lower for bradykinin. The levels of abovementioned seven metabolites in plasma and serum are shown in Fig. 2b.

To get insights into the difference in the metabolic profiles between plasma and serum, we counted the number of metabolites that were statistically different ($p < 0.05$) between plasma and serum in each group with different subject backgrounds (Fig. 2c). More than 100 metabolites showed significantly different levels between plasma and serum of all subject groups; among them, approximately 50 had more than 50 % changes, either higher or lower, in their levels (see Supplemental Table 3). Similar trends in numbers were observed for all four analyzed groups. Notable differences (i.e., more than twofold differences) between plasma and serum were observed for 24, 28, 31, and 21 metabolites in young males, old males, young females, and old females, respectively. Examples of these metabolites include aspartate, aspartylphenylalanine, glycerol-3-phosphate, and 2-palmitoylglycerol. To further understand pathway-based differences between plasma and serum, we identified the metabolic pathways whose components diverged the most between the two matrices by scoring the metabolites that were significantly different between the two. To do this, the number of statistically different metabolites in a pathway was divided by the total number of metabolites detected in the pathway, which was referred to as pathway occupancy. Figure 2d shows the average values of pathway occupancy for all four gender-age subject groups. The pathway occupancy of individual groups was almost the same as the average (data not shown). The pathways that displayed high levels of occupancy contain metabolites involved in blood coagulation, such as lysolipids (e.g., 1-stearoylglycerophosphoinositol), monoacylglycerols (e.g., 2-palmitoylglycerol), fatty acids

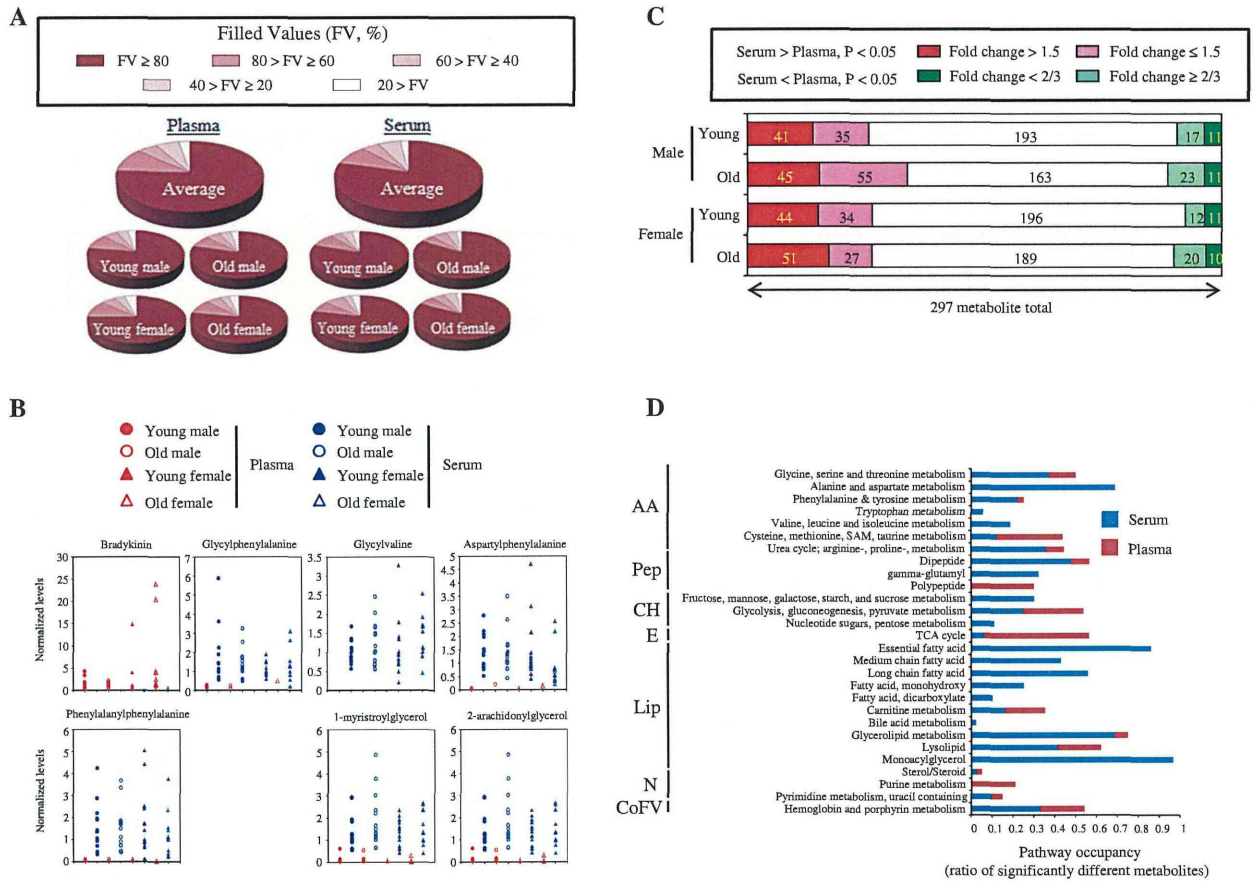

Fig. 2 Differences in the metabolite characteristics between plasma and serum. **a** The distribution of filled values (the percentages of metabolites detected in each subject group). Data obtained from plasma and serum are presented as the average values of all subject backgrounds or each subject background. **b** Metabolites showing significantly different levels between plasma and serum. Each *dot* represents the data of an individual subject. Data shown are human plasma (*red*) and serum (*blue*) samples from young males (*closed circle*), old males (*open circle*), young females (*close triangle*), and old females (*open triangle*). **c** The number of metabolites with statistically significant differences and with at least 50 % changes in their levels between plasma and serum. *Values within boxes* indicate the number of metabolites. **d** Pathway occupancy rates of statistically different metabolites between plasma and serum. *AA* amino acids, *P* peptides, *CH* carbohydrates, *E* energy metabolites, *Lip* lipids, *N* nucleotides, *CoFV* cofactors and vitamins. *Blue* the ratio of metabolites higher in serum than plasma, *red* vice versa

(e.g., eicosapentanoate), glycerophosphatidylcholine and its components (e.g., glycerol-3-phosphate), polypeptides (e.g., bradykinin), dipeptides (e.g., aspartylphenylalanine), and amino acids (e.g., aspartate).

3.3 Differences in the metabolite levels between ages and sexes (subject backgrounds)

Next, we analyzed the differences in the metabolite profiles between ages and sexes. Because age-associated differences in the metabolite profiles were more pronounced than gender-associated ones, we first focused on the differences between young and old subject groups. In agreement with the overall metabolic profiles shown in Fig. 1, the number of metabolites with statistically significant differences ($p < 0.05$) between young and old subjects (Fig. 3a) was greater in females than males. Specifically, 95 and 93 metabolites in plasma and serum, respectively, reached statistical significance in females, with 54 and 56 of which showing more than 50 % differences in their levels (see Supplemental Table 4). On the other hand, only 23 and 27 metabolites in plasma and serum, respectively, achieved statistical significance in males, with 12 and 16 of which showing more than 50 % level differences (see Supplemental Table 4). Plasma and serum samples demonstrated similar trends in the fold differences and statistical significance for both males and females. More than twofold differences between young and old subjects were observed for 4, 4, 25, and 20 metabolites in men's plasma and serum, and women's plasma and serum, respectively. Examples of these metabolites include pregnenolone sulfate (in both sexes) and 5alpha-pregnan-3beta, 20alpha-diol disulfate (only in females).

To get insights into the differences in the metabolic profiles between young and old subjects, we next determined the pathway occupancy of metabolites with significantly different levels between young and old groups (Fig. 3b). In females, a broad range of metabolic pathways for amino acids (such as alanine, asparagine, phenylacetylglutamine, and p-cresol sulfate) were predominant in the old population, whereas in the young population, metabolic pathways for fatty acids (such as palmitate and stearate) and sterol/steroids (pregnane metabolites, such as 5alpha-pregnan-3beta, 20alpha-diol disulfate) were dominant. In

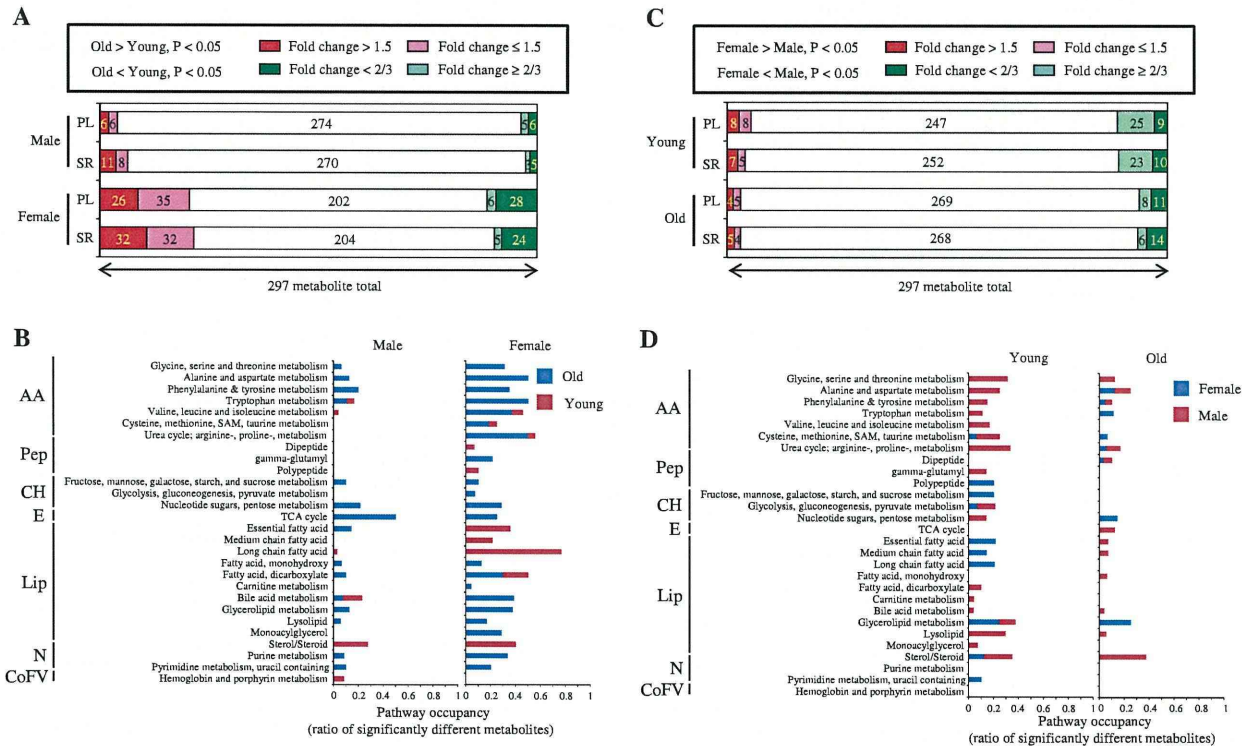

Fig. 3 Differences in the metabolite levels between ages and sexes. **a**, **c** The number of metabolites with statistically significant differences and with at least 50 % changes of the levels between young and old (**a**) or male and female (**c**) subjects. *Values within boxes* indicate the number of metabolites. *PL* plasma, *SR* serum. **b**, **d** Pathway occupancy rates of statistically different metabolites between young and old populations (**b**) or males and females (**d**). *AA* amino acids, *P* peptides, *CH* carbohydrates, *E* energy metabolites, *Lip* lipids, *N* nucleotides, *CoFV* cofactors and vitamins. *Blue* the ratio of metabolites higher in old subjects (**b**) or females (**d**) than young subjects (**b**) or males (**d**) respectively, *red* vice versa

males, specific types of amino acids (phenylacetylglutamine and p-cresol sulfate) produced by gut microflora showed age-associated differences, similar to females; however, most pathways had little significant difference between young and old males, except for the TCA cycle metabolites, such as citrate and malate, which showed higher levels in old subjects. These results indicate that age-associated differences in the metabolite profiles are more prominent in females than males.

Subsequently, we addressed the differences in the metabolite levels between male and female samples. In agreement with the overall metabolic profiles shown in Fig. 1, the number of metabolites with statistically significant levels differences ($p < 0.05$) between males and females (Fig. 3c) was greater in young subjects than old subjects. Specifically, 50 and 45 metabolites in plasma and serum, respectively, showed significant difference in young subjects, with 17 of both of which displaying more than 50 % level differences (see Supplemental Table 5). On the other hand, only 28 and 29 metabolites in plasma and serum, respectively, reached statistical significance in old subjects, with 15 and 19 of which showing more than 50 % level differences (see Supplemental Table 5). Plasma and serum samples demonstrated similar trends in the fold changes and statistical significance for both young and aged subject groups. More than twofold differences between males and females were observed for 5, 4, 7, and 8 metabolites in plasma and serum of young subjects, plasma and serum of old subjects, respectively. Examples of these metabolites included pyroglutamine (in both groups) and 5-alpha-pregnan-3beta, 20alpha-diol disulfate (in young subjects only).

Because sample subjects have significantly different BMIs between male and female, it remains possible that BMI is confounding factor of the gender-associated differences. To assess this possibility we selected young population, which have much severe difference in average BMI (26.9 for male and 37.0 for female). Young female subjects were divided into two groups as follows: normal BMI (range 24.9–35.4, which BMIs are within comparable range of those in male) and high BMI (range 42.8–49.7) (see Supplemental Fig. 1a). Of metabolites significantly different between normal BMI and high BMI female groups, only two each of metabolites (glutaroylcarnitine (C5) and cortisol for plasma and 3-(4-hydroxyphenyl)lactate and citrulline for serum) out of 50 and 45 gender-associated metabolites in plasma and serum, respectively, were BMI-dependent (Supplemental Fig. 1b). In addition, OPLS-DA analysis with young male, young female with normal BMI, and young female with high BMI demonstrated clear separation of male and female but not normal BMI and high BMI in both plasma and serum (Supplemental Fig. 1c). Taken all together, BMI of subject is not confounding factor of gender-associated difference in metabolite profiles.

We also described the pathway occupancy of the metabolites with significantly different levels between male and female samples (Fig. 3d). A larger number of metabolic pathways was highlighted in young subjects, even though the differences in occupancy rates between sexes were moderate compared to those between ages. Fatty acids (such as myristate and palmitoleate) were much more dominant in young females, whereas a broad range of amino acids (pyroglutamine and asparagine) were more dominant in young males. While sex steroid metabolites were moderately highlighted in both young and old subjects, the levels of androgen metabolites (4-androsten-3beta, 17beta-diol disulfate and 5alpha-androstan-3beta, 17beta-diol disulfate) were consistently higher in young and old males. In addition, the levels of progesterone metabolites (5alpha-pregnan-3beta, 20alpha-diol disulfate and pregnanediol-3-glucuronide) were only higher in young females, whereas the levels of pregnenolone metabolites (pregnen-diol disulfate and 21-hydroxypregnenolone sulfate) were only higher in old males.

3.4 Inter-individual variations in subject backgrounds

Inter-individual variations of the metabolite levels are critical factors for designing metabolomics studies on the exploration and/or qualification of biomarker candidates, since large inter-individual variations in healthy states could mask the changes of metabolite levels in response to diseases or drugs. Therefore, we determined inter-individual variations of the metabolite levels in each subject background by calculating RSD. The RSDs of the metabolite levels were found to be constant among all subject background groups (data not shown). As shown in Fig. 4, the RSDs of determined metabolites were largely distributed from 0 to 1.5 and showed almost similar patterns between plasma and serum samples. In total, 173 and 169 metabolites in plasma and serum, respectively, had a score of 0.5 or less.

3.5 Effect of freeze–thaw cycles on the metabolite profiles

Lastly, we examined the effect of freeze–thaw cycles on the stability of metabolites using plasma and serum samples from young males. The number of metabolites showing statistical significance ($p < 0.05$) is shown in Fig. 5a. While the overall difference between 2 and 10 freeze–thaw cycles was smaller than that between matrices or subject backgrounds, 43 and 19 metabolites in plasma and serum, respectively, showed statistically significant differences, with 7 and 3 of which displaying more than 50 % changes

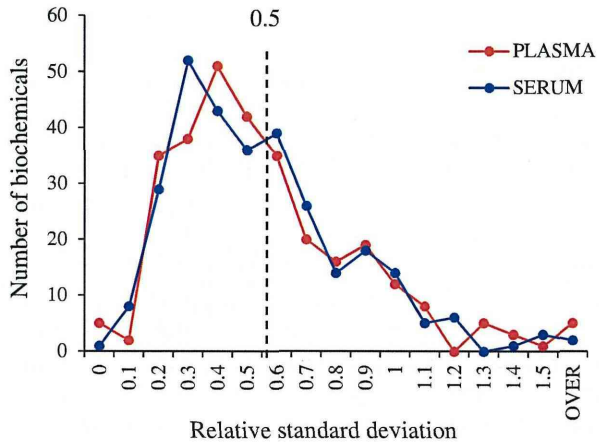

Fig. 4 Inter-individual variations of metabolites in subjects with the same background. Calculated relative standard deviation (RSD) values were rounded to 1 decimal place, and the number of metabolites listed at each RSD value was shown in sum. *Dotted lines* represent RSD values of 0.5 (arbitrary thresholds)

in their levels (see Supplemental Table 6). These results indicate that the plasma levels of metabolites were more sensitive than those in the serum were, and that the majority of their changes were enhanced by 10 freeze–thaw cycles. More than twofold differences between 2 and 10 freeze–thaw cycles were observed for 4 and 2 metabolites in plasma and serum, respectively. Examples of these metabolites include allantoin (in both plasma and serum) and bradykinin (in plasma only).

In addition, pathway occupancy was also analyzed to delineate sensitive metabolic pathways against freeze–thaw cycles (Fig. 5b). Compared to the serum samples, a larger number of metabolic pathways in plasma were affected; in particular, pathways that are associated with peptides (such as bradykinin), low-molecular-weight lipids (such as hexadecanedioate), and glycerolipid metabolites (such as choline) were affected more in plasma than serum. These results suggest that the sources of these metabolites, such as proteins and large lipids, may be broken down by freeze–thaw cycles. Notably, the metabolic pathway of cofactors and vitamins (e.g., heme, biliverdin, and (E,E)-bilirubin) was affected in both plasma and serum samples.

4 Discussion

In the present study, we demonstrate that the global metabolic profiles of two blood sample matrices (plasma and serum) were comparable; only a few metabolites were specific to either one or the other. Plasma and serum also exhibited compatible age- and gender-associated patterns in the overall metabolic profiles, suggesting both matrices compatibly reflect the variation of metabolite profiles caused by subject backgrounds. In addition, plasma and serum presented similar inter-individual variations of the measured metabolites among subjects with the same background. Together, these results suggest that serum and plasma are both useful matrices, with which metabolomics can be performed to discover and/or qualify biomarker candidates. However, more than one-third of the metabolites detected in this study showed significantly different levels between plasma and serum. This result underscores the need for a uniform matrix type when designing metabolomics studies to identify and/or evaluate biomarkers.

While our results suggest that both plasma and serum are suitable matrices for metabolomics studies, each of them has different characteristics. We found that the metabolites in serum were more stable against cycles of freezing and thawing than those in plasma were. In addition, Liu et al. (2010) previously demonstrated that the analytical peak areas in serum were less affected by 37 °C incubation of blood than those in plasma were. Based on these findings, we speculate that the metabolites are more stable in serum than plasma.

The characterization of differences in the metabolic pathways between matrices, subject backgrounds, and freeze–thaw cycles was also a focus of our present study. The pathways that were affected by matrices, subject backgrounds, and freeze–thaw cycles were identified by the pathway occupancy analysis (Fig. 6). The following core pathways are affected by variables: (A) pathways related to blood coagulation (differences between plasma and serum); (B) amino acids metabolized by gut microflora (differences between ages); (C) glucose catabolism (female-related differences between ages); (D) steroid hormone metabolism (common and age-specific differences between sexes); and (E) bilirubin synthesis (affected by freeze–thaw cycles).

Blood coagulation, which releases phospholipases and proteases by platelet activation (Zucker and Nachmias 1985), represents the major differences between plasma and serum. Metabolites produced by phospholipases, such as lysophospholipids and fatty acids, were found to be at higher levels in serum than plasma (Fig. 6a), in agreement with previous reports (Aoki et al. 2002; Yu et al. 2011). In addition, other lipid metabolites, including monoacylglycerol and glycerophosphorylcholine, were also detected in the present study. It is also noted that peptides were present at higher levels in plasma, whereas dipeptides and free amino acids were present at higher levels in serum.

Phenylacetylglutamine and p-cresol sulfate are catabolites of aromatic amino acids, phenylalanine and tyrosine, respectively. Bacteria of the gut microflora are responsible for the production of these aromatic amino acid derivatives (Smith and Macfariane 1996). In this study, phenylacetylglutamine and p-cresol sulfate were present at higher levels

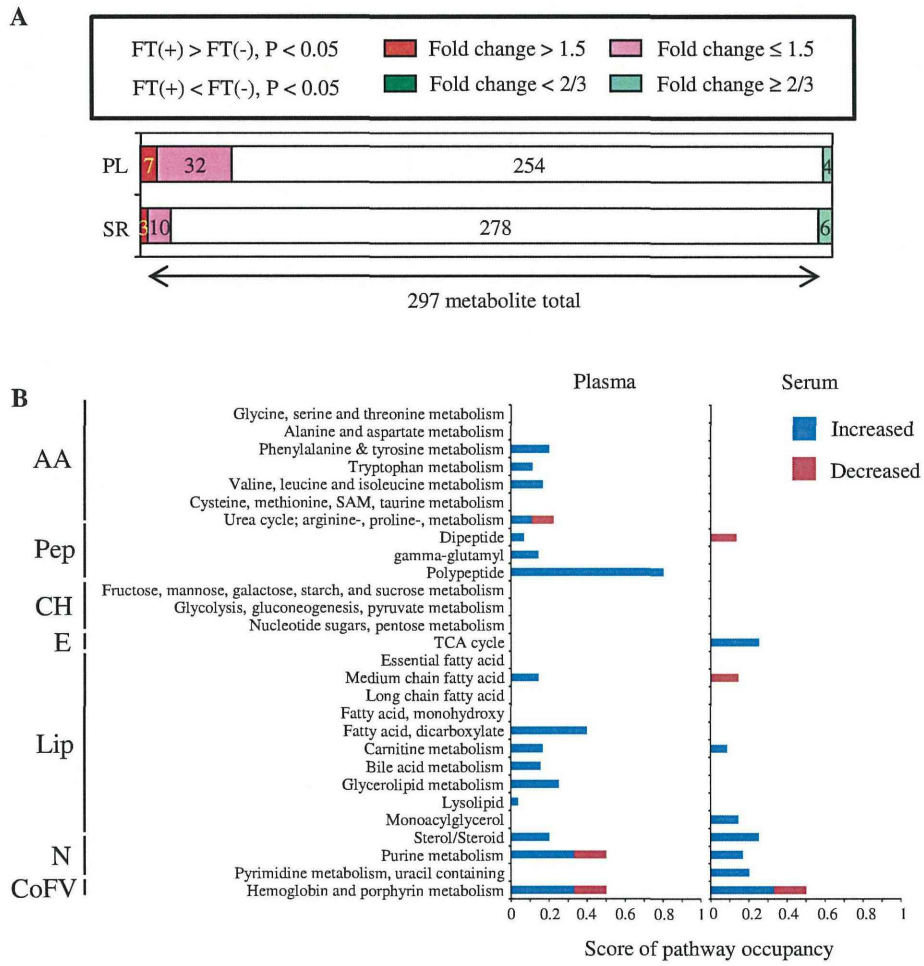

Fig. 5 Effect of freeze–thaw cycles on the metabolite levels. **a** The number of metabolites with statistically significant differences and with at least 50 % changes of the levels between 2 (FT(−)) and 10 (FT(+)) freeze–thaw cycles. *Values within boxes* indicate the number of metabolites. *PL* plasma, *SR* serum. **b** Pathway occupancy rates of statistically different metabolites either with or without freeze–thaw cycles. *AA* amino acids, *P* peptides, *CH* carbohydrates, *E* energy metabolites, *Lip* lipids, *N* nucleotides, *CoFV* cofactors and vitamins. *Blue* the ratio of metabolites higher after 10 freeze–thaw cycles than two cycles, *red* vice versa

in old subjects than in young individuals, without any age-associated decrease in the levels of their precursor aromatic amino acids in both males and females (Fig. 6b). While p-cresol sulfate has been reported as an age-associated biomarker (Lawton et al. 2008), our results suggest that phenylacetylglutamine, the catabolite of phenylalanine, may also serve as an age-associated biomarker.

Age-associated differences in females are the most profound among various comparisons of subject backgrounds (Fig. 1). Fatty acids are present at higher levels in young female subjects, whereas amino acids are at higher levels in old subjects (Fig. 6c). Because overnight fasting minimizes food-derived influences on the levels of amino acids, fatty acids, and those catabolized from glucose, glucose-related catabolism could be different between young and old female subjects. It has been reported that progesterone treatment increased lipogenesis from glucose, pyruvate, and lactate in the liver of pregnant rats (Lorenzo et al. 1986). In the present study, pregnanediol metabolites, the downstream metabolites of progesterone, showed much higher levels in young females than old females (Fig. 6d), suggesting that the decreases in progesterone levels depend on their age. Therefore, progesterone may play a role in the direction of glucose catabolism, resulting in female-specific differences in the metabolite profiles between young and old subjects. Since pregnenolone is the source of sex hormones, such differences may be associated with the drastic loss of female sex hormones upon reaching menopause.

Progesterone synthesis is regulated by estrogen (Endo et al. 1998). In agreement with decreased estrogen levels in post-menopausal women (Burger et al. 1999; Bjornerem et al. 2004), progesterone metabolites showed lower levels in old female subjects than young female subjects, and the levels in old females were comparable to those in males (Fig. 6d). In contrast, the decrease was quite limited for androgens, and their levels were still higher in old males than old females (Sowers et al. 2001; Muller et al. 2003), even though their levels were reported to be gradually decreased in an age-dependent manner. Together, these results indicate that the observation of higher levels of androgen metabolites in males than females was common between young and old subjects.

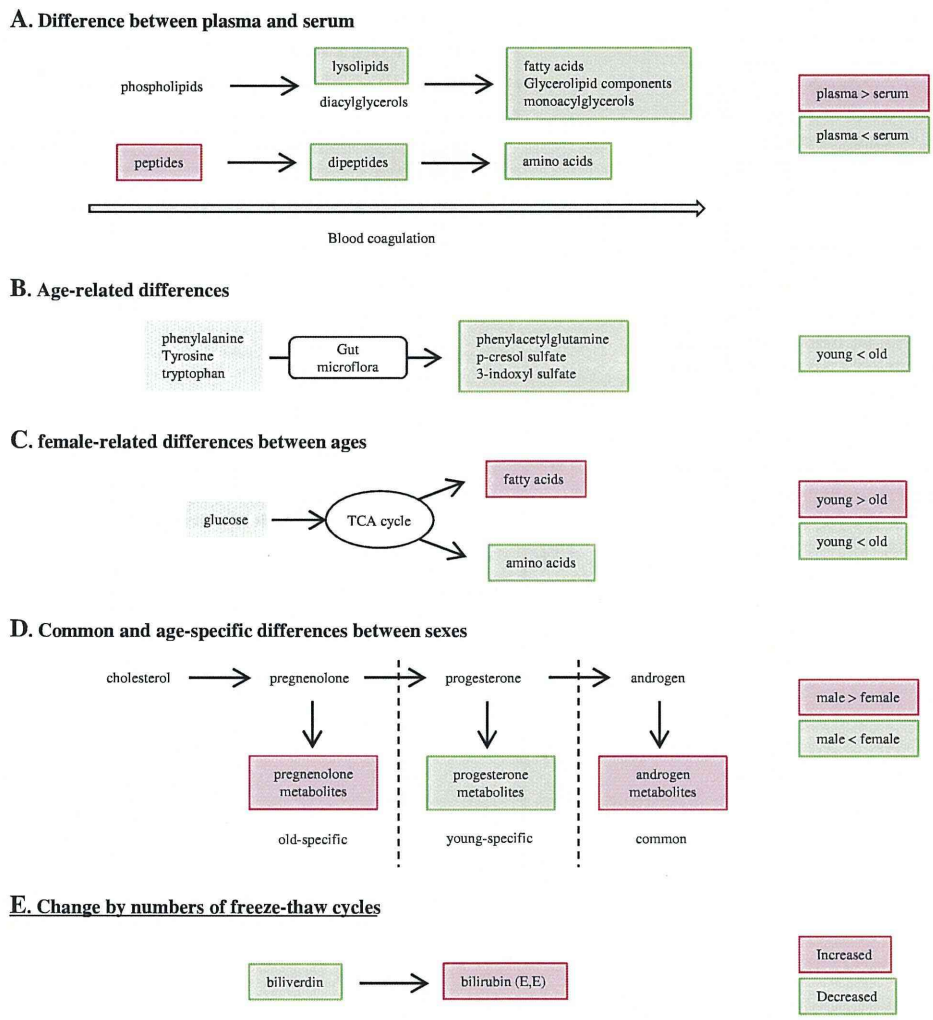

Fig. 6 Highlighted pathways in this study. Highlighted pathways contain specific metabolites showing differences between the subject groups

As for the changes by freeze–thaw cycles, heme degradation was the only pathway common between plasma and serum (Fig. 6e). Biliverdin and bilirubin were decreased and increased, respectively, by repeated freeze–thaw cycles. While it remains unclear as to whether biliverdin reductase is released into plasma or serum, the enzyme may be activated during freeze–thaw cycles and then catalyze biliverdin to bilirubin. On the other hand, peptides and several types of lipids were increased by more freeze–thaw cycles only in plasma, possibly due to the breakdown of much larger proteins and/or lipids by phospholipases and/or proteases, which may be removed from serum during the coagulation process.

Metabolites whose levels are not highly sensitive to differences in age or gender may have potential as biomarkers. In addition, biomarkers that are easy to detect and show low inter-individual variations might have even greater utility. In this study, we identified a subset of biochemicals sharing the following three characteristics (Supplemental Fig. 1a): ease of detection (average filled value, more than 80 %), low gender- or age-associated differences (less than 50 % changes and without statistically significant level differences), and low inter-individual variations (RSD, 0.5 or less). Among 297 metabolites detected in this study, 124 passed all three criteria in plasma and/or serum (Supplemental Fig. 1b; Supplemental Table 7). Of these 124 metabolites, 103 were shared by both plasma and serum; therefore, we suggest that these 103 metabolites are well-controlled in healthy adults and may be primary candidates for biomarkers. Alternatively, metabolites whose levels are drastically modulated by diseases or drugs could overcome the limitations of these background variations and serve as biomarkers. In the present study, Caucasians who had an overnight fast were employed as experimental subjects. It has been reported that nutrients and ethnicity also affect the metabolic profiles. For example, it was suggested that fruits and vegetables intake are strongly associated with the levels of

glycerophospholipids and sphingomyelines (Menni et al. 2013). Comparison of northern and southern Chinese populations using an NMR spectroscopy-based metabolome-wide association approach also demonstrated different levels of several amino acids and carbohydrates (Yap et al. 2010). Nevertheless, the differences associated with nutrients and/or ethnicity should also be taken into consideration for the exploration of biomarkers.

5 Concluding remarks

The discovery of biomarkers capable of forecasting disease states and efficacy/toxicity of therapeutic drugs is clinically important. While metabolomics has been applied to many research studies to identify such biomarkers, fundamental information regarding the metabolite profiles of different blood matrices and subject backgrounds is still limited. The findings of this study clearly suggest that plasma and serum are both useful matrices for exploring biomarkers among low-molecular-weight biochemicals and that the metabolites were more stable in serum than plasma. In addition, our results also show that several metabolites were scarcely detectable, had large age- and gender-associated differences, and possessed high RSD values, all of which are characteristics that should be taken into consideration when selecting biomarker candidates. Taken together, our present study provides useful fundamental information for exploring and selecting biomarkers in future clinical studies and may also help establish the regulatory standards for these studies.

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Plasma and Serum Lipidomics of Healthy White Adults Shows Characteristic Profiles by Subjects' Gender and Age

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Abstract

Blood is a commonly used biofluid for biomarker discovery. Although blood lipid metabolites are considered to be potential biomarker candidates, their fundamental properties are not well characterized. We aimed to (1) investigate the matrix type (serum vs. plasma) that may be preferable for lipid biomarker exploration, (2) elucidate age- and gender-associated differences in lipid metabolite levels, and (3) examine the stability of lipid metabolites in matrix samples subjected to repeated freeze-thaw cycles. Using liquid chromatography-mass spectrometry, we performed lipidomic analyses for fasting plasma and serum samples for four groups (15 subjects/group) of young and elderly (25–34 and 55–64 years old, respectively) males and females and for an additional aliquot of samples from young males, which were subjected to repeated freeze-thaw cycles. Lysophosphatidylcholine and diacylglycerol levels were higher in serum than in plasma samples, suggesting that the clotting process influences serum lipid metabolite levels. Gender-associated differences highlighted that the levels of many sphingomyelin species were significantly higher in females than in males, irrespective of age and matrix (plasma and serum). Age-associated differences were more prominent in females than in males, and in both matrices, levels of many triacylglycerols were significantly higher in elderly females than in young females. Plasma and serum levels of most lipid metabolites were reduced by freeze-thawing. Our results indicate that plasma is an optimal matrix for exploring lipid biomarkers because it represents the original properties of an individual's blood sample. In addition, the levels of some blood lipid species of healthy adults showed gender- and age-associated differences; thus, this should be considered during biomarker exploration and its application in diagnostics. Our fundamental findings on sample selection and handling procedures for measuring blood lipid metabolites is important for ensuring the quality of biomarkers identified and its qualification process.

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Introduction

Metabolomics is one of the "omics" platforms for analyzing comprehensive profiles of small molecule metabolites in cells, tissues, or biofluids such as blood and urine. Metabolomics provides a useful tool to analyze metabolite levels in physiological and biological states, and is therefore applied to explore biomarkers for disease diagnosis [1–4], and drug responses and toxicity [5–8]. Various metabolomic approaches are used, and among these, lipidomics includes the comprehensive analysis of lipid metabolites [9,10]. Lipid metabolites are not only components of cell membranes but are also involved in signal transduction [11,12]. Lipid metabolites are therefore considered potential biomarker candidates for disease diagnosis and drug responses. Indeed, recent lipidomic studies have shown that lipid metabolites such as eicosanoids and sphingolipids are biomarker candidates for cardiovascular events [1], traumatic brain injury [13], Alzheimer's disease [3,14], type 2 diabetes [15], and depression [16].

Blood is a commonly used biofluid for biomarker discovery because it is a "data-rich" source containing several thousands of hydrophilic and hydrophobic metabolites [17] that likely reflect many complex biological process in the body. In addition, collection of blood samples is a minimally invasive procedure as compared with collection of tissue samples by biopsy. Serum and plasma are two distinct matrices separated from blood after phlebotomy. Serum is prepared from whole blood following a clotting process. Plasma is obtained from whole blood in the presence of an anticoagulant, so that coagulation factors are not activated and thus no blood clot is formed. Because of the differences in the preparation of the two blood matrices, metabolite levels are expected to differ between plasma and serum. Indeed, previous studies, focusing primarily on hydrophilic metabolites, have found differences in the metabolite profiles of

plasma and serum, and discussed the advantage of each matrix for metabolite analysis [18–21]. As for lipid (hydrophobic) metabolites, we recently investigated the different levels of these molecules in plasma and serum by using non-fasting blood samples from healthy human subjects [21]. In that study, we found that the levels of thromboxane B_2 (TXB_2), 12-hydroxy-eicosatetraenoic acid (12-HETE), and 12-hydroxy-eicosapentaenoic acid (12-HEPE) tended to be higher in serum than in plasma, suggesting their release from activated platelets by the clotting process. In the non-fasted condition, however, relatively large inter-individual differences were observed for lipid metabolites levels in blood. On the other hand, matrix-associated differences in lipid metabolite levels remain to be evaluated in fasted subjects. Such data would be valuable because fasting blood is commonly used for biomarker discovery studies and dietary factors are known to affect lipid metabolite levels in blood [22].

To date, potential confounding factors that may affect lipid metabolite levels in the blood for biomarker exploration studies have not been thoroughly evaluated. Although it has been reported that the levels of lipid metabolites such as sphingomyelins (SMs) and phosphatidylcholines (PCs) in blood differ between genders [23,24], comprehensive data are lacking, especially on the exact molecular species and the extent of differences in their levels among samples derived from subjects from various backgrounds. In order to prevent false-positive or false-negative results in biomarker discovery, gender- and age-associated differences in the basal levels of individual lipid species should be analyzed in advance. Furthermore, it is important to determine an optimal matrix for analyzing lipid biomarkers and to clarify the stability of metabolites subjected to various sample handling and processing procedures. In this study, to obtain fundamental information on lipid metabolites in blood, we aimed to (1) investigate which of the two matrices, plasma or serum, is more suitable for lipid analysis, (2) elucidate gender- and age-associated differences in basal lipid metabolite levels, and (3) examine the stability of lipid metabolites following repeated freeze-thaw cycles of sample matrices. Toward these aims, we performed a lipidomic analysis by using liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) in fasting human plasma and serum samples for four groups (15 subjects/group) consisting of young (25–34 years old) and elderly (55–64 years old) subjects of both genders, and in repeatedly frozen and thawed plasma and serum samples from young male subjects. Because phosphoglycerolipids (PLs) and sphingolipids (SLs) have different physiological functions depending on their classes and fatty acid composition, we identified the exact species of each metabolite to understand gender- and age-associated differences in their levels.

Materials and Methods

Collection of Human Blood and Preparation of Plasma and Serum

Blood samples from healthy adults were purchased from PromedDX (Norton, MA). The samples were collected after obtaining written informed consent from all subjects. The ethics committee of the National Institute of Health Sciences authorized PromedDX as a validated provider of blood samples and exempted us from the committee's approval for use of the purchased blood samples. Venous blood was collected from 60 white subjects on the morning after fasting for 14 h. Participants were divided into four groups of 15 subjects each: young males (25–33 years old), elderly males (55–64 years old), young females (25–34 years old), and elderly females (55–63 years old) (Table 1). Fresh blood from each individual was collected and simultaneously drawn into 10 ml Vacutainer Serum Separator Tubes with a clot activator for serum and 10 ml Vacutainer Plasma Separator Tubes containing K_2-EDTA for plasma separation. Vacutainer tubes were purchased from Becton Dickinson (Franklin Lakes, NJ). Samples were centrifuged according to the manufacturer's instructions, and serum and plasma were separated within 2 h after collection of blood samples. The plasma and serum samples were immediately frozen and stored at −80°C. After shipment with dry ice from PromedDX, all frozen samples were thawed once on ice and divided into small aliquots before storing at −80°C until lipid extraction.

Extraction and Measurements of Lipid Metabolites

Lipid extraction and measurement of lipid metabolites by LC-MS(/MS) was performed as reported previously [21]. In brief, small aliquots of frozen plasma and serum were thawed on ice for 2 h. Our normal samples were thus frozen and thawed twice in total, including the dispensing process described above. Lipid metabolites were extracted from 100 µl of plasma or serum by using the method described by Bligh and Dyer (BD) [25] with a few modifications [21]. Lower organic layers were measured by ultra-performance liquid chromatography-time of flight mass spectrometry (UPLC-TOFMS; LCT Premier XE; Waters Micro-mass, Waters, Milford, MA) for analysis of phosphoglycerolipids (PLs), sphingolipids (SLs), and neutral lipids (NLs). To distinguish alkenylacyl and alkyl PL species with the same exact mass, a small aliquot of each BD sample was acid-hydrolyzed [26] and analyzed by UPLC-TOFMS. Upper aqueous layers were subjected to solid extraction to obtain polyunsaturated fatty acids (PUFAs) and their oxidative fatty acids (oxFAs), and then measured by UPLC-MS/MS using a 5500QTRAP quadrupole-linear ion trap hybrid mass spectrometer (AB Sciex, Framingham, MA) interfaced with an ACQUITY UPLC System (Waters, Milford, MA). Structural analysis of PLs and SLs was performed by LC-Fourier Transform Mass Spectrometry (LC-FTMS; LTQ Orbitrap XL, Thermo Fisher Scientific, Waltham, MA) as previously described [26], with a few modifications. Data-dependent MS^3 analysis was performed in the positive-ion mode to identify the long chain base of ceramides and cerebrosides.

Effect of Freeze-thawing on the Stability of Lipid Metabolites

To investigate the stability of lipid metabolites in the plasma and serum, we performed 8 additional freeze-thaw cycles (10 cycles in total) in plasma and serum samples of young males. Frozen plasma and serum samples were thawed on ice for 2 h and then re-frozen at −80°C for 30 min. After 10 cycles of freeze-thawing, lipid metabolites were extracted from the samples and analyzed by UPLC-TOFMS and UPLC-MS/MS as described above for normal samples.

Data Processing

UPLC-TOFMS data were processed using the 2DICAL software (Mitsui Knowledge Industry, Tokyo, Japan) [27]. The extracted ion peaks were normalized using internal standards (ISs). Metabolites eluted from 0.1 to 38.0 min (PLs, SLs, diacylglycerol [DG], and cholesterol [Ch]), and from 37.5 to 60 min (cholestryl ester [ChE], coenzyme Q10 [CoQ10], and triacylglycerol [TG]) by UPLC were separately normalized to 1,2-dipalmitoyl-[$^2H_6$]-*sn*-glycero-3-phosphocholine (16:0–16:0 PC-d6; Larodan Fine Chemicals, Malmo, Sweden) and 1,2-dioctanoyl-3-linoleoyl-*sn*-glycerol (8:0–8:0–18:2 TG, Larodan), respectively. Data for