Comparative blood and urine metabolomics analysis of healthy elderly and young male Singaporeans

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ABSTRACT:

Comparative metabolomics analysis of biofluids could provide information about the metabolic alterations in aging. To investigate the signature of multiple metabolic profiles associated with aging in an Asian population, we performed a pilot study in healthy Singaporeans, including 33 elderly and 33 young males. Fasting whole bloods were analysed by routine haematology; the serum and urine metabolome profiles were obtained using NMR-based non-targeted metabolomics analysis and targeted lipoprotein analysis.

Among the 90 identified compounds in serum and urine samples, 32 were significantly different between the two groups. The most obvious age-related metabolic signatures include decreased serum level of albumin lysyl, essential amino acids and derivatives, but increased level of *N*-acetyl glycoproteins and several lipids; elevated urine levels of TMAO, *scyllo*-inositol, citrate, and ascorbic acid, but decreased levels of several amino acids, acetate, etc. Among 112 lipoprotein subfractions, 65 were elevated, 2 were lower in the elderly group. These significantly age-varying metabolites, especially in the amino acid and fatty acid metabolism pathways, suggest the regulation of these pathways contributes to the aging process in Chinese Singaporeans. Further multi-omics studies including the gut microbiome and intervention studies in a larger cohort are needed to elucidate the possible mechanisms in the aging process.

KEYWORDS: Comparative metabolomics analysis, NMR, aging, lipoprotein

■ INTRODUCTION

Increasing lifespan without addressing aging associated health issues causes many socio-economic problems because aging is often accompanied with chronic disease, such as diabetes, hypertension and cardiovascular disease etc [1]. In recent years, attention has focused on the

development of approaches for health-span extension, including studies of genetics, epigenetics, diet and lifestyle [1]. Metabolomics, the universal detection and study of metabolites in a specific biological system, has become a powerful tool for unveiling metabolic details and screening important biomarkers of diseases, such as cancer, diabetes and cardiovascular disease [2]. However, more evidence on the signatures of metabolic alterations in relation to the normal aging process before disease diagnosis is needed for the design of anti-aging interventions. Although most metabolomics studies are conducted on disease models, several studies which have analysed biofluid samples such as blood, urine or faeces in mice or blood in humans, have reported specific metabolomic profiles that are affected by the aging process. For example, in a study in mice investigating the effect of age on both urinary and faecal metabolome, changes in crucial metabolic and signalling pathways were found, such as amino acids crucial for protein synthesis, short-chain fatty acids important for maintaining the intestinal microbiota, choline and betaine derivatives essential for cardiovascular health, and ketone bodies which are major modulators in liver health [3]. Another metabolomics murine study of plasma, liver and muscle showed that long chain acylcarnitines decreased, free fatty acids increased and amino acids decreased in the plasma of aged mice; in addition, glucose and fatty acid metabolism, and redox homeostasis pathways in both liver and muscle were affected by aging [4]. In a study analysing fasting serum samples from two human cohorts from UK and Germany (KORA F4 from Germany, n = 2162, age = 32–81 years; and TwinsUK from UK, n=724, age=19–82 years), the team identified eleven metabolites associated with aging in women and these included several acylcarnitines, diacyl phosphatidylcholines and sphingomyelins, which were linked to oxidative stress, cell morphology, fatty acid β-oxidation and vascular function [5]. To investigate the metabolic pathway regulating leukocyte telomere length, one of the most predictive biomarkers of aging, the team tested associations between 280 blood metabolites and leukocyte telomere length in both TwinsUK and KORA cohorts. Five novel metabolites in

blood, which belong to three different classes of lysolipids, gamma-glutamyl amino acids and xenobiotics, have been found to be independently correlated with leukocyte telomere length and functional measures of aging such as higher blood pressure and HDL cholesterol levels and poorer lung, liver and kidney function [6]. The change of these five metabolites are involved in the fatty acids metabolism alteration and oxidative stress increase, which suggests deregulation of these signalling pathways could be partly responsible for aging [6].

In a longitudinal metabolomics study of aging and sex, 2344 plasma samples from 1212 participants in the Wisconsin Registry for Alzheimer's Prevention (WRAP) study were analysed. Of the 1097 metabolites tested, 623 (56.8%) were found to be associated with aging, and included steroid lipids, fatty acid lipids and amino acids [7]. In the study investigating aging serum metabolome using two-time point data from KORA and CARLA studies, among the 122 metabolites analysed, five (C18, arginine, ornithine, serine and tyrosine) and four (arginine, ornithine, PC aa C36:3 and PC ae C40:5) significant metabolites were identified in women and men respectively [8]. In a metabolomics study carried out in 110 healthy Korean participants, plasma from elderly and young subjects were analysed using non-targeted chromatography–mass spectrometry (LC-MS) analysis. Alterations in fatty acid β-oxidation, glycerophospholipid metabolism, and sphingolipid metabolism were identified as significant metabolic pathways relevant to normal aging [9]. In a small cohort study conducted in 15 young and 15 elderly Japanese individuals, red blood cell and plasma metabolites were investigated using non-targeted LC-MS analysis. Fourteen blood compounds that are involved in ergothioneine, glycolysis, and methylation pathway were suggested to be aging-related [10].

Although metabolomic analysis is very promising for investigating mechanisms associated with aging, comprehensive information of human metabolic networks and how they are affected by aging and other factors is still not fully understood. Furthermore, most of the

reported human studies have been carried in Caucasian cohorts or only involved blood samples, and hence limit a full understanding of the human metabolic pool comprising complex transboundary relationships. To assist in the design of anti-aging interventions, a better understanding of the metabolic status of the rapidly increasing aging Asian population especially in Singapore, one of the fastest aging nations in the world, is urgently needed. Thus, in this study we aimed to conduct multiple metabolome comparisons in different biofluid samples from Singaporean cohort.

An NMR based non-targeted metabolomics platform was used as the first line screen to investigate urine and blood samples to obtain multiple metabolic profiles associated with aging in Singaporean males in our pilot study. Comparative analysis of the data from the urine and blood samples from elderly and young groups revealed changes in metabolic pathways such as amino acid and lipid metabolism. Moreover, lipoproteins play an important role in the pathogenesis of atherosclerosis and coronary heart disease. For example, LDL (Low-Density Lipoprotein) plays an import role in the development of atherosclerotic plaques, while HDL (High-Density Lipoprotein) plays an important role in reversing the cholesterol-transport pathway and thus provides protection from the development of atherosclerosis. Further understanding of their subclasses in the healthy elderly group will help to provide a better understanding of the effect of age on lipoprotein metabolism. Consequently, the comprehensive NMR based profiling for 112 lipoprotein subclasses were further analysed in this Chinese Singaporean cohort. Moreover, to better elucidate the metabolic pathways associated with aging, pathway analysis of differential metabolites in the two age groups and correlation analyses were conducted in the current study.

MATERIALS AND METHODS

Participants and sample collection

Healthy young (n=33; age 21-29) and generally healthy elderly (with controlled hypertension and lipid panel values) (n=33; above age 65; average age 69.3) Chinese male adults who had been lived in Singapore for at least 10 years were recruited at Nanyang Technological University and local elderly activity centres. The inclusion criteria were non-smokers and not obese with BMI<27.5 kg/m^2, with no history of diabetes, psychiatric/neurological disorders, or life-threatening diseases, and had not taken a course of antibiotics in the last 3 months. All participants gave their written informed consent. The study protocol was approved by the Institutional Review Board of the Nanyang Technological University (IRB approval number: IRB-2016-11-040).

In the dietary recall session, participants completed a 3-day dietary recall. An *ad libitum* meal was consumed by each participant. The Global Physical Activity Questionnaire developed by WHO [11] was used to screen participants' frequency and intensity of exercise and physical activity. METs (Metabolic Equivalents) were used to quantify physical activity level. Participants with METs<500 minus/week were categorised as "inactive", participants with METs>500 min/week were categorised as "active" in this study.

For blood sample collection, overnight fasting venous blood was drawn from each participant using the BD Vacutainer® tubes (BD, Singapore), including serum red cap tubes, SST II Advance K2-EDTA yellow cap tubes, and K2-EDTA lavender cap tubes by registered nurses or research staff certified for phlebotomy. Blood samples were centrifuged to separate plasma and serum according to manufacturer's instruction. One EDTA whole blood and one serum sample were sent to Quest Laboratories Pte Ltd (Singapore) for whole blood haematology determinations, lipid profile (total cholesterol, HDL- and LDL cholesterol, triglycerides),

immunohematology (wr-CRP) and glucose fasting analysis using Siemens ADVIA 1800 & ADVIA Chemistry XPT. Wilcox-rank test was used to calculate the p value. P values larger than 0.05 were reported as not significant. The remaining material was aliquoted and stored at -80°C freezer until further analysis.

For urine collection and storage, mid-stream urine was self-collected by the participant according to provided instructions. The collected urine samples were treated immediately in the lab with sodium azide (final concentration of 0.1%) to arrest bacterial growth prior to centrifugation at 2000g, 10 min at 4 ℃ to remove sediments. Aliquots (1ml) were stored at -80 ℃ prior to metabolomics analysis.

^{1}H NMR spectroscopy metabolomics analysis of serum and urine samples

Urine and serum samples were thawed at room temperature and prepared for analysis according to a published method with some modifications [12]. For each urine sample, a volume of 630 µl was mixed with 70 µl of urine buffer (1.5 M KH_2HPO_4, pH=7.4, 100% D_2O, 1.9 mM sodium azide and 5 mM trimethylsilyl[$^{2}H_4$] propionic acid (TSP) sodium salt). This mixture was centrifuged for 5 minutes at 13,000 g and 4ºC, and 600 µl of the supernatant was transferred into 5 mm outer diameter NMR tubes. ^{1}H NMR spectra were acquired at a 300K, on a Bruker NMR In Vitro Diagnostics Research (IVDr) system, using a standard pulse sequence with water suppression (noesypr1d, (RD)-90°-t1-90°-tm-90°- acquisition) at 600.13 MHz, a relaxation delay (RD) of 4 s, a mixing time (tm) of 10 ms, a 90º pulse set at around 11.3 µs, and 32 free induction decays (FIDs) using 64K data-points.

For each serum sample, a volume of 400 µl was mixed with 400 µl of serum buffer (0.075M NaH_2HPO_4, pH=7.4, 100% D_2O, 2 mM sodium azide and 0.08% TSP sodium salt). An aliquot (600 µl) of this mix was then transferred into 5 mm outer diameter NMR tubes. For each sample,

two ^{1}H NMR experiments were conducted at a temperature of 310 K. The one-dimensional (1D) NMR standard pulse sequence was used at 600.13 MHz, with a relaxation delay (RD) of 4 s, a mixing time (tm) of 10 ms, a 90° pulse set at around 10 μs, and 32 free induction decays (FIDs) using 64K data-points. The Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence with water presaturation, attenuated the broad signals from molecules with slow rotational correlation times such as the lipids and proteins which reduce resonance interferences in the spectrum. The CPMG pulse sequence (–RD-90°-(t-180°-t) n-ACQ) parameters were set up in the same way as those from the 1D NMR standard. In addition, the spin-echo delay (t) was set at 0.3 ms and the number of loops (n) was set at 128.

For all spectral acquisition, the FIDs were multiplied by an exponential factor to give a line broadening of 0.3 Hz, and were Fourier transformed to obtain the usual frequency spectrum (TOPSPIN 3.2 software, Bruker Biospin, Rheinstetten, Germany). The spectra were automatically phased, baseline corrected and calibrated using the TSP signal at δ 0 for urine samples, or the glucose signal at δ 5.23 for serum samples using a standard Bruker routine. The spectra were imported into MATLAB (version R2017a, the Math works, Natick MA, USA) and digitized to 20K data-points. The water peak resonance (δ 4.7-5.05) was removed from each spectrum and the spectra were aligned using an algorithm published by Veselkov et al [13]. The ^{1}H NMR spectra were normalised with the median fold change (MFC) prior to statistical analysis.

Statistical analysis: ^{1}H NMR data from serum and urine samples were analysed using a multivariate approach in SIMCA (SIMCA-P13 UMETRICS, Umea, Sweden). Principal Component Analysis (PCA) was performed to evaluate sample clustering behaviour and intra-group variation. Orthogonal Partial Least Square- Discriminant Analysis (OPLS-DA) was used to maximize the metabolic differences between the sample groups [14]. The OPLS-DA models

with a CV-ANOVA p-value<0.05 were predictive. ^{1}H NMR spectral peaks were also integrated using an in-house algorithm and Mann–Whitney–Wilcoxon (MWW) tests, and adjusted False Discovery Rate (pFDR) [15] were calculated.

Metabolite identification: Metabolite assignments in NMR data were performed by matching the chemical shifts and J-coupling information with NMR spectral databases (Human Metabolome Database) [16]. STOCSY (Statistical Total Correlation Spectroscopy) and 2-D ^{1}H-^{13}C and ^{1}H-^{1}H NMR experiments were also used to help the identification of unknown metabolites.

NMR Lipoprotein quantitation

A total of 112 lipoprotein subclass in serum were quantitated using the 600 MHz Bruker IVDr Lipoprotein Subclass Analysis B.I.LISA^{TM} (Bruker BioSpin GmbH, Germany) following Bruker's preparation and measurement protocols, whereby all the lipid signals acquired from standard serum 1D NMR spectra were decomposed into a set of 112 lipoprotein classes characterised by increasing density and decreasing size, namely: Very Low Density Lipoproteins (VLDL); Intermediate Density Lipoprotein (IDL); Low Density Lipoproteins (LDL); and High Density Lipoproteins (HDL), which also have a precursor-product relationship in the cascade of VLDL-IDL-LDL [17]. Parameters included cholesterol, phospholipids, triglycerides, apolipoprotein A1, A2, B and VLDL, IDL and LDL particle numbers. The main VLDL, IDL, LDL and HDL classes included five VLDL subclasses, six LDL sub-classes and four HDL-subclasses.

Pathway analysis and correlation analysis

To identify the most relevant pathways involved in the aging process, total detected metabolites and differential metabolites were submitted for pathway analysis in webtool MetaboAnalyst [18],

which combines results from powerful pathway enrichment analysis with the pathway topology analysis. Pearson correlations between albumin, N-acetyl glycoproteins with main metabolites in serum and urine, and main cytokines in blood were carried out.

The NMR data have been deposited to the MetaboLights [19] with the data set identifier MTBLS1553 (https://www.ebi.ac.uk/metabolights/MTBLS1553).

RESULTS

Aging is associated with whole blood haematology and lipid profile changes

The study population and clinical characteristics are shown in Table 1. To exclude the influence of gender, ethnicity and environment on metabolomic profiles, only healthy Chinese male adults who has been living in Singapore for at least 10 years, were recruited in this pilot study. A total of 33 elderly and 33 young ethnic Chinese subjects were recruited. As shown in Table 1, the clinical results of fasting blood samples are mostly within normal range. It was observed that elderly subjects had higher levels of cholesterol, triglycerides, glucose and wide range C-reactive protein (wr-CRP) compared to the healthy young subjects. In addition, some parameters in whole blood haematology were higher in elderly subjects as compared to young, such as MCV (mean corpuscular volume), neutrophils, MCH (mean corpuscular hemoglobin), RDW (red blood cell distribution width). In contrast, RBC (red blood cells) and lymphocytes were slightly lower in the elderly. Systolic and diastolic blood pressure increased with age. Systolic blood pressures of some elderly men showed signs of prehypertension and hypertension. Total cholesterol and LDL-cholesterol for several elderly subjects were slightly higher than the desirable range [20], but no hypercholesterolaemia was found in the current cohort.

^{1}H NMR spectra of serum and urine sample

In total, 90 metabolites and proteins were assigned in serum and urine samples. Their chemical shifts and peak multiplicity are listed in Table S1. Among the metabolites identified, 4-deoxythreonic acid, *scyllo*-inositol, pseudouridine, sumiki's acid and α/β GlcNAc were

Table 1 Study population and clinical characteristics

	Elderly	Young	P value
Sample size	33 men	33 men	
Age	69.3(3.65)	23.12(1.32)	<0.001
BMI (kg/m²)	23.33(2.46)	21.44(2.44)	0.002
Daily Physical Activity Level (MET score)	740(865.08)	1311.64(1357.12)	0.080
Systolic blood pressure (mmHg), Normal range 90-120	131.57(14.43)	113.73(8.56)	<0.001
Diastolic blood pressure (mmHg), Normal range 60-80	76.06(10.01)	65.37(7.12)	<0.001
Fasting Glucose (mmol/L), Normal range 3.9-6.0	4.93(0.64)	3.99(0.73)	<0.001
Triglycerides (mmol/L), Desirable range < 2.26	1.19(0.44)	0.79(0.30)	<0.001
Total cholesterol (mmol/L), Desirable range < 5.18; High ≥ 6.2	5.25(0.82)	4.74(0.80)	0.009
LDL-cholesterol (mmol/L), Desirable range < 3.37	3.19(0.78)	2.88(0.74)	0.074
HDL-cholesterol (mmol/L), Desirable range > 1.03	1.51(0.34)	1.51(0.31)	0.995
Total Cholesterol/HDL, Desirable range < 4.51	3.62(0.87)	3.24(0.67)	0.119
Haemoglobin (g/dL), Normal range 13.5-18.0	14.68(1.21)	15.06(0.72)	0.158
RBC ($\times10^{12}$/L), Normal range 4.5-6.5	4.82(0.45)	5.2(0.610)	0.004
TWBC($\times10^{9}$/L), Normal range 4.0-11.0	6.09(1.46)	5.41(1.20)	0.047
Neutrophils (units), Normal range 2.00-7.50	3.63(1.09)	2.63(0.74)	<0.001
Lymphocytes (unit), Normal range 1.50-4.00	1.73(0.50)	2.06(0.62)	0.022
Monocytes (unit), Normal range 0.20-0.80	0.50(0.18)	0.45(0.15)	0.317
Eosinophils (unit), Normal range 0.04-0.40	0.18(0.16)	0.23(0.16)	0.114
Basophils (unit), Normal range <0.21	0.03(0.03)	0.04(0.03)	0.117
Haematocrit (PCV), Normal range 0.40-0.54	0.44(0.03)	0.45(0.02)	0.234
MCV (fL), Normal range 78-98	91.64(5.25)	86.73(7.22)	<0.001
MCH (pg), Normal range 27-32	30.61(1.94)	29.24(2.69)	0.002
MCHC (g/dL), Normal range 31-36	33.42(0.82)	33.55(0.99)	0.651
RDW (%), Normal range 11.0-15.5	13.52(0.90)	12.98(0.97)	0.002
Platelets (unit), Normal range 140-440	236.21(58.55)	244.88(51.40)	0.445
wr-CRP (mg/L)	1.41(1.71)	0.39(0.57)	<0.001

Notes: Results are given as mean (standard deviation). BMI: Body mass index. MET: metabolic equivalent of task. HDL: high-density lipoprotein; LDL: low-density lipoprotein. RBC: red blood cells. TWBC: total white blood cells. MCV: mean corpuscular volume. MCH: mean corpuscular haemoglobin. MCHC: mean corpuscular haemoglobin concentration. RDW: red blood cell distribution width. wr-CRP: wide range C-reactive protein (Less than 1.0 mg/L Low Risk for CVD; Between 1.0-3.0 mg/L Average Risk for CVD; Greater than 3.0 mg/L Increased Risk for CVD; Greater than 5.0 mg/L Suggestion of infection/other sources of inflammation). Note: P value was calculated based on Wilcox-rank test. P values larger than 0.05 were reported as not significant.

identified by spiking standards in the urine samples. For serum and urine metabolite profiles, both multivariate analysis OPLS-DA and univariate analysis Mann–Whitney–Wilcoxon (MWW) tests were performed to evaluate the metabolic differences between the elderly and young groups. Raw p values and adjusted FDR p values were also calculated.

Serum metabolomics analysis in elderly and young group

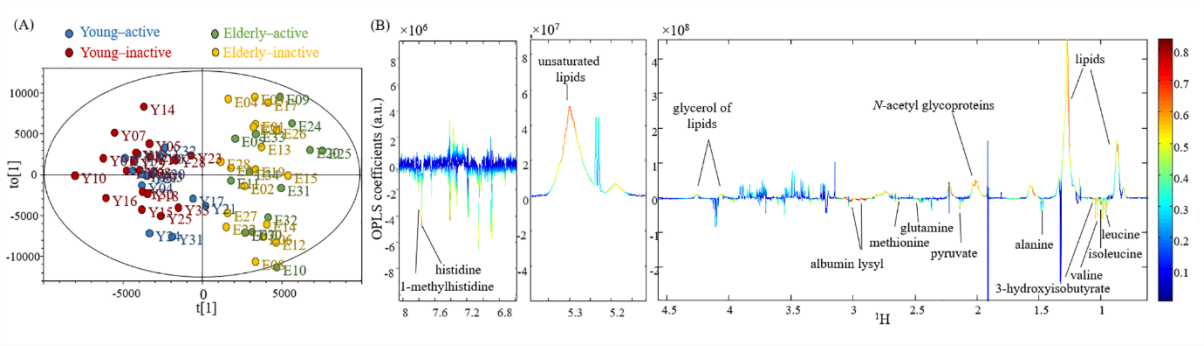

Figure 1. OPLS-DA Scores plots and loading plot of serum samples. A: OPLS-DA Scores plots of serum samples from young and elderly cohort. CV-ANOVA $p = 1.49 \times 10^{-14}$. 7-fold cross validation: R2Y(cum): 0.925, Q2(cum): 0.7. B: Loadings plot of OPLS-DA model of serum samples. Loadings plot displays variables responsible for separation in the score plot. The absolute values of OPLS correlation coefficients were color-coded. Positive peaks correspond to metabolites that have higher levels in elderly group compared to young group, and negative peaks correspond to metabolites that have lower levels in the elderly group compared to young group.

As shown in Figure 1, OPLS-DA analysis of ^{1}H NMR Carr-Purcell-Meiboom-Gill (CPMG) serum metabolome significantly discriminated between the elderly and young groups. The

compounds responsible for the discrimination are listed in Table S2. Based on Figure 1 and Table S2, the serum metabolome of elderly adults was characterized by higher levels of *N*-acetyl glycoproteins (NAG), lipids, glyceryl of lipids and unsaturated lipids. The serum metabolome of the elderly also showed lower levels of amino acids and organic acids including branched-chain amino acids (BCCA: isoleucine, valine, leucine), histidine, alanine, 1-methylhistidine, glutamine, methionine, 3-hydroxyisobutyrate, pyruvate, and protein albumin lysyl. As shown in Figure 1, there is not much difference between active and inactive subgroups in each age group.

Urine metabolomics analysis in elderly and young groups

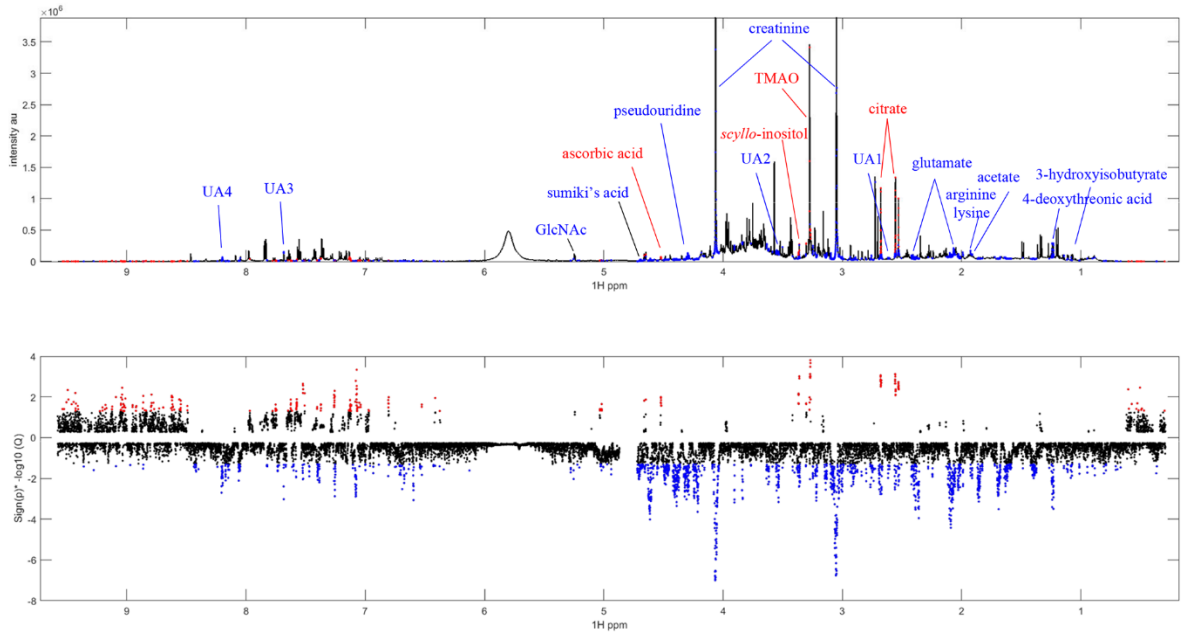

Figure 2. Urinary metabolome comparison between elderly and young subjects identified by ^{1}H NMR. This Manhattan plot shows the analysis of obtained NOESY features from urine samples. The signed negative log10 p-value is plotted against the chemical shift in ppm. The horizontal dashed line indicates the FDR correction significance level. FDR cut off value: 0.05. Red labelled peaks are the metabolites which have higher levels in the elderly group compared to young group, blue labelled peaks show the metabolites which have lower levels in the elderly group compared to young group.

Although multivariate data analysis was not able to discriminate the urine metabolome obtained from young and elderly participants, analysis using MWW test with FDR correction suggested a range of metabolites that were different between the young and elderly urine metabolome as shown in Figure 2 and Table S3. Based on the identified NOESY spectra, the urinary metabolome of elderly adults was characterised by higher levels of TMAO, *Scyllo*-inositol, citrate, and ascorbic acid, as well as relatively lower levels of creatinine, pseudouridine, 4-deoxythreonic acid, 3-hydroxyisobutyrate, arginine, lysine, acetate, glutamate, Sumiki's acid, N-acetylglucosamine (α/β GlcNAc), and unassigned metabolites UA1 to UA4.

Detailed characterisation of the lipoprotein profile alterations associated with aging

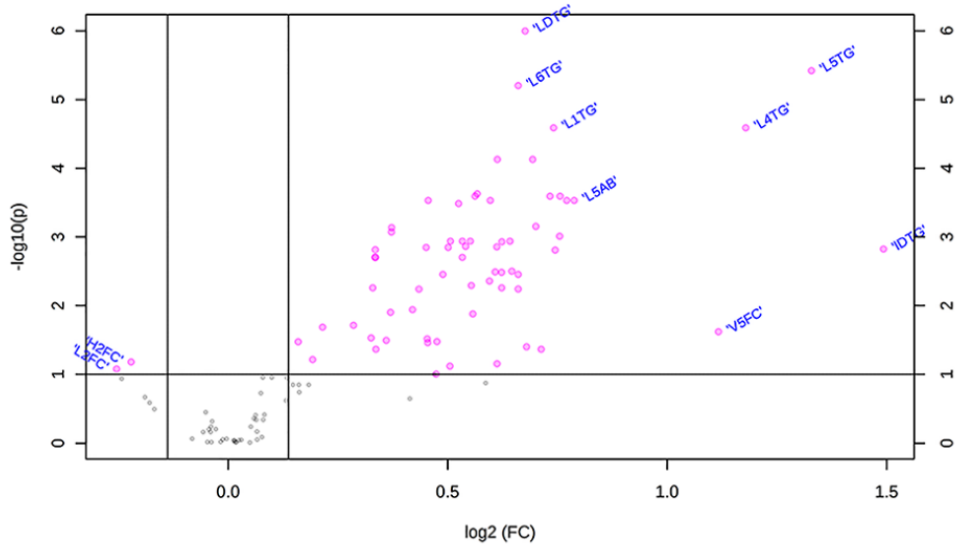

Figure 3. Volcano plot analysis of 112 lipoprotein subfractions between elderly and young. X-axis: fold change (elderly/young) threshold was set as 1.1, Y-axis: Wilcox rank test. FDR-adjusted P-value threshold was set as 0.1. H2FC: HDL-2-Free Cholesterol, L2FC: LDL-2-Free Cholesterol, LDTG: LDL-Triglycerides, L1TG: LDL-1-Triglycerides, L4TG: LDL-4-Triglycerides, L5TG: LDL-5-Triglycerides, L6TG: LDL-6-Triglycerides, L5AB: LDL-5-Apo-B, V5FC: VLDL-5-Free Cholesterol, IDTG: IDL-Triglycerides. Full list of names and relevant parameters are listed in Table S5.

From the one-dimensional NMR spectra, lipid signals were amongst the most discriminatory signal associated with aging, with the elderly having higher serum levels of most lipid signals relative to the young subjects (Figure 1). Changes in the lipid profile were also observed in the clinical characteristics (Table 1). These differences imply that lipoprotein metabolism potentially contributes to aging in this cohort. To further test the lipoprotein profile associated with aging, additional lipoprotein subfraction distribution analyses were conducted using the Bruker IVDr Lipoprotein Subclass Analysis B.I.LISA^{TM}. The full list of 112 lipoprotein subclasses is shown in supplement Table S4. In addition, Bruker NMR-detected triglycerides, cholesterol, HDL- and LDL-cholesterol significantly predicted the clinical results ($R^2 \sim 0.9$), which reflects the accuracy of the Bruker methodology (Figure S1).

Volcano plot analysis (Figure 3 and Table S5) showed that among the 112 lipoproteins identified, 65 subfractions were significantly higher in the elderly group, while 2 subfractions were significantly lower in the elderly group. Among the 7 main parameters (Triglycerides, Cholesterol, LDL-cholesterol, HDL-cholesterol, Apo-A1, Apo-A2 and Apo-B100), Triglycerides, Cholesterol, LDL-cholesterol, Apo-B100 and Apo-B100/Apo-A1 all showed higher levels in the serum of elderly, which is consistent with the clinical characteristics (Table 1).

Triglycerides distribution in HDL (HDTG), IDL(IDTG), LDL (LDTG) and VLDL (VLTG) and their subfractions HDL-1 to 4, LDL-1 to LDL-6, VLDL-1 to VLDL-5 were all higher in the elderly group (Table S5). In addition, the total concentration of ApoB carrying particles including total VLDL, LDL and IDL particles (TBPN, VLPN, LDPN, IDPN) all showed higher levels in the elderly serum, which is consistent with the increase of total Apo-B100 and Apo-B100 in VLDL, LDL and IDL. For cholesterol distribution, the total cholesterol, LDL-cholesterol, IDL-Cholesterol, VLDL-Cholesterol and VLDL-free Cholesterol were all higher

in the elderly. The subfraction of LDL and VLDL: LDL-4 to LDL-6, VLDL-1 to VLDL-5 all had a higher level of cholesterol and/or free cholesterol. However, the subfraction of LDL-2-free cholesterol (L2FC) in the elderly was lower than that for the young adults. Free cholesterol distribution in HDL-2 (H2FC) was observed to be less in the elderly. As for the change of phospholipids distribution, it was observed that the elderly had higher levels of phospholipids in IDL, LDL and VLDL, and in subfractions LDL-4 to LDL-6, VLDL-1 to VLDL-5.

Pathway analysis

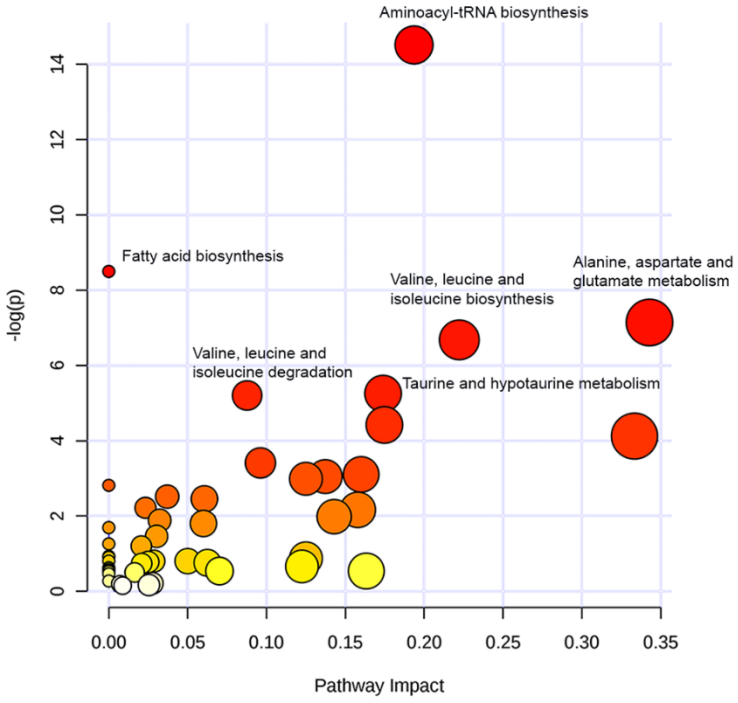

Figure 4. Pathway topology analysis in association with aging. Differential metabolites between elderly and young were analysed. Bubble area displays the impact of each pathway, with colour representing the significance from highest in red to lowest in white.

The metabolites that differed between the elderly and young groups were subjected to pathway analysis using MetaboAnalyst [18]. Pathway topology analyses with -log(P) are shown in Figure 4, the P values and pathway impact in association with Figure 4 are shown in supplementary

Table S6. As shown in Figure 4 and Table S6, six metabolic pathways (Raw p value <0.01), including aminoacyl-tRNA biosynthesis; fatty acid biosynthesis; alanine, aspartate and glutamate metabolism; BCCA biosynthesis; taurine and hypotaurine metabolism; valine, leucine and isoleucine degradation were revealed to be the most important pathways associated with the metabolic changes in the elderly group. These alterations in metabolic pathways can provide us some insights into the mechanisms involved in the aging progression.

■ DISCUSSION

In the present study, NMR based metabolomics and lipoprotein analysis were used as a first line screen to compare metabolomic profiles in serum and urine between an elderly and young Chinese male cohort in Singapore. Although the number of participants was relatively small, separation of the metabolome and lipoprotein profiles between elderly and young groups was evident. There was no observable significant difference between active and inactive subgroups in elderly and young cohort for metabolome and lipoprotein profiles, possibly due to the small sample size. The observed changed metabolites are involved in several pathways mainly including amino acid and fatty acid metabolism. Biological alterations in the aging process that lead to the metabolic changes were explored and discussed with reference to common aging theories, such as skeletal muscle wasting [21], cellular replicative senescence [22], telomere shortening, accumulation of glycated proteins (AGEs) [23].

Comparison of serum metabolome and association of amino acid and protein metabolism with aging

As identified by pathway analysis, several amino acid metabolic pathways were significantly different between elderly and young subjects, including proteinogenic amino acids metabolism (BCCA, alanine, aspartate and glutamate) and non-proteinogenic amino acids taurine and

hypotaurine metabolism. This is consistent with other reports that aging is associated with changes in amino acid and protein metabolism. For example, histidine in serum has been reported to decrease with age in KORA F4 Germany and TwinsUK cohorts [5]. Leucine and isoleucine was detected in lower levels in the elderly in a small healthy cohort in Japan [10]. Leucine was also shown to decrease with age in plasma samples in the WRAP study [7]. It was observed that serum levels of albumin in the elderly group were lower than that in the younger group in healthy Korean cohorts [9]. In agreement with these reported studies, we observed lower levels of all three BCAA in serum samples of elderly subjects as compared to young subjects. It has been well studied that dietary BCAA are absorbed from the intestines, their catabolism occurs initially in skeletal muscles and followed by further degradation in liver, kidneys, heart, muscle, adipose tissue and brain [24]. They not only serve as substrates for protein synthesis, energy production, but also regulate mRNA translation, simulate protein synthesis, inhibit proteolysis [24]. Low levels of BCAA can result in impaired growth and protein wasting [25], and especially leucine and isoleucine play a distinct role for supporting skeletal muscle activity in the elderly. Thus, their decrease in elderly serum as compared to young ($P < 0.001$, Table S2) in the current study suggests their decrease in blood probably be associated with muscle wasting during aging. The lower level of BCAA and other proteinogenic amino acids (alanine, aspartate, glutamate, histidine and methionine) are in accordance with the lower level of albumin in elderly subjects in the current study. This observation is consistent with the reported lower level of albumin in elderly in a non-targeted metabolomics study carried out in 110 healthy Korean participants [9]. The concurrent decrease of proteinogenic amino acids and albumin in the elderly group indicates a decreased protein synthesis associated with aging. As shown in Table 2, albumin lysyl strongly and positively correlated with amino acids such as histidine, 1-methylhistidine, glutamine, 3-hydroxyisobutyrate and methionine ($r > 0.5$); moderately and positively correlated with valine, leucine ($0.3 < r < 0.5$), which further supports

the correlation between amino acid and albumin in serum. Since the initial step of BCAA catabolism in muscles releases glutamine and alanine into blood [24], it is reasonable to observe lower levels of glutamine and alanine in the elderly as seen in the current study. Since 3-hydroxyisobutyrate is an intermediate in the valine degradation, the lower level of it in the elderly is also in accordance with lower level of valine.

Table 2. Compounds correlated with the albumin lysyl

Compounds	correlation	p-value	FDR
histidine	0.69417	3.94E-10	8.67E-09
1-methylhistidine	0.67249	2.15E-09	2.40E-08
TG	-0.67229	2.19E-09	2.40E-08
glutamine	0.58337	6.49E-07	5.71E-06
glyceryl of lipids	-0.51439	1.90E-05	0.000123
3-hydroxyisobutyrate	0.5137	1.95E-05	0.000123
creatinine	0.50428	2.93E-05	0.000155
methionine	0.50236	3.18E-05	0.000155
N-acetyl glycoproteins	-0.47426	9.86E-05	0.000434
valine	0.45838	0.000179	0.000717
Apo-B100	-0.45017	0.000242	0.000886
leucine	0.39847	0.001338	0.00429
Apo-B100/Apo-A1	-0.39782	0.001365	0.00429
IL-8	-0.35189	0.005036	0.014772
acetate	-0.34561	0.005936	0.016324
4-deoxythreonic acid	0.33318	0.008142	0.021072
IL-7	-0.32166	0.010791	0.026379
citrate	-0.30807	0.014855	0.0344

In addition to the role of proteinogenic amino acids, one of the main histidine metabolic pathways is to form the dipeptide carnosine (β-alanyl-L-histidine) with alanine. Carnosine has antioxidant properties and is able to suppress oxidative damage, protein glycation, and scavenging of toxic age-related molecules [26]. It is therefore not surprising that there are lower levels of histidine in elderly subjects in our study. Similarly, methionine serves as a sulphur source and principal methyl donor. It is important in detoxification reactions and serves as a precursor for the synthesis of cysteine and glutathione [27]. Cysteine is an important precursor

for taurine [28] which has many physiological functions, including reducing apolipoprotein B100 and lipids. Glutathione is a crucial antioxidant that can reduce oxidative stress in the human body [29]. It is therefore interesting to note that the lower level of methionine in the elderly in our study is consistent with the lipid profile.

The effect of BCAA on improving glucose consumption and utilization has been widely demonstrated [30]. The lower levels of BCAA in the serum of elderly subjects are consistent with higher glucose fasting levels in elderly serum. In addition, the BCAA level is affected by the dietary protein intake, but we didn't observe significant differences in dietary protein intake percentage and total calorie intake in this study. In our previous study, it has been shown that calorie intake index exerts a dominant effect on metabolic perturbations irrespective of dietary regime [31]. Since there was no observable significant difference in protein and total calorie intake, the amino acid metabolism difference may be attributable to the host physiological metabolism associated with aging. Moreover, since only healthy male elderly and young Singaporeans were recruited, we assume that the observed metabolic changes between elderly and young groups play an important role in the general aging process.

Since 1-Methylhistidine is a biomarker for the consumption of meat [32], the lower level of 1-Methylhistidine in elderly subjects indicates a lower habitual meat consumption, which highlights the need for a detailed food frequency questionnaire in future work. Pyruvate is the end product of glycolysis, and a key intermediate in several metabolic pathways in cells. Aberrant pyruvate metabolism plays an prominent role in several human diseases [33]. The change of pyruvate levels in elderly indicates the reprogramming of energy supply and metabolism in the aging process [34].

In addition to the muscle wasting in aging, theories of aging also include the accumulation of glycated proteins (AGEs) [23]. GlycA levels, in units of μmol/L glycoprotein *N*-acetyl methyl

groups, have been reported to be associated with the concentrations of inflammatory cytokines such as IL-6, tumour necrosis factor (TNF)-α and C-reactive protein [35]. Consequently, GlycA is suggested as a clinical biomarker of systemic inflammation, and has been associated with cardiovascular disease risk and cancer [35]. In our current healthy cohort, we observed significant elevated level of *N*-acetyl glycoprotein (NAG) in the elderly group. In fact, there was a strong positive correlation between NAG and Apo-B100/Apo-A1, glyceryl of lipids, TG, LDL-Chol/HDL-Chol ($r>0.5$), and a strong negative correlation between NAG and HDL-Chol ($r<-0.5$). There was a moderate positive correlation between NAG and wr-CRP, IL-7, IL-8, TNF-α, Apo-B100 ($0.3<r<0.5$), moderate negative correlation between Apo-A1, albumin lysyl, glutamine ($-0.5<r<-0.3$) (Table 3). These data indicate an association between NAG and the change of lipid and protein metabolism and systemic inflammatory responses in aging.

Table 3. compounds correlated with the *N*-acetyl glycoprotein

Compounds	correlation	p-value	FDR
HDL-Chol	-0.61871	6.51E-08	1.22E-06
Apo-B100/Apo-A1	0.61482	8.29E-08	1.22E-06
glyceryl of lipids	0.56655	1.30E-06	1.25E-05
TG	0.56475	1.43E-06	1.25E-05
LDL-Chol/HDL-Chol	0.55764	2.06E-06	1.51E-05
Apo-A1	-0.48587	5.42E-05	0.000341
albumin lysyl	-0.47073	9.89E-05	0.000544
IL-8	0.41034	0.000837	0.004093
Apo-B100	0.40211	0.001088	0.004786
glutamine	-0.36647	0.003136	0.012545
TNF-α	0.34717	0.005309	0.019465
wr-CRP	0.32522	0.009301	0.03148
IL-7	0.31931	0.010745	0.033771
citrate	0.29393	0.019377	0.056838
acetate	0.28984	0.021215	0.058341
4-deoxythreonic acid	-0.27079	0.031828	0.082378

Comparison of urine metabolite profiles between elderly and young group

Lower levels of proteinogenic amino acids were observed in elderly urine samples, including arginine, lysine, glutamate, and intermediate 3-hydroxyisobutyrate. This is associated with the decreased amino acid metabolism in elderly subjects. In fact, several important biomarkers differed between elderly and young subjects, for example, trimethylamine *N*-oxide (TMAO) which is a marker of atherosclerosis, cardiovascular disease and metabolic syndrome [36]. High urinary excretion of TMAO is associated with insulin resistance and metabolic syndrome [36]. In addition, it is also a gut microbiota dependent metabolite, and inhibition of trimethylamine production from gut microorganisms has been suggested for the treatment of atherosclerosis and cardiometabolic diseases [37]. Further investigation of its metabolism in the gut and its role in host–microbiota metabolic axis could prove valuable for anti-atherosclerosis strategies. *Scyllo*-inositol levels in the brain has been shown to increase with aging [38]. In our study, *scyllo*-inositol levels were higher in urine samples from the elderly.

Ascorbic acid (Vitamin C) is an essential nutrient in the human diet. It is considered an antioxidant and functions as a reducing agent and coenzyme in several metabolic pathways. Since humans can't synthesize ascorbic acid, the high ascorbic acid level in elderly subjects in the current study is assumed to be due to the intake of Vitamin C containing food or supplements. Citric acid (citrate) which is a key metabolite of energy metabolism is formed in the tricarboxylic acid cycle and is obtained from foods and supplements. It is also an intermediate in cellular oxidative metabolism [39]. The higher level of citrate in elderly compared to the young in the current study can therefore be attributed to the difference in diet and host metabolism.

Creatinine is a breakdown product of creatine phosphate during muscle use or meat consumption and can be considered as a muscle index. Lower level of creatinine in elderly is

associated with decline in muscle index. However, we cannot rule out the contributions of decreased glomerular filtration rate (GFR) in the decreased urinary creatinine level in elderly subjects as previously reported [40]. Moreover, another potential biomarker of kidney function [41], pseudouridine, was also detected in lower levels in current elderly group, and this is consistent with the decreased level of creatinine.

Several important beneficial metabolites were detected in lower levels in elderly urine samples in our study, such as *N*-acetylglucosamine, which is a monosaccharide derivative of glucose with an important role in cell signalling [42], anti-oxidant and immune- modulating effects [43], and is a widely accepted treatment for osteoarthritis [44]. Acetic acid is one of the main short chain fatty acids (SCFAs) produced by gut microbiota and is the only SCFA that circulates systemically in the host in significant amounts. It plays a role in reducing the risk of heart disease and hypercholesterolemia [45]. It is therefore considered relevant that lower levels of acetate were detected in the urine samples from the elderly in our study.

Sumiki's acid (5-hydroxymethyl-2-furoic acid) in urine is a major metabolite of hydroxymethylfurfural (HMF). HMF is naturally generated in low concentrations in heated food, especially sugar-containing foods. Higher quantities of HMF are found naturally in coffee and dried fruit [46]. The concentration of Sumiki's acid would be related to individual food consumption patterns. As for 4-deoxythreonic acid, it is a normally occurring carboxylic acid in humans, and probably a product of L-threonine metabolism [47]. It is found in significantly elevated levels in patients with type 1 diabetes [47], but no report related to aging was found. It is therefore interesting to note the lower level of 4-deoxythreonic acid in this study and to suggest that it could be related to the overall change of amino acid metabolism, especially L-threonine metabolism.

Lipid metabolism associated with aging

In addition to the protein and amino acid metabolism, significant differences in lipid and lipoprotein metabolism were observed in this study. Lipids are essential for health, but increased cholesterol and triglyceride concentrations in blood are associated with an increased incidence of cardiovascular disease [48]. Lipoproteins, as transporters of lipids from intestine and liver to other tissues, are heterogeneous particles composed of proteins and lipids. Their distribution in the blood reflects the dynamic response of the host to external conditions such as diet, lifestyle and environment [49]. It has been consistently demonstrated that apolipoprotein B and LDL particle numbers are more accurate indices of cardiovascular risk as compared to LDL-C or non-HDL-C [50]. Thus, detailed characterisation of the lipoprotein profile was carried out in the current cohort to associate the lipoprotein change with the aging process. Elevated levels of triglycerides were observed in all HDL, IDL, LDL and VLDL and most of their subfractions in the elderly compared to the young subjects. In addition, increased cholesterol and phospholipids were also observed in IDL, LDL, VLDL and especially in the more pro-atherogenic small dense LDL particles.

Of the total discriminatory subfractions between the elderly and young subjects, most of the lipid subfractions (50/67) belong to the LDL and VLDL lipid class (Table S5), ranging from large, more light particles (LDL-1, VLDL-1) to small, dense ones (LDL-6, VLDL-5). For the LDL subfractions, LDL-4 to LDL-6 particle numbers were higher in the elderly subjects. This suggests an increased endogenous LDL lipoprotein metabolism, which can facilitate the transfer of triglycerides synthesized in the liver to muscle and adipose tissue, and transfer of cholesterol from the liver to peripheral tissues [17]. There was a trend towards an increase of cholesterol distribution in small dense LDL particles (LDL-4 to LDL-6), which are considered as more pro-atherogenic than large LDL particles [17], but less cholesterol distribution in large

light LDL-2 (L2FC) (Table S5). Although there is no significant difference in total HDL-cholesterol between elderly and young, free cholesterol in HDL-2 (H2FC) was lower in elderly subjects (Figure 3 and Table S5). In addition, a change of HDL structure in elderly was noted with higher levels of triglyceride in total HDL-triglyceride and all four HDL-subfractions in elderly. Triglyceride-rich HDL is the target of hepatic lipase, and hence could become smaller and unstable and even lose its apo-A, and thereby no longer able to reverse cholesterol transports [51].

These observations all imply that lipid and lipoprotein metabolic profiles could be sensitive indicators of aging. An association between lipid metabolism and aging, in which the serum concentrations of TG, and LDL in the elderly was higher than young group has been reported [9, 52], but the role of lipid metabolism in aging is still not well defined. Cellular senescence, a state of irreversible growth arrest, has been suggested as a cellular model of organismal aging. Senescent cells accumulate *in vivo* with increasing age and at sites of age-related pathology [53]. An accumulation of 19 polyunsaturated triacylglycerols in cellular replicative senescence state of BJ fibroblast cells has been reported [54]. It is suggested to be a cellular mechanism to prevent lipotoxicity under increased oxidative stress conditions and indicate that regulation of specific lipid species has a central role during replicative senescence. Similarly, increased triacylglycerols and changed lipid metabolism in the current elderly cohort could play a similar protective role.

Phospholipids are major components of cell membranes and higher levels were found in the elderly group in the current study. It is known that a common feature of cellular senescence is an increased cell surface [53]. Naru et al [55] found that *in vitro* senescent human dermal fibroblasts cells had increased levels of a particular phosphatidylcholine species. In another study, senescent human peritoneal mesothelial cells were found to store and secrete substantially more

phosphatidylcholines than young cells [56]. Our results are also in agreement with another large-scale targeted metabolic profiling study [5] in which there was a significant increase in specific phosphatidylcholines and sphingomyelins. It is suggested that the elevated serum levels of phospholipid in elderly is also involved in protection against oxidative stress [5], however, further investigation is needed to understand the mechanism involved. Detailed characterization of lipidomic and markers of senescence in a larger cohort will provide insight for the underlying mechanism.

Limitation and follow up investigation

Since the study focused on the metabolism of normal aging, obese subjects or those with a history of diabetes, psychiatric/neurological disease and other diseases were excluded. This strict exclusion criteria led to difficulties in recruiting completely healthy elderly individuals and hence the sample size is small [9]. Large-scale longitudinal studies are needed to confirm the signature of metabolic alterations in relation to aging in this pilot study.

Another limitation of this study is that it is largely descriptive in nature, which is characteristic of most metabolomics studies, since metabolomics is a tool for generating rather than validating a hypothesis. Further studies that focus on elucidation of the missing link between the differential metabolites and their biological functions, the causal effects of changes or their origins are essential. In addition, several metabolites that differed in the elderly when compared to the young subjects are produced by gut microbiota, especially amino acids, TMAO and SCFA. Consequently, metabolomic and metagenomic analysis of the gut microbiome would be useful to elucidate the biological function of these metabolites and correlate with other aging biomarkers in term of host–microbiota metabolic interactions.

Moreover, aging is a dynamic process that accumulates unfavourable structural and functional changes which lead to a progressive loss of physiological integrity of an organism. It is a complex phenotype due to the involvement of multiple factors, and involves an integrated network from genes, proteins and metabolites. Many of the theories proposed in the past decades interlinked with each other in one way or another and it is probable that each contribute in part to aging [57]. Thus, high throughput analysis of large-scale networks by multi-omics approaches will provide a deeper understanding of the processes that drive aging.

■ CONCLUSIONS AND FUTURE WORK

In summary, the comparative metabolomics analysis of blood and urine samples from elderly and young male Singaporeans revealed some age-related metabolic signatures, such as decreased serum level of albumin lysyl, several essential amino acids and their derivatives, but increased level of *NAG*, total lipids, glyceryl of lipids and unsaturated lipids, and the change of triglycerides, phospholipid, cholesterol distribution in the lipoprotein profile. In urine samples, there are elevated urine levels of TMAO, *scyllo*-inositol, citrate, and ascorbic acid, but relatively decreased levels of several amino acids and their derivatives, SCFA acetate, and other metabolite such as creatinine, pseudouridine, 4-deoxythreonic acid, sumiki's acid, *N*-acetylglucosamine. Pathway analysis showed significant difference in amino acids metabolism (BCCA, alanine, aspartate, glutamate, taurine and hypotaurine metabolism) and lipid metabolism.

This work not only provides an important reference dataset for understanding the relative variation in blood and urine metabolome of the Singaporean Chinese male population, but also adds to the growing evidence of the importance of amino acid and lipid metabolism in aging. In addition, the metabolome is related to diet choice and the gut microbiome. Further analysis associated with gut microbiome and dietary choice will be carried out. A systems

interconnected network analysis with combination of metabolomics, metagenomics and transcriptomics will enable a deeper understanding of these changes in the biological pathways with age and provide possible information that could assist in the design of anti-aging interventions strategies or preventive measures in the Singaporean Chinese population.

■ ASSOCIATED CONTENT

Supporting Information

The following supporting information is available free of charge at ACS website http://pubs.acs.org

Table S1. NMR signals assignment in serum and urine samples; Table S2. Metabolites discriminating elderly and young in serum samples identified by ^{1}H NMR in OPLS-DA model; Table S3. ^{1}H NMR urinary metabolites discriminating elderly and young group; Table S4. List of 112 subfraction of lipoprotein in Bruker IVDr Lipoprotein Subclass Analysis B.I.LISA™; Table S5. List of statistically significant serum lipoprotein subfractions (measured by B.I.LISA analysis) between elderly and young through volcano plot analysis; Table S6. Pathway analysis and the detected pathways using MetaboAnalyst; Figure S1. Linear regression analysis of the Bruker IVDr Lipoprotein Subclass Analysis B.I.LISA™ (in mg/dL) and clinical measurements (in mmol/L) of total triglycerides (TG) (A), total cholesterol (B), HDL-cholesterol (HDL-Chol) (C) and LDL-cholesterol (LDL-Chol) (PDF).

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Notes

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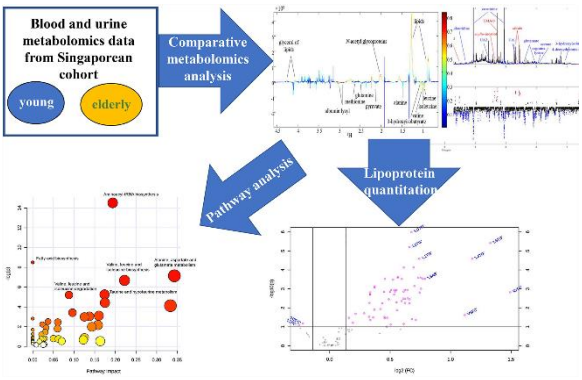
Blood and urine metabolomics data from Singaporean cohort
young
elderly
Comparative metabolomics analysis
Pathway analysis
Lipoprotein quantitation