Am J Transl Res 2021;13(11):12495-12508
www.ajtr.org /ISSN:1943-8141/AJTR0136366

Original Article

^{1}H NMR metabolomic profiling of human cerebrospinal fluid in aging process

Huan-Tang Lin[1,2,3], Mei-Ling Cheng[4,5,6], Chi-Jen Lo[4], Wen-Chuin Hsu[2,7], Gigin Lin[8,9], Fu-Chao Liu[1,2]

[1]Department of Anesthesiology, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan; [2]College of Medicine, Chang Gung University, Taoyuan 333, Taiwan; [3]Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan; [4]Metabolomics Core Laboratory, Healthy Aging Research Center, Chang Gung University, Taoyuan 333, Taiwan; [5]Department of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan; [6]Clinical Metabolomics Core Laboratory, Chang Gung Memorial Hospital, Taoyuan, Taiwan; [7]Department of Neurology, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan; [8]Department of Medical Imaging and Intervention, Institute for Radiological Research, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan; [9]Clinical Phenome Center, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

Received June 15, 2021; Accepted September 21, 2021; Epub November 15, 2021; Published November 30, 2021

Abstract: The molecular process of biological aging might be accompanied by significant metabolic derangement, especially in the central nervous system (CNS), since the brain has an enormous energy demand. However, the metabolic signature of the aging process in cerebrospinal fluid (CSF) has not been thoroughly investigated, especially in the Asian population. In this prospective cohort study on CSF metabolomics using proton nuclear magnetic resonance (NMR) spectroscopy, fasting CSF samples from 75 cognitively unimpaired patients aged 20-92 years without diabetes or obesity, undergoing spinal anesthesia for elective surgery were analyzed. Several metabolites in CSF samples were identified as having a significant association with the aging process in cerebral circulation; among the metabolites, the levels of alanine, citrate, creatinine, lactate, leucine, tyrosine, and valine significantly increased in old patients compared to those in young patients. The combined CSF metabolite alterations in citrate, lactate, leucine, tyrosine, and valine had a superior correlation with the aging process in all age groups. In conclusion, our pilot study of aging CSF metabolomics in the Taiwanese population presents significantly altered CSF metabolites with potential relevance to the aging process. These metabolic alterations in CSF samples might imply increasing anaerobic glycolysis, mitochondrial dysfunction, and decreasing glucose utilization in cerebral circulation in aged patients.

Keywords: Aging process, cerebrospinal fluid, metabolomics, nuclear magnetic resonance

Introduction

With an increase in world population and an improvement in life expectancy, there has been a worldwide increase in the aging population. According to a 2020 United Nations report on global population aging, the number of people aged above 65 years worldwide is projected to be more than double by 2050, reaching 1.5 billion persons or 16 percent of the total population [1]. The increasing trend of global aging has become a serious socioeconomic challenge due to chronic diseases such as cardiovascular disease, diabetes, cancer, and neurodegenerative diseases accompanying population aging.

Aging is a complex process that is featured by a gradual decline in cellular and organ functions, and is modulated by multiple factors including genetics, environment, diet, and lifestyle [2]. Although accumulated oxidative stress and DNA damage both contribute to aging, progressive metabolic dysfunction is a more generalized hallmark of biological aging [2]. Previous research on aging has identified aging pathways such as mTOR and AMPK to be involved, which are major targets of anti-aging interventions, including rapamycin, metformin, exercise training, and intermittent fasting, aimed at regulating these metabolic pathways [3].

The human brain is extremely energy-consuming and metabolizes approximately one-quarter

of systemic glucose for energy production, although it only comprises 2% of the total body mass [4]. During brain aging, compromised bioenergetics, neuroplasticity, oxidative stress, and neuroinflammation in the brain render the aging brain vulnerable to neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD) and ischemic stroke [5]. Age-related accumulation of oxidative stress may lead to functional decrements in cerebral energy metabolism, including glucose transport, mitochondrial oxidative phosphorylation, DNA repair, and redox regulation [6]. Previous experiments have shown that mice with reduced glucose transporter 1 (GLUT1) levels display an age-dependent decline in cerebral blood flow and an increased blood-brain barrier (BBB) permeability [7]. Additionally, brain hypoperfusion and BBB leakage in elderly individuals can contribute to diminished nutrient import and toxin removal, leading to cognitive decline [8]. Furthermore, progressive brain insulin resistance during the aging process may share a common feature of metabolic derangements observed in patients with diabetes, obesity, and dementia [9-11].

Chronic oxidative stress, bioenergetic deficits, and neuroinflammatory changes are major contributors to cognitive decline and have been detected in neurodegenerative diseases such as AD as well as in normal aging [12]. Functional neuroimaging studies using PET-based measurements in cognitively normal adults have found that aging produces glucose hypometabolism in the brain [13]. In addition, a series of population-based brain autopsy studies in elderly people without known neurological diseases also consistently reported the presence of misfolded protein aggregates such as amyloid plagues and the loss of brain volume in the majority of these aging brains [14]. In the Baltimore brain autopsy cohort, it was found that glucose hypometabolism in the brain may precede AD, with evidence that the severity of AD is associated with higher glucose concentration in brain tissue, reduced glycolytic flux, and lower GLUT3 levels [15]. Given that neurodegenerative diseases are common in the elderly and the disease features are widespread in the aged brain, even in patients without cognitive dysfunction, it is plausible to conclude that normal brain aging might form a continuum with neurodegenerative diseases [14].

Metabolomic analysis provides abundant information on small metabolite alterations and provides clear insight into the metabolic perturbations in the aging process. Previous metabolomics studies on aging mostly focused on plasma, serum, and urine samples obtained from model organisms and humans, most of which found that aging-related metabolites are largely associated with carbohydrates, lipids, amino acids, and redox metabolism [16]. Studies of aging metabolomics in plasma samples have found that ceramide, fatty acids, methionine, and nitric oxide pathways are associated with the aging process and are the main regulators of health span in healthy adults [17]. Metabolomic analysis of neurodegenerative diseases focusing on AD found that sphingolipid perturbations affecting tau phosphorylation and amyloid pathology are associated with preclinical AD and AD pathology at biopsy [18].

Cerebrospinal fluid (CSF) exchanges metabolites between the cerebral and systemic circulation; however, transcellular transport is limited by the specialized tight junctions of the BBB [19]. Therefore, CSF metabolomics may reflect brain-specific metabolite alterations during the aging process and provide more comprehensive cerebral metabolomic information [20, 21]. However, the metabolomic dysregulation in CSF samples during the aging process had not been published until recently by Carlsson et al. in February 2021. In a small Swedish study conducted by Carlsson et al., 23 claimed healthy subjects (aged 30-74 years) underwent CSF metabolomic analysis for health aging using liquid chromatography mass spectrometry [22]. They found ten metabolites with a significant association with aging: acetylcarnitine, glutarylcarnitine, hippurate, 5-hydroxytryptophan, isoleucine, ketoleucine, methionine, and pipecolate, all of which were observed to have increasing levels in the elderly. On the contrary, methylthioadenosine and 3-methyladenine showed decreasing levels in older patients [22]. However, due to limited case numbers and case information (only sex and age), further cohort studies are required to gain a deeper insight into the CSF metabolomics of health aging. Therefore, we presented our metabolomic study to profile the CSF metabolic

alterations during the aging process in cognitively unimpaired patients using NMR spectroscopy.

Materials and methods

In this metabolomic study, we compared the metabolomic profiles of CSF samples in cognitively unimpaired patients in different age groups to examine possible biomarkers of the aging process in cerebral circulation. This clinical study was registered in the Clinical Trials Registry (ClinicalTrials.gov Identifier: NCT 04315038) and was permitted by the Institutional Review Board of Chang Gung Medical Foundation, Taiwan (approval number: 201801931A3). Before enrollment into the study, the study protocol was clearly explained to every participant, and written informed consent was obtained from each patient.

Study population

In this study, we enrolled cognitively unimpaired participants (without neurological or psychiatric diseases) scheduled for elective surgery who underwent optional spinal anesthesia at Linkou Chang Gung Memorial Hospital, an international medical center in Northern Taiwan, and divided them into three different age groups: young (age 20-30 years), middle-aged (45-55 years), and old (aged 70-100 years). We excluded patients with a known history of diabetes and obesity because insulin resistance in these patients might confound the metabolomic analysis of the aging process. From June 1, 2019 to March 31, 2020, a total of 81 participants completed the initial evaluation and received CSF sampling for metabolomic analysis. All enrolled participants belonged to the American Society of Anesthesiologists physical status classification of ≤3. They were admitted for elective surgeries such as urologic or orthopedic surgery (for the lower extremity) and fasted for ≥8 hours before CSF sampling. Cognitive assessment was based on the exclusion of neurological or psychiatric diseases and perioperative conversation, and mild cognitive impairment might be overlooked. After the initial examination, we excluded six participants with fasting blood glucose >126 mg/dL or BMI >30 kg/m^2 for further analysis because these patients were generally recognized as having diabetes or obesity according to the diagnostic criteria [23]. The final analyzed cohort was divided into young, middle, and old age groups with 25, 26, and 24 patients in each group, respectively. Their demographics, including age, sex, body height, body weight, and hospital records were recorded. Biochemical data, such as plasma glucose and creatinine levels, were recorded from the latest laboratory results before CSF sampling.

CSF sample collection

After obtaining adequate informed consent, spinal anesthesia was performed with a 26-gauge spinal needle at the L4 and L5 interspace. Once there was successful outflow of clear CSF from the spinal needle, 1.2 mL CSF was drained into a polypropylene tube before administration of spinal anesthesia and stored as aliquots at -80°C for further analyses. No complications or patient discomfort were reported during the whole CSF sample collection procedures.

Sample preparation and NMR spectra acquisition

Most of the CSF sample preparation and NMR spectra processing were the same as in our previous CSF metabolomic analysis [21]. The 630 μL CSF samples were mixed with 70 μL of solution (1 mM TSP, 3 mM NaN_3 in D_2O).

The CSF NMR spectra were acquired at Bruker Avance III HD 600 MHz (Bruker Biospin GmbH, Rheinstetten, Germany). This spectrometer possessed 5 mm probe ($^1H/^{13}C/^{15}N$) and a SampleJet system. The spectra were automatically acquired and processed by using Topspin software (version 3.6.2; Bruker Biospin GmbH, Rheinstetten, Germany).

The Carr-Purcell-Meiboom-Gill pulse sequence with water suppression was set up for spectra data acquisition. The broad signals from proteins were filtered by an 80 ms T_2 relaxation time. The 32 transients CSF NMR spectra were acquired with 64 k data points in a 20 ppm. The NMR spectral region (between 9.50 and 0.50 ppm) was segmented into separated bins with a bin width of 0.01 ppm and the water region (5.10-4.20 ppm) was removed by using AMIX software (version 3.9.14; Bruker Biospin GmbH, Rheinstetten, Germany). The final multivariate analysis was performed by using software (SIMCA-P+, version 13.0; Umetrics, Umea, Sweden) under Pareto scaling conditions.

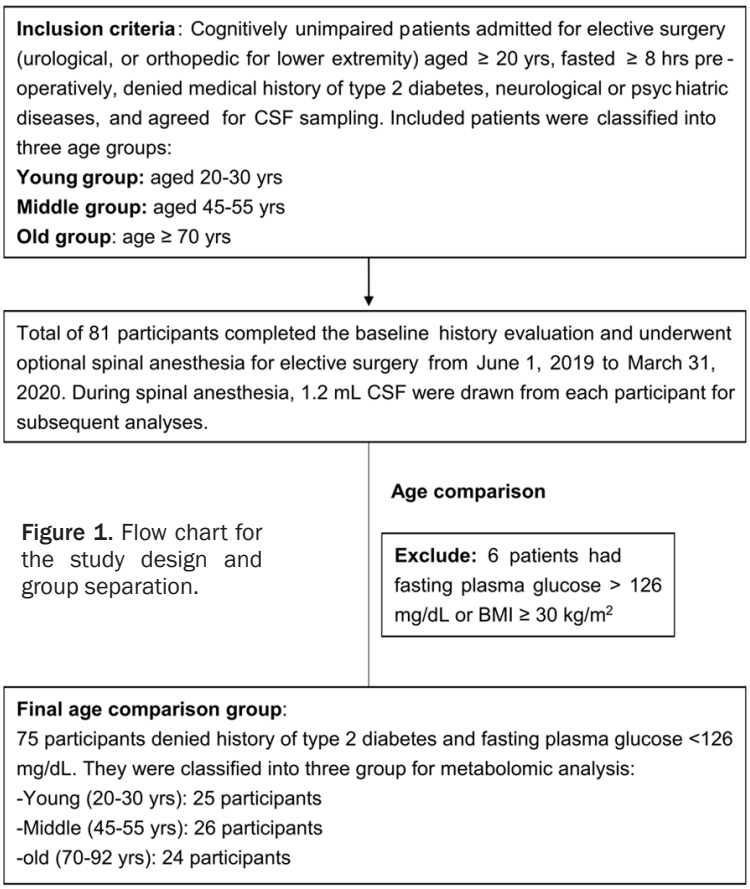

Figure 1. Flow chart for the study design and group separation.

Statistical analysis

Each metabolite was specified in the analyzed NMR spectrum by comparing the chemical shifts and multiplicity patterns of listed metabolite in the Human Metabolome Database (HMDB) or the laboratory library [25]. We then selected significant variables in the Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) discriminative scores using a threshold level of 0.05, and a correlation coefficient of ±0.396. Additionally, the predicted values of the variable Y from the constructed OPLS-DA model were applied to calculate the Akaike information criterion (AIC) value for further fitting comparison. The metabolites were then analyzed and compared with their calculated AIC values and fold changes. We then applied an online tool, MetaboAnalyst 5.0, to accomplish the other metabolomic analyses including heatmap and enrichment analysis [26].

The recorded data are presented as a percentage for qualitative variables (sex and medical diseases) and as means ± SD for continuous variables. The statistical analyses in our study were based on acquired NMR signal integration of each metabolite, and between-group comparisons were performed using the Student's t-test or χ^2 tests (for two-group comparison) and analysis of variance (ANOVA) (for multiple-group comparison). The between-group differences of a specific metabolite were compared using the OPLS-DA coefficients in NMR signals, and the variable in OPLS-DA score plots was compared with goodness of fit (R^2X, R^2Y, and Q^2).

In this study, CSF profiling of the aging process in the cerebral circulation was the primary outcome. The overall structure for specifying the primary outcome was to identify CSF metabolites that we could use to distinguish between the young and old age groups and then identify metabolite combinations that could distinguish every age group. We constructed combinations of significant metabolites and compared their odds ratios (ORs) and *P*-values to differentiate these age groups. Since it is evident that diabetes, obesity, and other neurological diseases might confound our analysis, we excluded patients with these diseases and utilized multivariate logistic regression to adjust for significant variables including sex, BMI, serum creatinine, and medical history of hypertension. All statistical analyses were performed using SAS software (version 9.4; SAS Institute Inc. NC, USA), and a two-sided *P* value <0.05 was defined as statistically significant.

Results

Patient groups and their demographic comparison

Our final cohort included 75 patients, and we divided them into three age groups: 25 patients in the young group, 26 in the middle group, and 24 in the old group. The study protocol is illustrated in **Figure 1**. The demographic and bio-

Table 1. Demographic comparison among young, middle-aged, and old patients

Group	Young (20-30 y/o) (n=25)	Middle (45-55 y/o) (n=26)	Old (70-92 y/o) (n=24)	*P* value#
Male sex, N (%)	4 (16.00%)	11 (42.31%)	17 (70.83%)	<0.001*
Age (mean ± SD, years)	24.92±3.72	51.31±3.86	78.50±7.29	<0.001*
BMI (kg/m^2)	21.06±2.79	24.15±3.22	23.96±2.97	<0.001*
Fasting blood glucose (mg/dL)	93.05±20.31	94.26±10.33	108.90±21.52	0.115
Serum creatinine (mg/dL)	0.64±0.20	0.75±0.21	1.05±0.56	0.003*
Hypertension	0 (0%)	0 (0%)	15 (62.50%)	<0.001*
Hyperlipidemia	0 (0%)	2 (7.69%)	0 (0%)	0.325
Obesity	0 (0%)	0 (0%)	0 (0%)	NA

Abbreviation: NA, non-applicable. #*P* value was calculated using Chi-square test for categorical variables and the analysis of variance (ANOVA) for continuous variables. **P*<0.05.

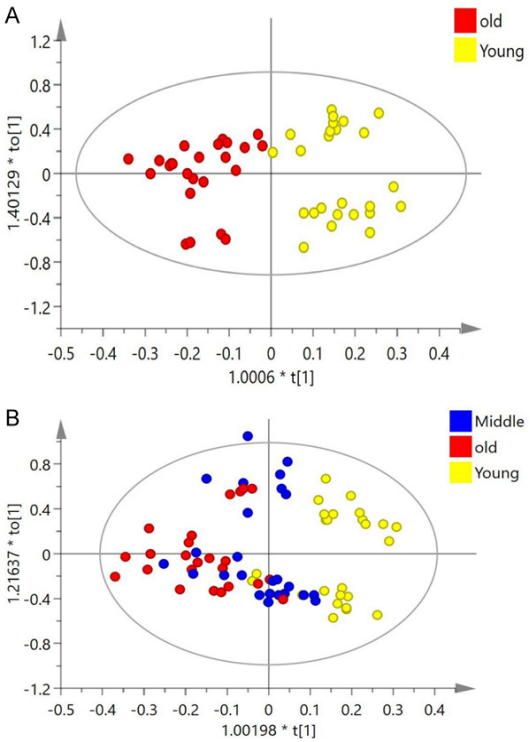

Figure 2. Orthogonal partial least-squares discriminant analysis (OPLS-DA) score plots in CSF samples obtained from (A) young patients versus old patients (reliability: R^2X=0.952, R^2Y=0.82, Q^2=0.643), (B) comparison between young, middle, and old patients (reliability: R^2X=0.923, R^2Y=0.34, Q^2=0.22). The OPLSDA plots show a clear separation between young and old group in CSF samples.

chemical parameters of the enrolled participants are presented in **Table 1**. Demographic comparison of the CSF samples showed that patients in the old age group had significantly higher male percentage, BMI, and serum creatinine level compared to the other age groups. Moreover, older patients had a medical history of hypertension compared to the other groups. Due to these significant differences, subsequent analyses were adjusted for sex, BMI, serum creatinine, and hypertension.

OPLS-DA score plots of NMR signals and the OPLS-DA coefficient loading plot

The OPLS-DA score plots of the NMR spectroscopy in CSF samples from older, middle-aged, and young patients are compared in **Figure 2.** The OPLS-DA score plots showed a clear discrimination between the old and young groups in CSF samples (reliability: R^2X=0.952, R^2Y=0.82, Q^2=0.643). The OPLS-DA coefficient loading plots of NMR signals in CSF samples from the old and young groups are shown in **Figure 3**. The significant metabolites discriminating between the old and young groups were annotated in the NMR spectra. The higher abundance of alanine, acetate, citrate, creatinine, glycine, lactate, leucine, and valine was observed in the old group, while higher abundance of glucose and histidine was observed in the young group.

Metabolomic comparison between old patients and young patients

The comparison of NMR signal integration in CSF samples between old and young groups is shown in **Table 2**, and comparisons between the other groups are listed in **Table 3**. The NMR signal integration in old group had significantly higher levels of alanine, citrate, creatinine, glycine, lactate, leucine, and valine

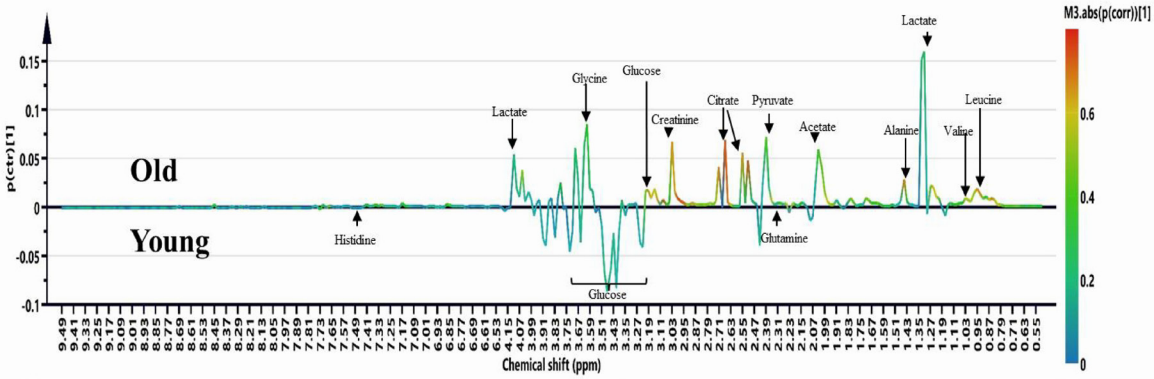

Figure 3. The OPLS-DA coefficient loading plots of NMR signals in CSF samples obtained from young age patients versus old age patients.

Table 2. Comparison of CSF metabolites in young versus old patients

Metabolites in CSF	Chemical shift (ppm)	NMR signal integration (mean ± SD) ($\times 10^{-3}$ a.u.)		Adjusted fold change[a]	Adjusted *P* value[a,#]
		Old	Young	Old/Young	Old vs. Young
Increased metabolite levels in old patients compared to young patients					
Alanine	1.455	134.7±8.7	66.2±12.3	2.035	<0.001*
Glycine	3.556	572.3±70.4	366.6±98.8	1.561	0.071
Leucine	0.998	94.1±6.8	61.6±9.6	1.527	0.004*
Tyrosine	6.872	33.7±2.7	24.0±3.9	1.403	0.031*
Citrate	2.537	1309.4±63.1	955.1±89.2	1.371	<0.001*
Valine	1.028	78.9±4.1	58.0±5.6	1.361	0.002*
Acetate	1.910	145.5±17.4	108.4±24.5	1.342	0.184
Creatinine	3.037	456.1±16.6	348.1±23.4	1.310	<0.001*
Pyruvate	2.366	244.3±20.6	192.6±29.0	1.268	0.119
Lactate	4.107	7689.8.1±376.8	6201.5±531.9	1.240	0.016*
3-hydroxyisovalerate	1.193	131.8±12.9	110.1±18.2	1.197	0.294
Phenylalanine	7.410	56.9±3.6	48.0±5.1	1.184	0.125
Isoleucine	0.992	24.6±2.3	21.6±3.3	1.137	0.425
2-hydroxybutyrate	0.893	108.0±17.0	107.6±23.9	1.003	0.989
Glutamine	2.428	1060.2±74.6	1059.1±105.4	1.001	0.992
Decreased metabolite levels in old patients compared to young patients					
Histidine	7.722	35.6±2.7	35.8±3.7	0.993	0.958
Formate	8.450	19.1±1.4	20.4±2.0	0.936	0.571
Isobutyrate	1.065	32.7±6.8	38.9±9.6	0.839	0.564
Acetone	2.223	78.4±22.1	98.1±31.2	0.798	0.576
Glucose	5.233	1953.3±513.4	2528.5±724.8	0.773	0.483

[a]*P* value was adjusted for sex, body mass index (BMI), serum creatinine, and hypertension. [#]*P* value was calculated using two sample *t* test. *$P<0.05$.

compared to young patients (with adjusted fold change >1.2 and *P* value <0.05). **Figure 4** shows the metabolite heatmaps in CSF samples of old and young patients for each metabolite according to their coefficients of NMR signals. The age-related metabolite variations between these groups could be visualized in the heatmaps combined with Hierarchical Cluster Analysis. Significantly higher abundance of citrate, lactate, leucine, tyrosine, and valine was observed in old patients (**Figure 4A**).

Table 3. Comparison of CSF metabolites in middle age patients to young or old patients

Comparison	Young age vs. Middle age		Middle age vs. Old age	
Significantly changed metabolites in CSF	Adjusted fold change[a]	Adjusted *P* value[a,#]	Adjusted fold change[a]	Adjusted *P* value[a,#]
Alanine	1.179	0.091	1.419	0.041*
Citrate	1.174	0.019*	1.089	0.393
Creatinine	1.165	0.004*	1.152	0.018*
Formate	1.121	0.069	0.816	0.037*
Glucose	0.892	0.389	0.934	0.829
Glycine	1.821	0.057	1.225	0.288
Isoleucine	1.343	0.032*	0.832	0.299
Lactate	1.067	0.258	1.165	0.076
Leucine	1.920	0.001*	1.070	0.605
Tyrosine	1.402	0.013*	1.119	0.397
Valine	1.436	<0.001*	0.966	0.725

[a]Adjusted for sex, body mass index (BMI), and hypertension. [#]*P* value was calculated using two sample *t* test. *$P<0.05$.

Metabolite combinations for correlating with aging process in cerebral circulation

To identify biomarkers correlating with our primary outcome of the aging process in cerebral circulation, we constructed metabolite combinations using CSF metabolites with a significant discrimination between old and young patients. We then compared their AIC values (**Table 4**) and adjusted ORs (**Table 5**) for discriminating every age comparison (young vs. middle, middle vs. old, and young vs. old age groups) using stepwise ANOVA and multivariate analysis. Among these combinations, a combination of citrate, lactate, leucine, tyrosine, and valine had comparatively low AIC values and significant adjusted ORs in every age comparison for discriminating the aging process.

Enrichment analysis and altered metabolic pathways

Figure 5 shows the results of enrichment analysis of altered metabolic pathways for these significantly altered CSF metabolites during the aging process. Among the altered pathways, alanine metabolism and glutathione metabolism showed significant enrichment ratios in CSF samples. Since these significant metabolites discriminating young age and old age patients are all involved in glycolysis and the mitochondrial tricarboxylic acid (TCA) cycle, their specific metabolic change during the aging process in cerebral circulation is depicted in **Figure 6**. The observed significantly increased alanine, lactate, and citrate levels in fasting CSF samples of older patients might suggest increased anaerobic glycolysis, mitochondrial dysfunction (lower TCA cycle activity), and decreased glucose utilization in aged brain circulation.

Discussion

This cohort study aimed to profile the CSF metabolomic signature of the aging process in cognitively unimpaired patients in the Taiwanese population using proton NMR spectroscopy. The metabolomic analysis of fasting CSF samples showed significantly increased alanine, citrate, creatinine, lactate, leucine, tyrosine, and valine levels in elderly patients than in young patients, and the combination of citrate, lactate, leucine, tyrosine, and valine demonstrated superior correlation with the aging process in all age groups. The profiled CSF metabolome of the aging process revealed glucose hypometabolism and increased oxidative stress in the cerebral circulation, implying higher anaerobic glycolysis and mitochondrial dysfunction during aging.

In our results, the identified CSF metabolites with high correlation with the aging process were mainly composed of branch-chained amino acids (BCAAs) (leucine, valine), aromatic amino acids (AAAs) (tyrosine), and carbohydrates (citrate, lactate). The higher CSF leucine levels in the elderly might be explained by the increased CSF concentration of leucine-rich

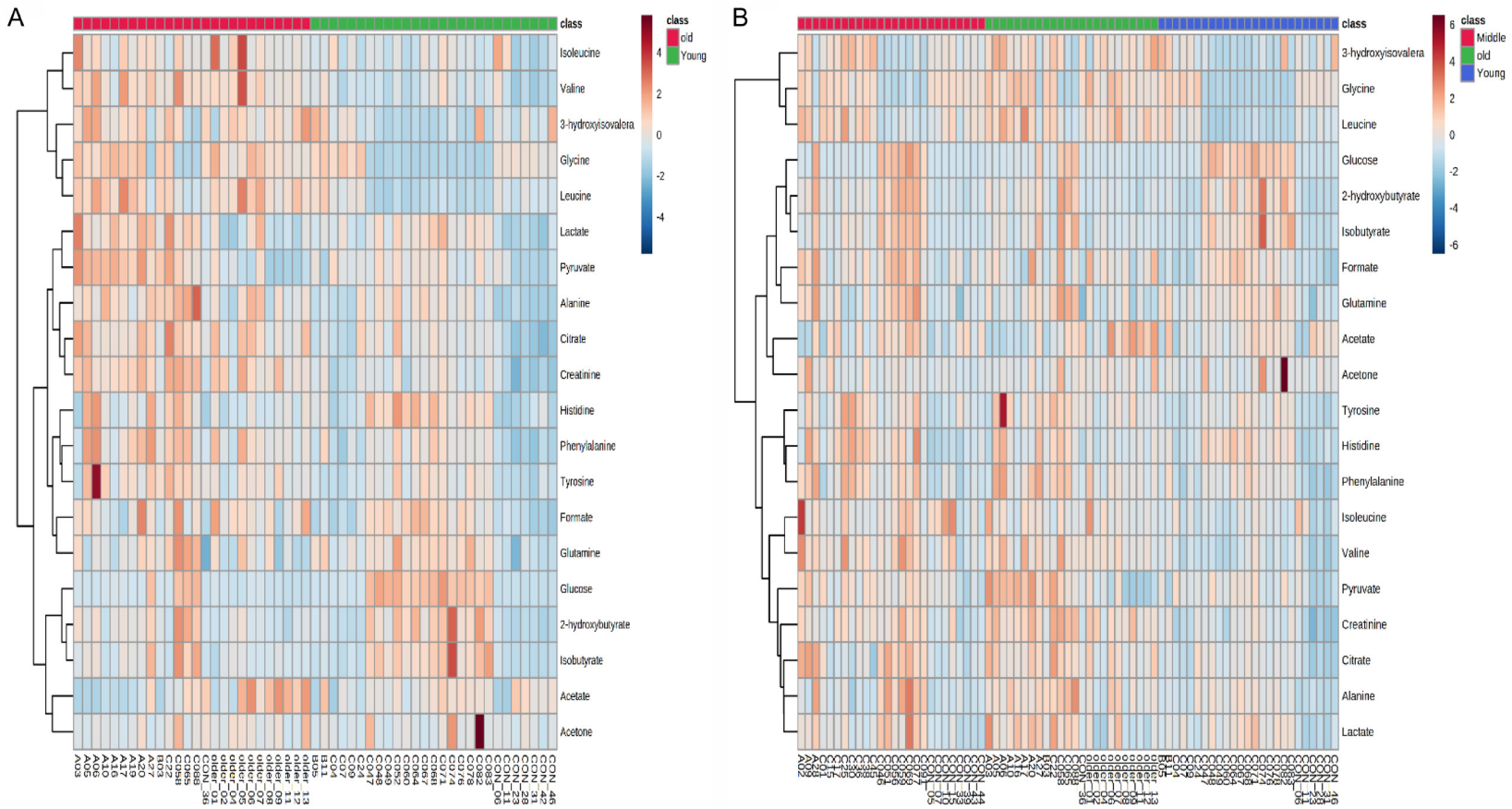

Figure 4. Metabolite heatmaps in CSF samples from (A) young vs. old patients, and (B) young, middle, and old patients.

Table 4. CSF metabolite combinations for correlating aging process based on Akaike Information Criterion (AIC) estimation

CSF metabolite combinations	Akaike Information Criterion (AIC)[#]		
Comparison	Young vs. Middle	Middle vs. Old	Young vs. Old
CSF Glucose	72.94	60.59	55.06
Alanine	68.40	57.49	38.49
Citrate	66.08	60.83	37.27
Creatinine	62.08	55.77	32.71
Lactate	71.21	59.86	50.95
Leucine	61.27	59.91	43.69
Tyrosine	63.52	59.89	43.66
Valine	55.66	60.74	30.18
Leucine, Tyrosine	55.18	71.65	28.32
Citrate, Valine, Leucine	49.07	71.23	28.56
Citrate, Leucine, Tyrosine, Valine	49.98	71.78	27.59
Citrate, Lactate, Leucine, Tyrosine, Valine	48.97	73.67	28.94
Alanine, Citrate, Lactate, Leucine, Tyrosine, Valine	49.63	70.94	29.69
Alanine, Citrate, Creatinine, Lactate, Leucine, Tyrosine, Valine	51.06	63.01	29.64

[#]AIC value was calculated using logistic regression model.

Table 5. Association of altered CSF metabolites with aging process

Comparison	Young vs. Middle	Middle vs. Old	Young vs. Old
Significantly changed metabolites in CSF	Adjusted OR[a,#]	Adjusted OR[a,#]	Adjusted OR[a,#]
CSF glucose	0.999	0.999	0.999
Alanine	1.014	1.011	1.043*
Citrate	1.002*	1.001	1.009*
Creatinine	1.018*	1.012*	1.032*
Lactate	1.001	1.001	1.006*
Leucine	1.043*	1.012	1.049*
Tyrosine	1.130*	1.036	1.182*
Valine	1.079*	0.989	1.197*
Leucine, Tyrosine	2.950*	2.374	2.794*
Citrate, Valine, Leucine	2.094*	2.219	3.591*
Citrate, Leucine, Tyrosine, Valine	2.371*	2.134	3.571*
Citrate, Lactate, Leucine, Tyrosine, Valine	2.062*	2.717*	3.519*
Alanine, Citrate, Lactate, Leucine, Tyrosine, Valine	2.316	3.125*	3.178*
Alanine, Citrate, Creatinine, Lactate, Leucine, Tyrosine, Valine	2.319*	2.933	2.858*

Abbreviation: OR, odds ratio. [a]Adjusted for age, sex, body mass index (BMI), and hypertension. [#]Odds ratio was calculated using logistic regression model. *$P<0.05$.

α2-glycoprotein in advanced age found in previous human and animal studies which is associated with synaptic dysfunction and cognitive decline [27]. The accumulation of BCAAs and AAAs in fasting CSF in elderly patients might imply decreased catabolism of BCAAs and AAAs, glucose hypometabolism, and mitochondrial dysfunction in the cerebral circulation [28]. Lactate is a source of neuronal energy and it is produced by anaerobic glycolysis of glucose in astrocytes. CSF lactate level reflects anaerobic metabolism in the brain and high CSF lactate levels has been found to have correlation with cognitive decline in AD patients [29]. The increase in CSF lactate concentration in the elderly patients in our results might reflect the increase of anaerobic glycolysis and decline of glucose utilization in their cerebral

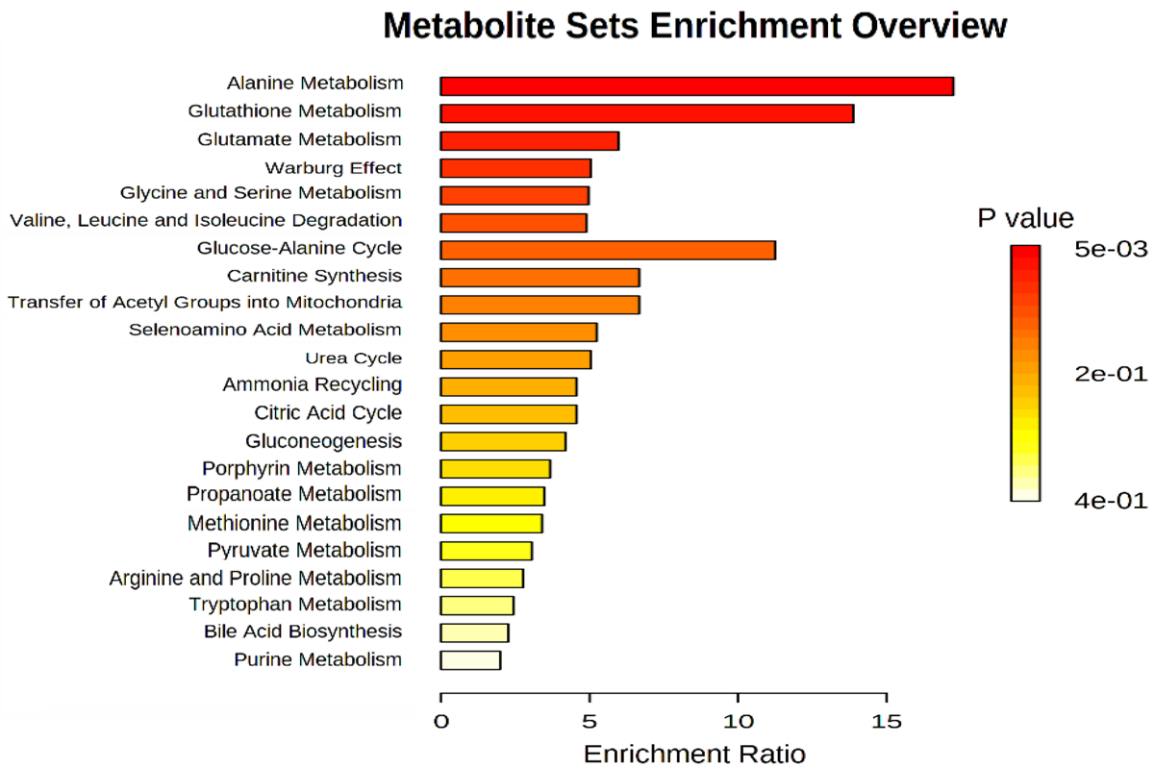

Figure 5. Enrichment analysis of involved metabolic pathways for significant CSF metabolites during aging process.

circulation during fasting [13]. On the other hand, citrate is an intermediate of the mitochondrial TCA cycle, and CSF citrate accumulation in the elderly might imply lower TCA cycle activity than glycolytic rate thus leading to decreased citrate utilization in aging brain cells [30].

The other significantly altered metabolites during the aging process in our study were alanine and creatinine. Alanine participates in gluconeogenesis, and increasing alanine levels in CSF samples of older patients might reflect lower glucose utilization in the aged brain during fasting. Altogether, the increased alanine, lactate, and citrate levels in fasting CSF samples of older patients might suggest increased anaerobic glycolysis, mitochondrial dysfunction, and decreased glucose utilization in aged brain circulation. Our finding is in line with previous PET analysis of aging brain that decreased whole-brain glucose utilization, oxygen consumption, and decreased aerobic glycolysis during normal aging in humans [13]. Increased CSF creatinine levels with age in our result might be associated with muscle breakdown, declined renal function, and BBB breakdown in elderly patients, which has been observed in CSF metabolomic analysis of AD patients [31]. The above results might imply higher oxidative stress and decreased oxidative phosphorylation in the aged brain circulation, but further examination is required to validate our observations.

Our identified CSF metabolic alterations are somewhat different from previous observed metabolic alterations in patients with neurodegenerative diseases such as AD and PD. In a case-control study comparing AD patients and cognitively healthy controls with paired plasma and CSF samples, they found dysregulated systemic energy metabolism, CNS-specific tryptophan pathway and creatinine alterations in AD [31]. Their results showed higher creatinine level in CSF of AD patients, but the levels of several AAAs (tryptophan and phenylalanine) and BCAAs (isoleucine and leucine) had significant negative associations with CSF amyloid pathology [31]. Another case-control study in AD patients using PET and CSF lactate levels to evaluate brain energy metabolism also found increased CSF lactate levels and reduced glucose consumption in AD patients [32]. Regar-

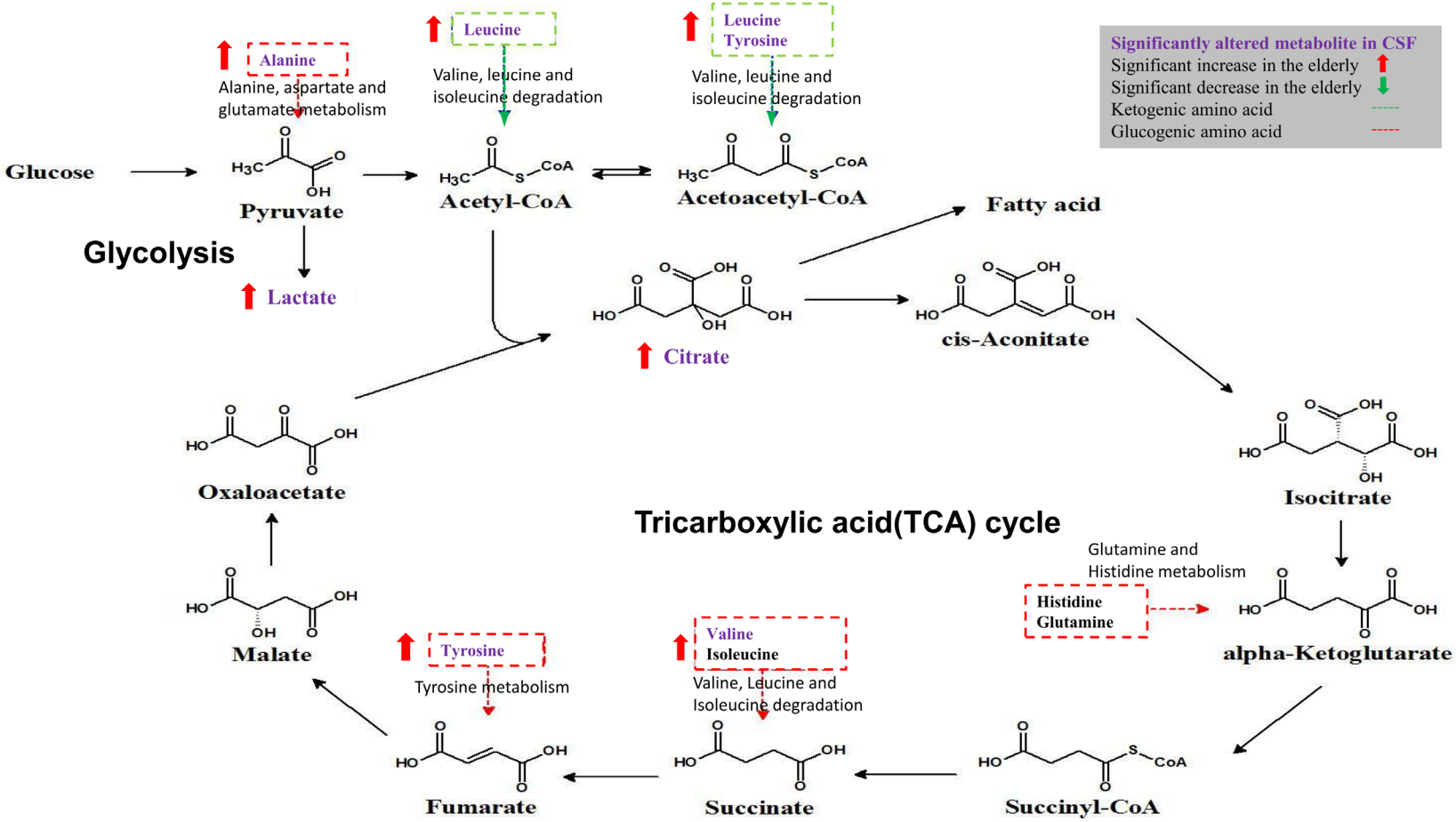

Figure 6. Schematic representation of significant CSF metabolite alterations and involved metabolic pathways in aging process.

ding PD, small case-control studies comparing PD patients and healthy controls found decreased alanine, lactate, mannose, and creatinine levels but increased isoleucine, ketoleucine, and leucine in CSF of PD patients, which is different from what we found in aging population [33-35]. BBB breakdown might explain the partial alignment of our findings with previous CSF metabolomics in AD. Using high-resolution MRI and CSF/plasma albumin ratio as measurements of BBB integrity, BBB breakdown has been identified in patients with advanced age and patients with AD [8, 36]. However, the integrity of BBB is not verified in this CSF metabolomic study and it will be examined in our further project.

To the best of our knowledge, this CSF metabolomic study is the first to profile the metabolomic signature of health aging in cerebral circulation in an Asian population, and our metabolomic analysis has expanded case numbers and case information compared to previous report on CSF metabolomics of healthy aging [22]. Our CSF sampling methodology during routine anesthesia procedure enabled us to detect real-time metabolite alterations in the cerebral circulation without additional discomfort or risks. Additionally, we minimized potential bias by excluding patients with diabetes and obesity and adjusting for significant confounders.

However, this study had several limitations. First, we only collected CSF samples from the study participants, so we could not obtain further information from other samples, such as plasma. Second, our enrolled participants had significant between-group differences in sex, BMI, serum creatinine, and chronic diseases, which might compromise our analysis even though we adjusted for these confounders. Besides, due to the complex nature of aging process and many factors that could affect the CSF metabolome, such as socioeconomic circumstances, diet, lifestyle, sleeping, and medications, our results require further validation in larger cohort or longitudinal study to eliminate these confounders. Third, there might be sex-related differences in the aging process, but we could not address this hypothesis in the current study design and that issue will be explored in a further analysis [37, 38]. Moreover, our results of CSF profiling of the aging process might reflect the combined effects of metabolic dysfunction, decreased CSF turnover, and BBB breakdown in the elderly, but we could not quantify these variables due to methodological limitations [39, 40]. Our heterogeneous result compared to earlier publications might be due to different patient selection, technical differences, and population limitations. Therefore, future multi-omics studies or larger cohort in different populations are required to validate our results and to establish possibly causal relationships in the aging process.

Conclusion

In this prospective cohort of metabolomic profiling of human CSF samples from cognitively unimpaired patients in the Taiwanese population, we presented novel insights into metabolic dysregulation in cerebral circulation during aging. The dysregulated metabolites in CSF samples in our study are involved in amino acid metabolism, energy metabolism, and synaptic transmission. The profiled metabolic changes during the aging process might imply enhanced anaerobic glycolysis, mitochondrial dysfunction, and higher oxidative stress in the cerebral circulation. Furthermore, a combined alteration of citrate, lactate, leucine, tyrosine, and valine displayed a superior correlation with the aging process in cerebral circulation, which may deserve further investigation for causal relationships.

Acknowledgements

The work was partially funded by grants from the Ministry of Science and Technology (MOST 108-2314-B182A-059-MY2) and Chang Gung Memorial Hospital (CMRPG3J1561-2). The authors wish to thank the statistical assistance and the support of Clinical Informatics and Medical Statistics Research Center, Chang Gung University for the study analysis and data interpretation. The metabolomics analysis using NMR spectroscopy was carried out at the Metabolomics Core Laboratory, Healthy Aging Research Center (HARC), Chang Gung University, and Clinical Metabolomics Core Laboratory, Chang Gung Memorial Hospital.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Fu-Chao Liu, Department of Anesthesiology, Chang Gung Memorial Hospital, 5 Fu-Shin Street, Kwei-Shan, Taoyuan 333, Taiwan. Tel: +886-3-3281200 Ext. 2324; Fax: +886-3-3281200 Ext. 2793; E-mail: ana5189@cgmh.org.tw

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