and/or supporting information.

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