relatively shorter and less unsaturated TGs and of relatively longer and highly unsaturated TGs. In addition, feeding and fasting condition also bi-directionally influenced the levels of relatively shorter and less unsaturated DGs and TGs and of relatively longer and highly unsaturated DGs and TGs. The effects of feeding condition are relatively smaller on the levels of sphingolipids than phospholipids, neutral lipids, and PUFAs and their metabolites. Taken together, our present study provides useful, fundamental information for exploring and validating lipid biomarkers in future preclinical studies and may also help to establish the regulatory standards for these studies.

Supporting Information

File S1 Supplemental tables. Table S1. Nutrient composition of CRF-1 in 100 g. **Table S2.** Sample information. **Table S3.** Identified lipid molecules in plasma. **Table S4.** Normalized levels of lipid molecules in each sample group.
(XLSX)

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Author Contributions

Conceived and designed the experiments: KS YK KM Y. Saito. Performed the experiments: KS MI MU Y. Senoo KT. Analyzed the data: KS MI MM Y. Senoo. Contributed reagents/materials/analysis tools: MI KM. Contributed to the writing of the manuscript: KS Y. Saito.

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ORIGINAL ARTICLE

Differences in metabolite profiles between blood matrices, ages, and sexes among Caucasian individuals and their inter-individual variations

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Abstract Endobiotic metabolites are associated with biological processes in the body and therefore may serve as biomarkers for disease states or therapeutic efficacy and toxicity. However, information is limited regarding how differences between blood matrices, patient backgrounds, and sample handling affect human metabolite profiles. Our objective was to obtain metabolite profiles from Caucasian individuals, based on different matrices (plasma and serum), subject backgrounds (male/female and young/old), and storage conditions (2 or 10 freeze–thaw cycles). In total, 297 metabolites were detected by LC/MS and GC/MS, and more than 75 % of them were highly represented in all sample groups. The multivariate discriminant analysis (OPLS-DA as a model) singled out the matrix type as the most important variable influencing global metabolic profiles; that is, more than 100 metabolites were significantly different based on the matrix type. The influence of subject backgrounds on global metabolic profiles was consistent between plasma and serum. Age-associated differences were more predominant in females than males, whereas gender-associated differences were more prevalent in young subjects than old individuals were. The relative standard deviation of metabolite levels in subjects with the same background ranked from 0.1 to 1.5. Moreover, the changes of metabolite levels caused by freeze–thaw cycles were limited, and the effect was more prominent in plasma than serum. These data demonstrate the impact of matrix, age, gender, and freeze–thaw cycles on the metabolite profiles and reveal metabolites affected by these factors. Thus, our results provide would useful fundamental information for exploring and qualifying biomarkers for clinical applications.

Keywords Metabolomics · Endobiotic metabolite · Plasma and serum · Age · Gender · Freeze–thaw cycle

1 Introduction

Biomarkers reflecting the severity or the presence of diseases are useful tools for their diagnosis and treatment (Gowda et al. 2008; Zineh and Huang 2011). Discovering biomarkers that can forecast therapeutic efficacy and toxicity of drugs is also becoming clinically important for developing new drugs and avoiding adverse events. Endobiotic metabolites, which reflect both genetic and environmental factors, represent the biological processes in the metabolic system of cells, organs, as well as bodies (Psychogios et al. 2011; He et al. 2012), and are therefore expected to be suitable biomarker candidates. Metabolomics is a useful tool for high-throughput biomarker identification, because it can measure a wide range of metabolites at once (Hollywood et al. 2006; Wishart 2007).

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To date, however, the fundamental information regarding the profiles of the stability and variance of human blood metabolites remains limited, thereby retarding biomarker exploration.

Plasma and serum, two matrices that are fractionized from blood and that contain abundant circulating metabolites, can be easily obtained with low invasiveness. While both plasma and serum are commonly used in metabolomics studies for biomarker exploration, several groups have reported differences between their metabolite levels. By determining the levels of 72 metabolites in human plasma and serum, Liu et al. (2010) demonstrated that while most amino acids were present at higher levels in serum, pyruvate and citrate were observed to be at higher levels in plasma. In addition, a large population study has reported higher serum levels of several amino acids, such as arginine, serine, phenylalanine, and glycine (Yu et al. 2011). Moreover, the levels of phosphatidylcholine, erythritol, creatinine, hexadecanoic acid and glutamine were correlated with life expectancy for small-cell lung cancer in plasma but not in serum (Wedge et al. 2011). Liu et al. (2010) also showed that the levels of metabolites in serum were less affected by incubation of blood specimens at 37 °C, compared to those in plasma, suggesting higher stability of serum metabolites at 37 °C. To date, the impact of handling and storage on a wide range of metabolites from blood and serum remains unclear. In addition, the information regarding the metabolite profiles in association with subject background, such as sex and age, is also limited. Previously, several analyses of the human plasma serum metabolome demonstrated gender- and age-associated differences in the metabolite profiles (Lawton et al. 2008; Mittelstrass et al. 2011; Yu et al. 2012). However, because these studies combined all ages when comparing the metabolite profiles between sexes, we speculate that precise gender-associated differences were confounded by age-associated differences, and vice versa. Therefore, there remains an unmet need to reveal gender- and age-associated differences in the metabolite profiles using human subjects. Inter-individual variations in each metabolite level should be elucidated using subjects with the same background, since high inter-individual variations could mask metabolite level changes that reflect disease progression and drug response. Nevertheless, comprehensive metabolomics studies of these differences would warrant accelerated exploration and evaluation of biomarkers for clinical applications.

In the present study, using a global metabolomics approach, we determine the levels of 297 endogenous low-molecular-weight biochemicals (mostly hydrophilic), including amino acids, carbohydrates, and lipids, in plasma and serum samples obtained from human subjects categorized by either age or sex. To minimize the possibility of unexpected variations affecting the differences we focused on, we controlled subjects' age (young population, 25–34 years old; and old population, 55–64 years old), ethnic genetics (healthy Caucasians), and food intakes (overnight fasting). To examine the variables tested in this study (matrix, gender, and age), data were processed by the multivariate statistical analysis, i.e., orthogonal partial least squares discriminant analysis (OPLS-DA) modeling, and matrix type gave the clearest separation. Plasma and serum both presented clear gender- and age-associated differences. Based on our data, we addressed the metabolic profile differences between plasma and serum samples, young and old populations, or males and females, as well as inter-individual variations of the metabolite levels in subjects with the same background. In addition, we also examined the effect of freeze–thaw cycles on the levels of metabolites in plasma and serum samples. Overall, our current study provides fundamental information for future biomarker exploration and qualifications.

2 Materials and methods

2.1 Collection of human blood and preparation of plasma and serum

Blood samples were purchased from ProMedDx (Norton, MA). ProMedDx collected samples after informed consent was obtained rightly from all participants; the ethics committee of the National Institute of Health Sciences authorized the company as a validated provider and exempted us from the committee's approval for the use of purchased blood samples. Venous blood was collected from 60 healthy Caucasian volunteers in the morning after fasting for 14 h. Participants were categorized into 4 groups as follows: young males (25–33 years old), old males (55–64 years old), young females (25–34 years old), and old females (55–63 years old). Each group included 15 individuals, except for the old female group, which had 14 individuals due to the presence of EDTA in serum samples of 1 individual. Subject information is displayed in Supplemental Table 1. Fresh blood from each individual was simultaneously drawn into 10-mL Vacutainer Plasma Separator Tubes containing K2-EDTA (Becton–Dickinson, Franklin Lakes, NJ) and 10-mL Vacutainer Serum Separator Tubes with clot activators (Becton–Dickinson). Following the manufacturer's instructions, samples were centrifuged, and serum and plasma were separated within 2 h of blood collection and then immediately frozen. Upon receiving samples from PromedDX, all samples were thawed on ice, divided into aliquots, and refrozen at −80 °C until sample extraction. An aliquot of plasma and serum samples from young males was subjected to 10

freeze–thaw cycles, with thawing and freezing being done on ice for 2 h and at −80 °C for 30 min, respectively. Samples were subsequently stored at −80 °C.

2.2 Determination of endobiotic metabolite levels

The non-targeted metabolic profiling instrumentation employed for this analysis combined three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS) (Evans et al. 2009; Bourdonck et al. 2009). For each plasma and serum sample, protein was precipitated and low molecular weight compounds were extracted with methanol that contained four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for analysis on the three platforms. Aliquots, dried under nitrogen and vacuum-desiccated, were subsequently either reconstituted in 50 µL 0.1 % formic acid in water (acidic conditions) or in 50 µL 6.5 mM ammonium bicarbonate in water, pH 8 (basic conditions) for the two UHPLC/MS/MS analyses or derivatized to a final volume of 50 µL for GC/MS analysis using equal parts bis-trimethyl-silyl-trifluoroacetamide and solvent mixture acetonitrile: dichloromethane: cyclohexane (5:4:1) with 5 % triethylamine at 60 °C for 1 h.

For UHLC/MS/MS analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Millford, MA) instrument with separate acid/base-dedicated 2.1 mm × 100 mm Waters BEH C18 1.7 µm particle columns heated to 40 °C and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer (Evans et al. 2009). Extracts reconstituted in formic acid were gradient eluted at 350 µL/min using (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in methanol (0 % B to 70 % B in 4 min, 70–98 % B in 0.5 min, 98 % B for 0.9 min), whereas extracts reconstituted in ammonium bicarbonate used (A) 6.5 mM ammonium bicarbonate in water, pH 8, and (B) 6.5 mM ammonium bicarbonate in 95/5 methanol/water (same gradient profile as above) at 350 µL/min. The MS instrument scanned 99–1000 *m/z* and alternated between MS and MS2 scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5 % diphenyl/95 % dimethyl polysiloxane fused silica column with helium as the carrier gas and a temperature ramp from 60 to 340 °C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific, Inc.) operated at unit mass resolving power with electron impact ionization and a 50–750 atomic mass unit scan range (Bourdonck et al. 2009). Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as associated MS spectra, and were curated by visual inspection for quality control using software developed at Metabolon Inc. (DeHaven et al. 2010).

Data extraction of raw MS files from both platforms was performed as described previously (DeHaven et al. 2010). Peaks were identified using Metabolon's proprietary peak integration software, and metabolites were identified by automated comparison of the ion features in experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, in-source fragments, and MS/MS spectra. The quality control and curation processes were designed to not only ensure accurate and consistent identification of true chemical entities but also remove systematic artifacts, misassignments, and background noises. Processing of raw ion feature data yielded 297 endobiotic metabolites of known identity (75 metabolites from GC/MS, and 128 and 94 metabolites from negative and positive ion mode of LC/MS, respectively). Since this study spanned multiple days, samples from each experimental category were randomized across run days and, following data collection, a data normalization step was performed to correct variations resulting from instrument inter-day tuning differences. For monitoring of data quality and process variation, several technical replicate samples created from a homogeneous pool containing a small amount of all study samples were injected throughout the run, interspersed among the experimental samples in order to serve as technical replicates for calculation of precision. In addition, process blanks and other quality control samples are spaced evenly among the injections for each day, and all experimental samples are randomly distributed throughout each day's run. The median relative standard deviation (RSD) was 11 % for technical replicates and 6 % for internal standards. Each metabolite was corrected in run-day blocks by registering the medians to equal one and normalizing each data point proportionately. For samples with missing values for a metabolite, the minimum observed value of the metabolite among all samples was applied as the missing values. RSD of each metabolite was determined by dividing standard deviation of each metabolite by the mean of that metabolite in specific sample groups. Comparison of the metabolite levels among groups was performed by *t* test analyses (the paired *t* test, comparison between plasma and serum or samples subjected to freeze–thaw cycles; and the Welch's *t* test, comparison between young and old subjects or males and females) to assess statistical differences. In this study, $p < 0.05$ represents statistical significance and it

was used for the pathway occupancy analysis. The average values, standard deviation and RSD obtained from normalized levels of each metabolite, filled values (% of detectable samples), as well as the categories and pathways of each metabolite, were displayed in Supplemental Table 2.

2.3 OPLS-DA analysis

Metabolite data, following run-day normalization and minimum value imputation, were loaded into SIMCA-P+ 12 (Umetrics, Umea, Sweden), pareto-scaled, and analyzed using OPLS-DA to visualize the variance among the groups evaluated in this study. The OPLS-DA results were given as score plots to represent the similarity of overall metabolic profiles.

2.4 Pathway occupancy analysis

To construct pathway occupancy maps, pathways represented by more than four metabolites were picked and scored with statistically different metabolites within specific pathways ($p < 0.05$, scored as 1). The scored values were divided by the number of metabolites within specific pathways, resulting in the ratio of occupied metabolites that reached statistical significance within a pathway.

3 Results

3.1 Global profiles of low-molecular-weight biochemicals in plasma and serum of young and old males and females

To generate an overview of group-based variances of global metabolic profiles in different matrices (plasma and serum), subject backgrounds (young and old males and females), and sample storage (2 or 10 freeze–thaw cycles), the OPLS-DA model was applied. Because the examination of the effect of freezing and thawing on metabolic profiles was limited to the subset of plasma and serum from young males, data from this subset were excluded from modeling. As shown in Fig. 1, the plasma and serum samples clustered into two distinct groups separated mainly by component 1 (R2Y = 0.448 and Q2 = 0.29). Within each cluster of plasma and serum sample groups, young and old sample groups clustered into two groups separated mainly by component 2. By age-based clustering, young male and female sample groups were separated distinctly from each other, whereas old sample groups showed no clear separation between sexes. Overall, the trend of clustering for ages and sexes was similar between plasma and serum. In addition, age-associated changes of the metabolic profiles were more pronounced in females than males.

3.2 Differences in the metabolite levels between sample matrices

Our results show that the difference in the overall metabolic profiles between plasma and serum was the greatest. Of 297 metabolites we measured, around 25 % were detected in less than 80 % of the samples with a given group. As shown in Fig. 2a, four individual gender-age groups and their averages were assessed for filled values of each metabolite (the percentage of detectable samples within a group), which were found to be almost the same between plasma and serum. Only five peptides (bradykinin, glycylphenylalanine, glycylvaline, aspartylphenylalanine, and phenylalanylphenylalanine) and two lipids (1-myristroylglycerol and 2-arachidonylglycerol) showed markedly higher filled values (≥80 %) in either plasma or serum than the other matrix (≤40 %). Specifically, the filled values in serum were much higher for glycylphenylalanine, glycylvaline, aspartylphenylalanine, phenylalanylphenylalanine, 1-myristroylglycerol, and 2-arachidonylglycerol but were

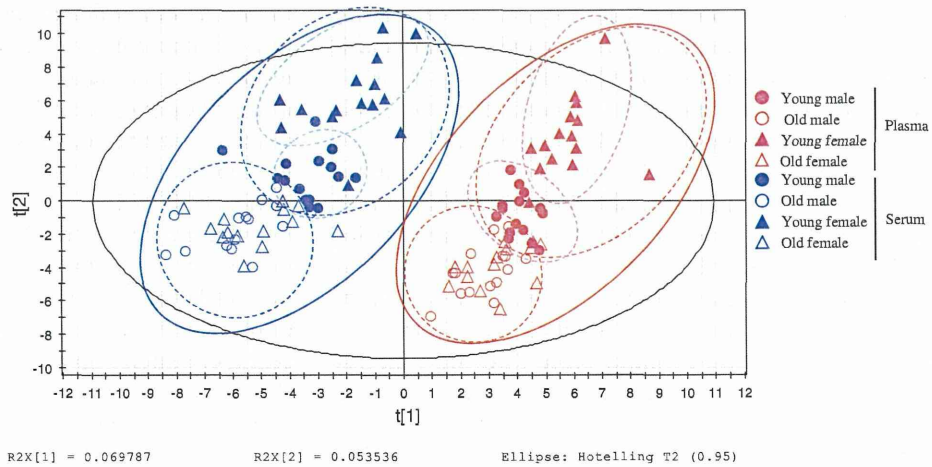

Fig. 1 OPLS-DA model of overall metabolic profiles. Data obtained from human plasma (*red*) and serum (*blue*) samples of young males (*closed circle*), old males (*open circle*), young females (*close triangle*), and old females (*open triangle*) were analyzed. The goodness-of-fit parameter R2 and the predictive ability parameter Q2 were 0.448 and 0.297, respectively

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

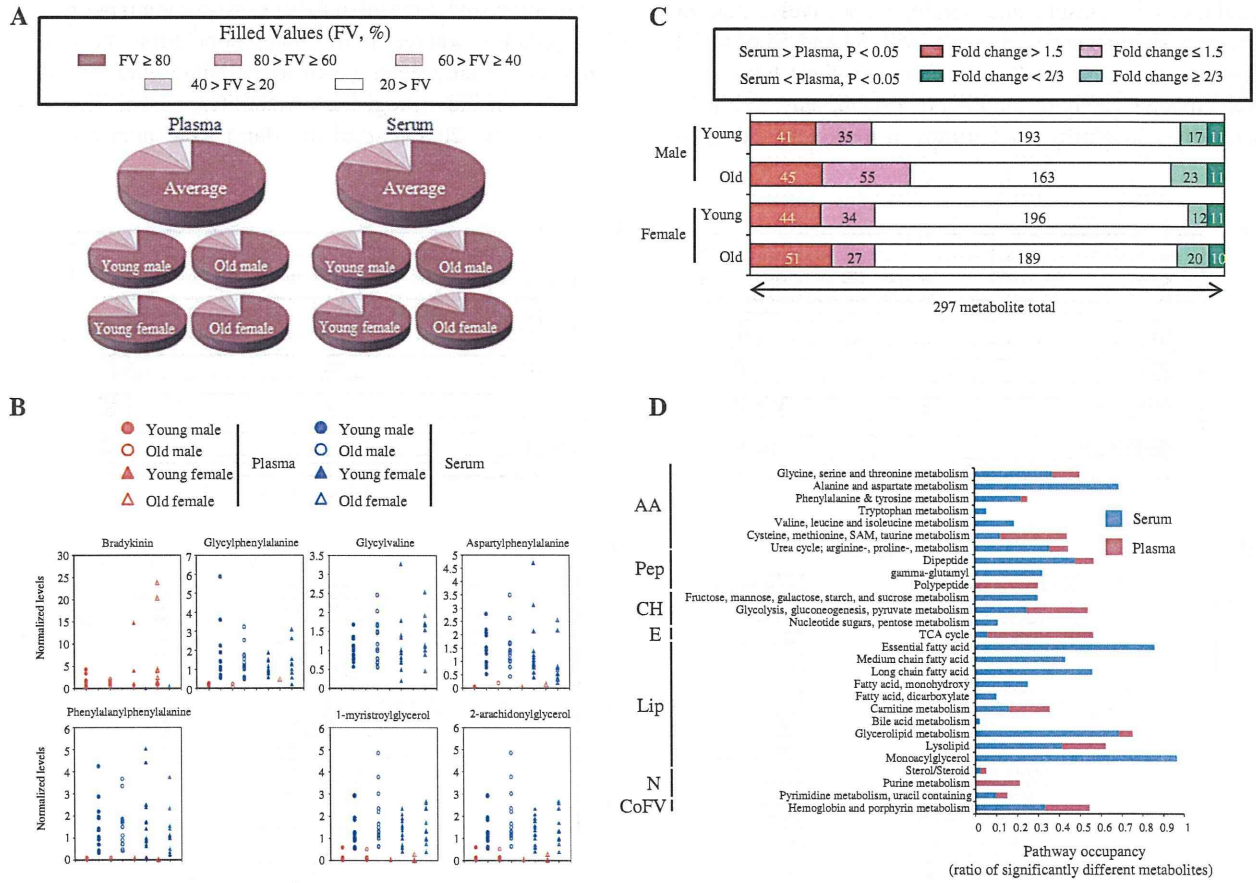

Fig. 2 Differences in the metabolite characteristics between plasma and serum. **a** The distribution of filled values (the percentages of metabolites detected in each subject group). Data obtained from plasma and serum are presented as the average values of all subject backgrounds or each subject background. **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

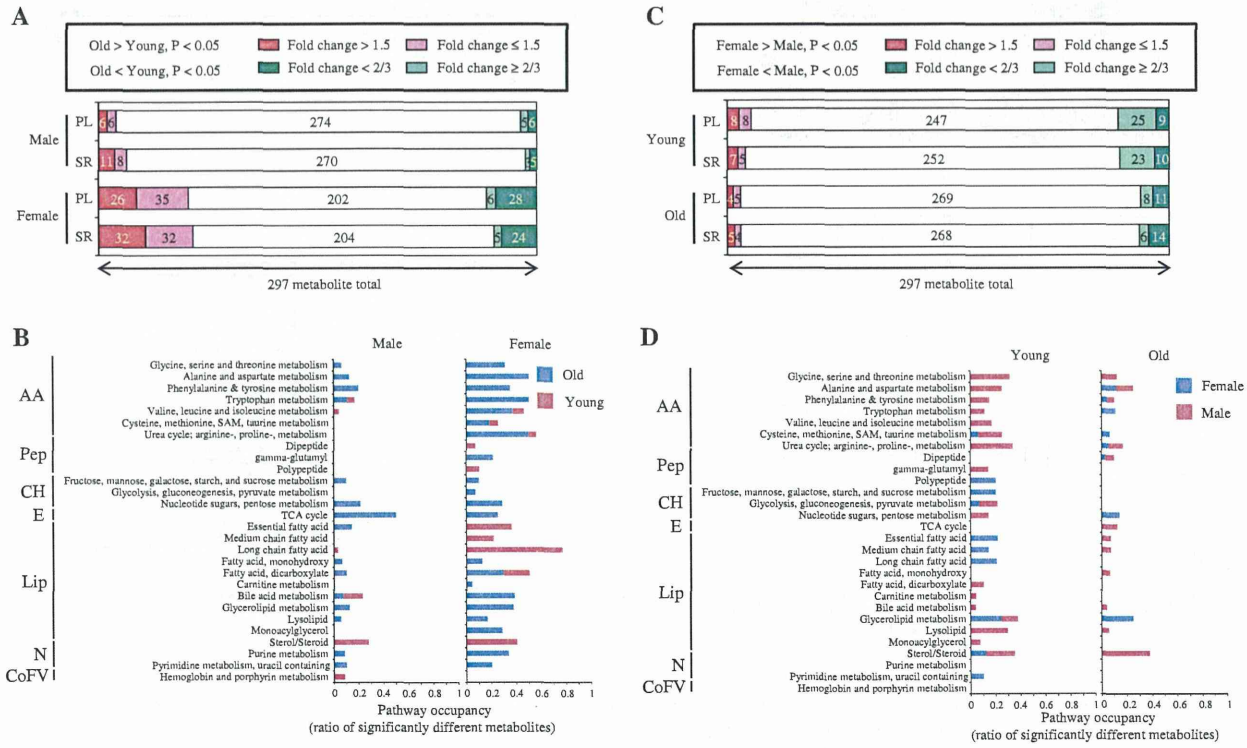

Fig. 3 Differences in the metabolite levels between ages and sexes. **a**, **c** The number of metabolites with statistically significant differences and with at least 50 % changes of the levels between young and old (**a**) or male and female (**c**) subjects. *Values within boxes* indicate the number 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

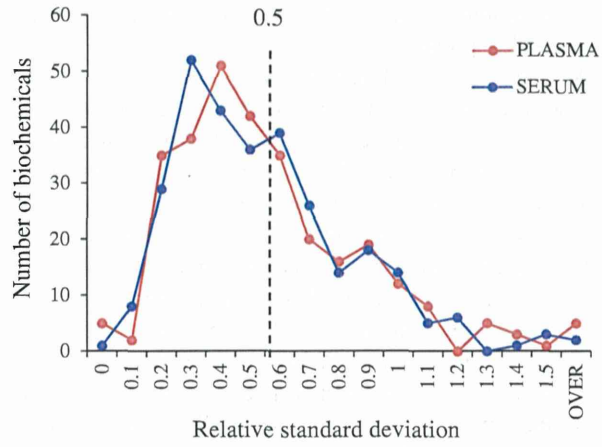

Fig. 4 Inter-individual variations of metabolites in subjects with the same background. Calculated relative standard deviation (RSD) values were rounded to 1 decimal place, and the number of metabolites listed at each RSD value was shown in sum. *Dotted lines* represent RSD values of 0.5 (arbitrary thresholds)

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

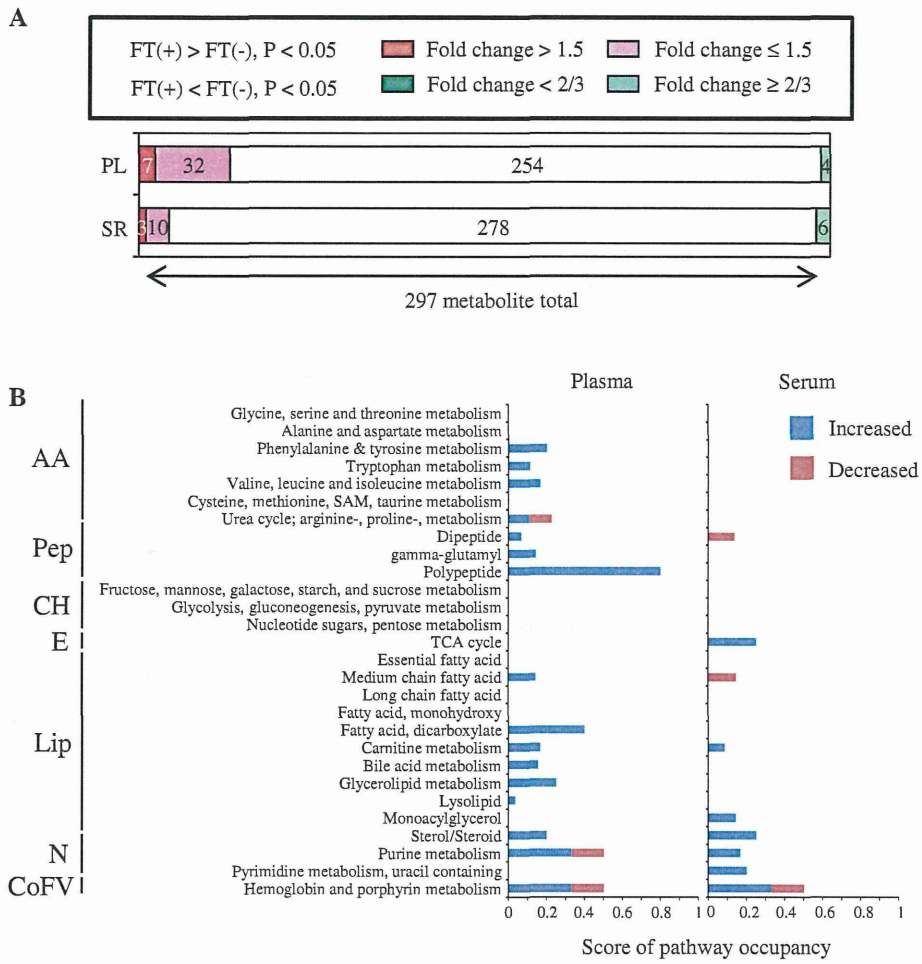

Fig. 5 Effect of freeze–thaw cycles on the metabolite levels. **a** The number of metabolites with statistically significant differences and with at least 50 % changes of the levels between 2 (FT(−)) and 10 (FT(+)) freeze–thaw cycles. *Values within boxes* indicate the number of metabolites. *PL* plasma, *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.