spectrometry for their qualitative and quantitative analyses. Although the range of measurable lipid classes varies among assay platforms, a lipidomic approach usually can measure over 100 lipid molecules from plasma as well as from tissues (e.g., liver tissues) [19,20]. Recently, we characterized and measured 253 lipid molecules in our assay platforms by using human blood plasma [12]. In the present study, we employed the same platforms and examined the effects of multiple factors on circulating lipids, including sex, age, and feeding conditions (feeding, length of fasting period, and diurnal time of sample collection) in the plasma of rats, which is the most-frequently used animal model in preclinical studies. In total, we determined and examined 262 lipid molecules (68 phospholipids, 20 sphingolipids, 138 neutral lipids, and 36 polyunsaturated fatty acids [PUFAs] and their metabolites). Multivariate statistical analysis, i.e., orthogonal partial least squares discriminant analysis (OPLS-DA), demonstrated that the plasma lipid profiles of rats are predominantly affected by feeding conditions, followed by sex and age. No component separating length of fasting period or diurnal time of sample collection was observed. In addition, we also addressed the effects of multiple factors on individual circulating lipid molecules.

Materials and Methods

Animals

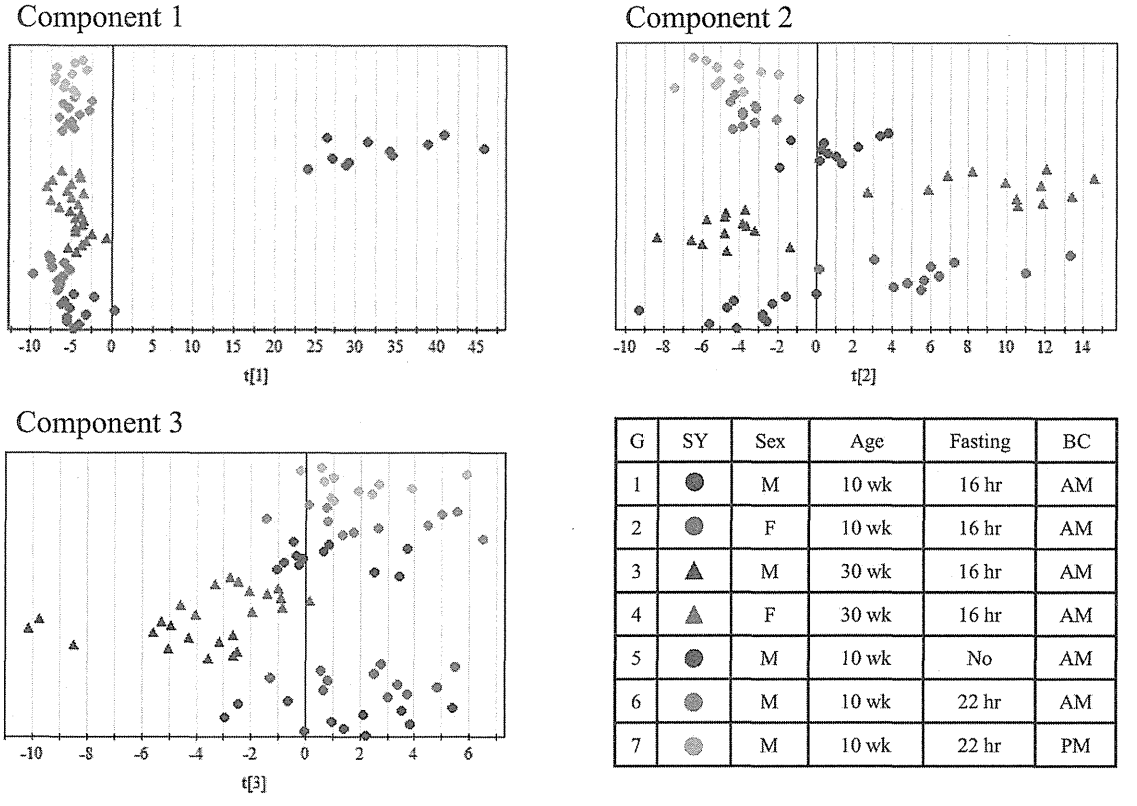
Male and female Sprague-Dawley rats (8 weeks old) were purchased from Charles River Japan (Kanagawa, Japan) and housed until they were 10 or 30 weeks old. The animals were housed in a 12-hr light/dark cycle and were allowed food (CRF-1, nutrient composition were described in Table S1; Oriental Yeast, Tokyo, Japan) and water ad libitum. The plasma samples were obtained in the presence of EDTA-Na from rats after the indicated fasting periods and were collected at the indicated times (Fig. 1A and Table S2). The plasma samples were frozen immediately and stored at −80°C. The use of animal specimens was approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences (Tokyo, Japan).

Lipid extraction

Lipid extraction was performed as described previously [12]. In brief, lipids were extracted from 90 μL of plasma by using the Bligh and Dyer's method with a few modifications. The lower organic phase was used for measurement of phospholipids, sphingolipids, and neutral lipids. The upper aqueous phase was subjected to solid extraction and was used for measurement of PUFAs and their metabolites. Hexadeuterated 16:0/16:0 PC (16:0/16:0-d6 PC; Larodan Fine Chemicals, Malmo, Sweden) and tetradeuterated leukotriene B4 (LTB4-d4; Cayman Chemical, Ann Arbor, MI) were added as internal standards before extraction.

Measurement of phospholipids, sphingolipids, and neutral lipids

Phospholipids, sphingolipids, and neutral lipids were measured using liquid chromatography-time-of-flight mass spectrometry (LC-TOFMS; ACQUITY UPLC System [Waters, Milford]-LCT Premier XE [Waters, Milford]), as described previously [12]. The samples from each experimental group were random-



G	SY	Sex	Age	Fasting	BC
1	●	M	10 wk	16 hr	AM
2	●	F	10 wk	16 hr	AM
3	▲	M	30 wk	16 hr	AM
4	▲	F	30 wk	16 hr	AM
5	●	M	10 wk	No	AM
6	●	M	10 wk	22 hr	AM
7	●	M	10 wk	22 hr	PM

Figure 1. OPLS-DA model of overall profiles of lipid molecules. The goodness-of-fit parameters R2X, R2Y, and Q2 values are as follows; 0.469, 0.159, and 0.149 for component 1, 0.094, 0.143, and 0.127 for component 2, and 0.034, 0.107, and 0.088 for component 3. M, male; F, female; wk, week; SY, symbol; BC, time of blood collection.
doi:10.1371/journal.pone.0112266.g001

Table 1. Lipid classes identified and numbers of individual lipid molecules in rat plasma.

Lipid types	Detected ion mode	Lipid classes	Number of molecules
		lysophosphatidylcholine (LPC)	9
		lysophosphatidylethanolamine (LPE)	1
		phosphatidylcholine (PC)	40
Phospholipid	Negative	ether-type PC (ePC)	4
		phosphatidylethanolamine (PE)	4
		ether-type PE (ePE)	3
		phosphatidylinositol (PI)	7
		sphingomyelin (SM)	14
Sphingolipid	Negative	ceramide (Cer)	5
		hexosylceramide (HexCer)	1
		cholesterol/cholesterolester (Ch/ChE)	26
Neutral lipid	Positive	diacylglycerol (DG)	10
		triacylglycerol (TG)	101
		coenzyme Q (CoQ)	1
poly unsaturated fatty		arachidonic acid (AA) and its metabolites	18
acids (PUFAs)	Negative	eicosapentaenoic acid (EPA) and its metabolites	8
and their metabolites		docosahexaenoic acid (DHA) and its metabolites	10
		total	262

doi:10.1371/journal.pone.0112266.t001

ized across the run. Raw data obtained by LC-TOFMS were processed using the 2DICAL software (Mitsui Knowledge Industry, Tokyo, Japan), which allows detection and alignment of the ion peaks of each ionized biomolecule obtained at the specific m/z and column retention time (RT). The main parameter of 2DICAL was set as described previously with a few modifications [12]. To extract the ion peaks of phospholipids (lysophosphatidylcholine [LPC], lysophosphatidylethanolamine [LPE], PC, ether-type PC [ePC], PE, ePE, phosphatidylinositol [PI]) and sphingolipids (sphingomyelin [SM], Cer, hexosylceramide [HexCer]), the RT range was from 2.0 to 38.0 min in the negative ion mode; while for ion peaks of neutral lipids (cholesterol/cholesterol ester [Ch/ChE], DG, triacylglycerol [TG], coenzyme Q), the RT range was from 2.0 to 60.0 min in the positive ion mode. The intensities of each extracted ion peak were normalized to those of the internal standard (16:0/16:0-d6 PC). To monitor experimental quality throughout extraction, measurement, and data extraction, the relative standard deviation (RSD) of the internal standard (16:0/16:0-d6 PC) was calculated (7.34%). Extracted ion peaks were subjected to identification of lipid molecules by comparison of the ion features, including RT, m/z, preferred adducts, and in-source fragments, of the experimental samples with those of our reference library of lipid molecule entries, as described previously [12]. Processing of extracted ion peaks yielded 226 lipid molecules (88 and 138 lipid molecules from negative ion mode and positive ion mode, respectively; Table 1 and Table S3).

Measurements of PUFAs and their metabolites

PUFAs and their metabolites were measured by targeted approach using LC-MS/MS (ACQUITY UPLC System-5500QTRAP quadrupole-linear ion trap hybrid mass spectrometer [AB Sciex, Framingham, MA]), as described previously [12]. The samples from each experimental group were randomized across the run. Targeted lipid molecules were annotated by comparison of RT, parent ion, and MS/MS ion fragments with standard lipid molecules using MultiQuant Software (Version 2.1, AB Sciex). The intensities of each ion peak from targeted lipid molecules were normalized to those of the internal standard (LTB4-d4). To monitor experimental quality throughout extraction, measurement, and data processing, the RSD of the internal standard (LTB4-d4) was calculated (18.22%). Processing of targeted lipid molecules yielded 36 lipid molecules (Table 1 and Table S3).

Data processing

All data obtained of individual lipid molecules were normalized to the median values of all measured samples as 1. The average values ± standard deviations of the normalized levels of each lipid molecule in each study group are presented in Table S4. For overall comparison, data were loaded into SIMCA-P+12 (Umetrics, Umea, Sweden), pareto-scaled, and analyzed using OPLS-DA to visualize the variance among the groups in the present study. Comparison of individual metabolite levels among groups was performed by the Welch's t-test to assess statistical differences. In the present study, $p<0.05$ represents statistical significance.

Results

Effects of multiple factors on global profiles of lipid molecules in rat plasma

To compare the differences caused by multiple factors (sex, age, and feeding conditions) on the determined 262 lipid molecules, the OPLS-DA model was applied. In the present study, we employed

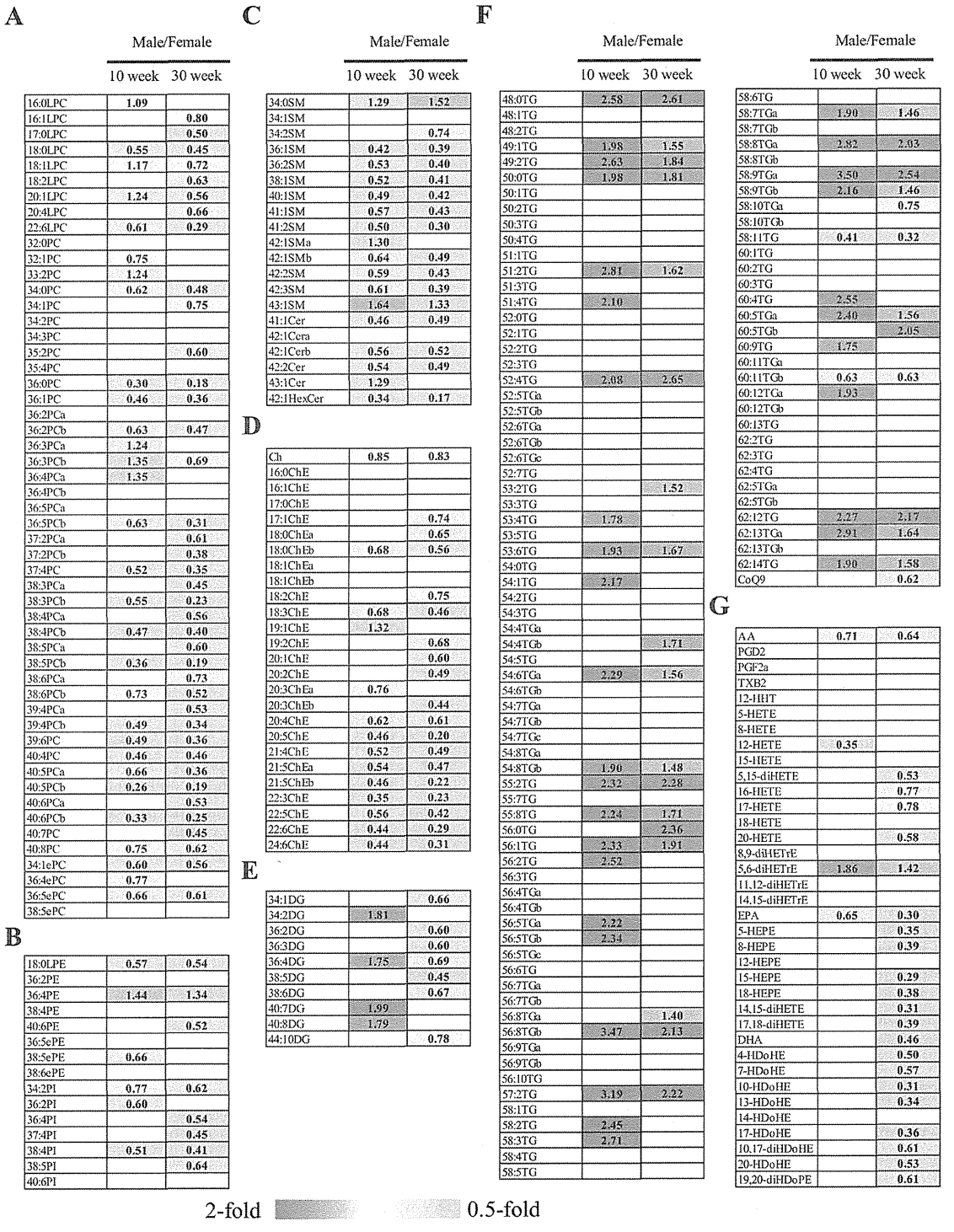


Figure 2. Heat maps of lipid molecules among fasted male and female rats. The heat maps were generated with statistically significant different lipid molecules ($p<0.05$) of lysophosphatidylcholines and phosphoatidylcholines (A), other phospholipids (B), sphingolipids (C), cholesterol/cholesterolesters (D), diacylglycerols (E), triacylglycerol and coenzyme Q9 (F), and arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and their metabolites (G). The numbers indicate the fold change of levels of individual lipid molecules in male over female at 10 and 30 weeks old. The alphabet of the lipid class is used to distinguish 2 lipid species possessing the same formula. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; ePC, ether-type PC; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; ePE, ether-type PE; SM, sphingomyelin; Cer, ceramide; HexCer, hexosylceramide; Ch, cholesterol; ChE, cholesterolester; DG, diacylglycerol; TG, triacylglycerol; CoQ9, coenzyme Q9; AA, arachidonic acid; PG, prostaglandin; TX, thromboxane; HETE, hydroxyeicosatetraenoic aicd; diHETE, dihydroxyeicosatetraenoic acid; diHETrE, dihydroxyeicosatrienoic acid; EPA, eicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; DHA, docosahexaenoic acid; HDoHE, hydroxydocosahexaenoic acid; diHDoHE, dihydroxydocosahexaenoic acid; diHDoPE, dihydroxydocosapentaenoic acid.

doi:10.1371/journal.pone.0112266.g002

Table 2. Sex-based differences in the number of lipid molecules at significantly higher levels.

		10 week		30 week		Common	
Lipid classes	**Total lipid molecules**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Phospholipids	68	8	28	1	46	1	24
Sphingolipids	20	4	13	2	14	2	13
Neutral lipids	138	38	15	27	30	22	2
PUFAs and metabolites	36	1	3	1	21	1	2
Total	262	51	59	31	111	26	41

doi:10.1371/journal.pone.0112266.t002

fasted conditions to examine the effect of sexes and ages, because food intake affects lipidomic profiles and therefore fasted condition is prefer to identify biomarkers from lipid molecules in preclinical study. We used all 7 groups listed in Figure 1 and Table S2. As shown in Fig. 1, component 1 separated fed (purple circle) and fasted (all others) rats; component 2 separated female (red) and male (blue, purple, green, and light blue) rats,; and component 3 separated 10-week-old (dot) and 30-week-old (triangle) rats. There is no component separating fasting duration (16 hr [blue dot] vs. 22 [green dot]) or time of blood collection (10 AM [green dot] vs. 4 PM [light blue dot]).

Differences in the fasted levels of lipid molecules between sexes

To gain insight into sex-dependent differences in plasma lipid molecules, we next compared the levels of individual lipid molecules between fasted male and female rats. Of the 262 lipid molecules we determined, 110 and 142 lipid molecules were significantly different between male and female at 10 and 30 weeks old, respectively (Table 2). Of these lipid molecules, 67 lipid metabolites were commonly changed among 10- and 30-week-old rats (Fig. 2). Higher levels of most of the SMs (9/14 molecules), such as 36:1 and 36:2 SM, were consistent among 10- and 30-week-old female rats (Table 2 and Fig. 2C). In addition to the SMs, PUFAs containing ChEs are higher in both 10- and 30-week-old female rats (Fig. 2D). On the other hand, higher levels of PUFAs and their metabolites, such as eicosapentaenoic acid (EPA) and 5-hydroxyeicosatetraenoic acid, were almost specific for 30-week-old female rats. Furthermore, most of the PUFAs and their metabolites that showed higher levels in 30-week-old female rats are EPA, docosahexaenoic acid (DHA), and their metabolites (Fig. 2G).

Differences in the fasted levels of lipid molecules between ages

Subsequently, we compared the levels of individual lipid molecules between 10- and 30-week-old rats. In fasted male rats, 76 lipid molecules were significantly different, and the levels of 61 molecules were high in 10-week-old rats whereas the levels of 15 molecules were high in 30-week-old rats (Table 3). The phospholipids that presented higher levels at 10 weeks in male rats were LPCs and PCs (Fig. 3A and B). In addition, most of the PUFAs and their metabolites that showed higher levels at 10 weeks in male rats than those at 30 weeks were EPA, DHA, and their metabolites (Fig. 3G). In contrast, in fasted female rats, 101 lipid molecules were significantly different, and the levels of 98 were higher at 30 weeks than at 10 weeks (Table 3). Most of these lipid molecules are neutral lipids (72 out of 101). As shown in Fig. 3F, levels of relatively shorter and less unsaturated TGs, such as 48:0, 50:0, and 52:1 TGs, were higher at 30 weeks than at 10 weeks in female rats. In contrast, levels of relatively longer and highly unsaturated TGs, such as 56:10, 58:11, and 60:13 TGs, were lower at 30 weeks than at 10 weeks in male rats. Only 16 lipid molecules, such as 36:4 and 40:6 PE, showed significantly different levels between 10- and 30-week-olds in both male and female rats (Fig. 3).

Differences in the levels of lipid molecules among feeding conditions

To gain insight into the effects of feeding conditions on plasma lipid molecules, we next compared the levels of individual lipid molecules between rats that were fed and fasted, 16-hr fasted and 22-hr fasted, and from which blood was collected at 10 AM and 4

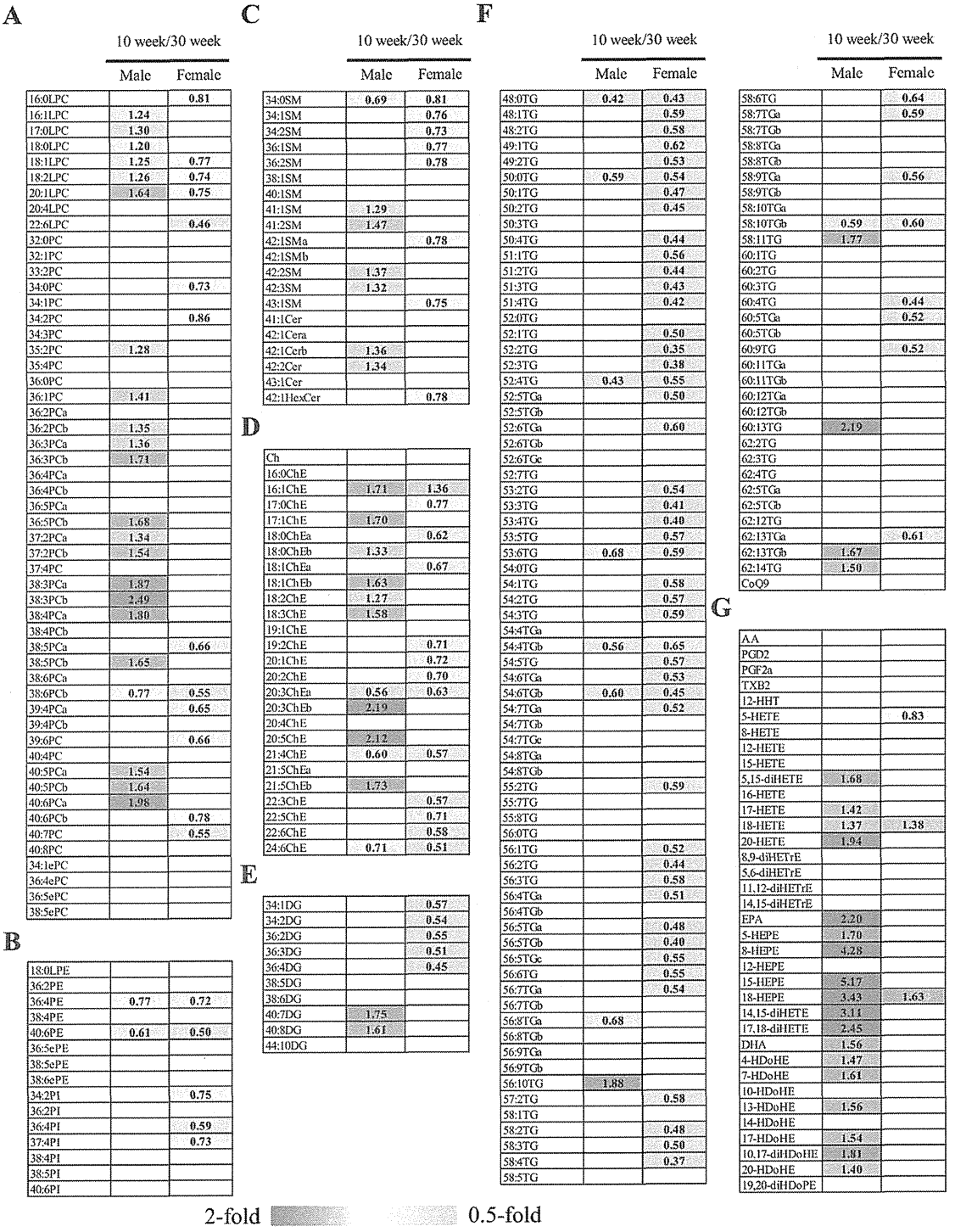


Figure 3. Heat maps of lipid molecules among fasted rats of different age groups. The heat maps were generated with statistically significant different lipid molecules ($p<0.05$) of lysophosphatidylcholines and phosphoatidylcholines (A), other phospholipids (B), sphingolipids (C), cholesterol/cholesterolesters (D), diacylglycerols (E), triacylglycerol and coenzyme Q9 (F), and arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and their metabolites (G). The numbers indicate the fold change of levels of individual lipid molecules in 10-week-old over 30-week-old male and female rats. The alphabet of the lipid class is used to distinguish the 2 lipid species possessing the same formula. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; ePC, ether-type PC; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; ePE, ether-type PE; SM, sphingomyelin; Cer, ceramide; HexCer, hexosylceramide; Ch, cholesterol; ChE, cholesterolester; DG, diacylglycerol; TG, triacylglycerol; CoQ9, coenzyme Q9; AA, arachidonic acid; PG, prostaglandin; TX, thromboxane; HETE, hydroxyeicosatetraenoic aicd; diHETE, dihydroxyeicosatetraenoic acid; diHETrE, dihydroxyeicosatrienoic acid; EPA, eicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; DHA, docosahexaenoic acid; HDoHE, hydroxydocosahexaenoic acid; diHDoHE, dihydroxydocosahexaenoic acid; diHDoPE, dihydroxydocosapentaenoic acid.
doi:10.1371/journal.pone.0112266.g003

Table 3. Age-based differences in the number of lipid molecules at significantly higher levels.

		Male		Female		Common	
Lipid classes	**Total lipid molecules**	**10 week**	**30 week**	**10 week**	**30 week**	**10 week**	**30 week**
Phospholipids	68	21	3	0	18	0	3
Sphingolipids	20	6	1	0	8	0	1
Neutral lipids	138	16	11	1	71	1	10
PUFAs and metabolites	36	18	0	2	1	2	0
Total	262	61	15	3	98	2	14

doi:10.1371/journal.pone.0112266.t003

Table 4. Differences in the number of lipid molecules at significantly higher levels among rats subjected to different feeding conditions.

		Fasting		Duration of fasting		Blood collection time	
Lipid classes	**Total lipid molecules**	**Fasted**	**Fed**	**16 hr**	**22 hr**	**10 AM**	**4 PM**
Phospholipids	68	2	44	0	0	6	0
Sphingolipids	20	4	3	0	0	1	0
Neutral lipids	138	13	96	0	0	4	6
PUFAs and metabolites	36	16	5	0	0	0	0
Total	262	35	148	0	0	11	6

doi:10.1371/journal.pone.0112266.t004

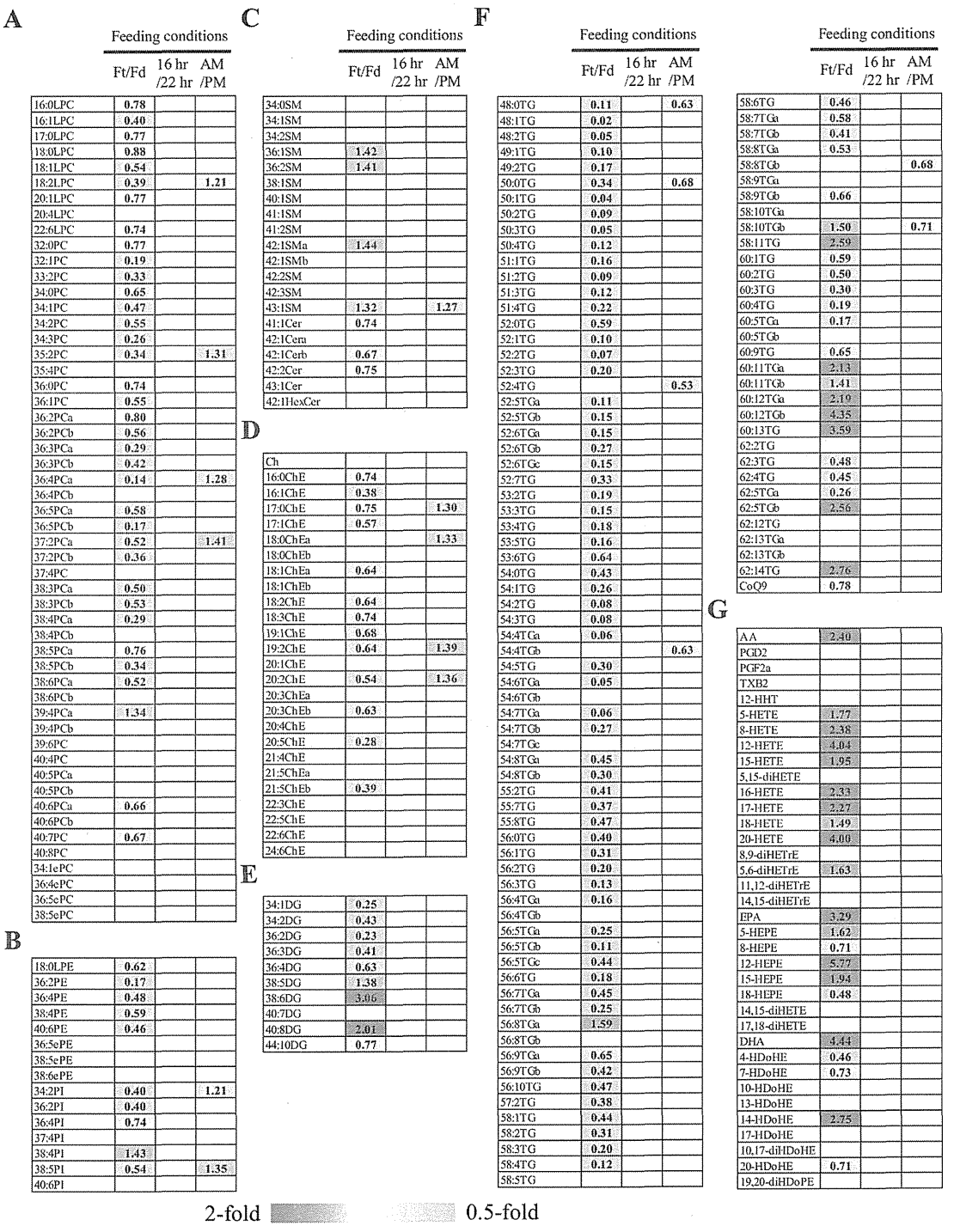


Figure 4. Heat maps of lipid molecules among rats subjected to different feeding conditions. The heat maps were generated with statistically significant different lipid molecules ($p<0.05$) of lysophosphatidylcholines and phosphoatidylcholines (A), other phospholipids (B), sphingolipids (C), cholesterol/cholesterolesters (D), diacylglycerols (E), triacylglycerol and coenzyme Q9 (F), and arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and their metabolites (G). The numbers indicate the fold change of levels of individual lipid molecules in fasted over fed male rats (Ft/Fd), 16-hr fasted over 22-hr fasted male rats (16 vs. 22 hr), and blood collected from male rats at 10 AM over at 4 PM (AM/PM). The alphabet of the lipid class is used to distinguish 2 lipid species possessing the same formula. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; ePC, ether-type PC; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; ePE, ether-type PE; SM, sphingomyelin; Cer, ceramide; HexCer, hexosylceramide; Ch, cholesterol; ChE, cholesterolester; DG, diacylglycerol; TG, triacylglycerol; CoQ9, coenzyme Q9; AA, arachidonic acid; PG, prostaglandin; TX, thromboxane; HETE, hydroxyeicosatetraenoic aicd; diHETE, dihydroxyeicosatetraenoic acid; diHETrE, dihydroxyeicosatrienoic acid; EPA, eicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; DHA, docosahexaenoic acid; HDoHE, hydroxydocosahexaenoic acid; diHDoHE, dihydroxydocosahexaenoic acid; diHDoPE, dihydroxydocosapentaenoic acid.
doi:10.1371/journal.pone.0112266.g004

PM. The results of the comparative analysis showed that 183 lipid molecules were significantly different between fed and fasted male rats (Table 4). More than half of the lipid molecules in phospholipids, neutral lipids, and PUFAs and their metabolites were significantly different between fed and fasted male rats. Forty-four out of 46 significantly different phospholipids and 96 out of 109 significantly different neutral lipids were higher in fed rats than in fasted rats (Table 4). As shown in the heat map presented in Fig. 4E and F, levels of relatively longer and highly unsaturated DGs and TGs, such as 40:8 DG, 58:11, 60:13, and 62:14 TGs, were higher in fasted rats than in fed rats. On the other hand, 16 out of 21 significantly different PUFAs and their metabolites were higher in fasted rats than in fed rats (Fig. 4G). In contrast to the other lipid classes, only 7 out of 20 sphingolipids were statistically significant and their fold changes were less than 50% (Fig. 4C). No significantly different level of lipid molecules was observed between 16 hr-fasted and 22 hr-fasted male rats (Fig. 4). In addition, only 17 lipid molecules, such as 18:2 LPC and 35:2 PC, showed significantly different levels among different blood collection conditions (10 AM vs. 4 PM; Fig. 4).

Discussion

In the present study, we compared effects of multiple factors on the global profiles of lipid molecules in rat plasma. Based on overall comparison by OPLS-DA, feeding condition has a predominant role in the global profiles of lipid molecules, followed by sex and age. The number of statistically different levels in the lipid molecules between fasted and fed male rats reached 70% of the total lipid molecules determined, indicating a massive impact of feeding on lipid molecule levels. Sex-based differences in the levels of 42% of the lipid molecules tested were observed at 10 weeks of age and in 54% of the lipid molecules tested, at 30 weeks of age; whereas, age led to differences in the levels of 29% of the lipid molecules tested in male rats and 39% of lipid molecules in female rats. In contrast, overall comparison did not show any component contributing to separation among length of fasting period (16 hr vs. 22 hr) or diurnal time of sample collection (10 AM vs. 4 PM). In addition, there was no statistically significant difference in the levels of lipid molecules among the length of fasting period. Only 6.5% of the total lipid molecules determined showed significantly different levels among diurnal time of sample collection. These observations indicate that feeding or fasting condition, sex, and age are dominant factors influencing the global profiles of lipid molecules in rat plasma, while length of fasting period and diurnal time of sample collection has no or minor effects.

Sexual differences in hepatic gene expression, including cytochrome P450s, are well known in rodents [21,22]. In the present study, we demonstrated that profiles of lipid molecules in plasma also sexually vary in rats. Levels of SMs and PUFAs containing ChEs were higher in both 10- and 30-week-old female rats. In humans, the levels of SM are higher in female than in male subjects throughout their lifespan, suggesting the sex-based differences in the plasma levels of SMs are conserved among these species [12,14,23]. The reason for higher levels of SMs in female rats has been proposed to be due to the nutritional preferences of women, because there are strong associations between fruit and vegetable intake and the serum levels of d18:1/26:1 SM [23]. In the present study, however, we housed rats with same diet between male and female. Thus, in addition to nutritional preference, there could be alternative factors responsible for the sexually different levels of SMs in plasma. On the other hand, the present study demonstrated that cholesterol and PUFAs were present in higher levels in female than in male rats, although the difference in the levels of most PUFAs levels in 10-week-old rats was not significant. In addition, the expression levels of hepatic acyl-coenzyme A:cholesterol transferase 2 were approximately 3-fold higher in female than in male subjects [24]. Thus, the higher levels of PUFA-containing ChEs in female rats might be due to higher levels of their substrates, as well as their synthesizing enzymes.

Unlike SMs and PUFA-containing ChEs, the levels of EPA, DHA, and their metabolites were higher in 30-week-old female but not in 10-week-old female rats. It remains unclear why the higher levels of EPA, DHA, and their metabolites in female rats were specific for the 30-week-old age group. However, in a previous study, the serum estradiol level in female rats was elevated from an undetectable level at 3 months of age, to 90 pM at 18 months of age [25]. Estrogens have been demonstrated to induce the synthesis of DHA in rat [26]. Thus, one possible mechanism for higher levels of PUFAs and their metabolites in 30-week-old female rats is the estrogen effect.

In the present study, we employed 10-week-old (young adult) and 30-week-old (matured adult) rats for examining the profiles of lipid molecules in plasma. Our study demonstrated that levels of relatively shorter and less unsaturated TGs (48:0, 50:0, and 52:1 TGs) were higher in 30-week-old female rats than in 10-week-old rats, but that levels of relatively longer and highly unsaturated TGs (56:10, 58:11, and 60:13 TGs) were not higher. It has been reported that blood TG levels increase between 10 weeks and 30 weeks in rats and that this increase is much more drastic in female than male rats [27]. Thus, relatively shorter and less unsaturated TGs dominantly contribute to age-associated increases in TGs observed in female rats, but the physiological mechanism for this remains unclear. However, it has been reported that estrogen treatment in turkey liver resulted in increased levels of TGs with total carbon numbers of 53, but in decreased levels of TGs with total carbon numbers of 57 [28]. Thus, estrogen might regulate the synthetic ratio of acyl side chains in TGs through an unknown mechanism in the liver, possibly leading to an increase in shorter, less unsaturated TGs in female plasma.

We have demonstrated that feeding and fasting condition represented a major contribution on the profiles of lipid molecules in plasma. The majority of lipid molecules that are significantly different between fasted and fed rats are food derivatives, which are at lower levels in fasted rats. On the other hand, relatively longer and highly unsaturated DGs and TGs, such as 40:8 DG, 58:11, 60:13, and 62:14 TGs, increased in fasted rats. In addition, PUFAs also increased in fasted rats. Because DGs and TGs are synthesized from glycerol and fatty acids, increased levels of PUFAs might contribute to increased levels of relatively longer and highly unsaturated DGs and TGs.

Unlike other lipid classes, sphingolipids were relatively stable among fasted and fed rats. Feeding condition, as well as food preference, is major concern of clinical tests. Recently, sphingolipids have emerged as biomarker candidates for cardiovascular events, traumatic brain injury, and depression [29–31]. Thus, stability of the levels of sphingolipids throughout feeding and fasting might be advantageous in the application of sphingolipids as biomarkers in clinical tests, although, the effect of gender on the levels of sphingolipids should be considered.

In conclusion, we demonstrated the effects of multiple factors on lipidomic profiles in rat plasma. Our results demonstrated that feeding condition and subjects' sex and age are dominant factors modulating the levels of lipid molecules in rat plasma. The levels of most sphingolipids and PUFAs and their metabolites are sexually different. Age of female rats modulates differently the levels of

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

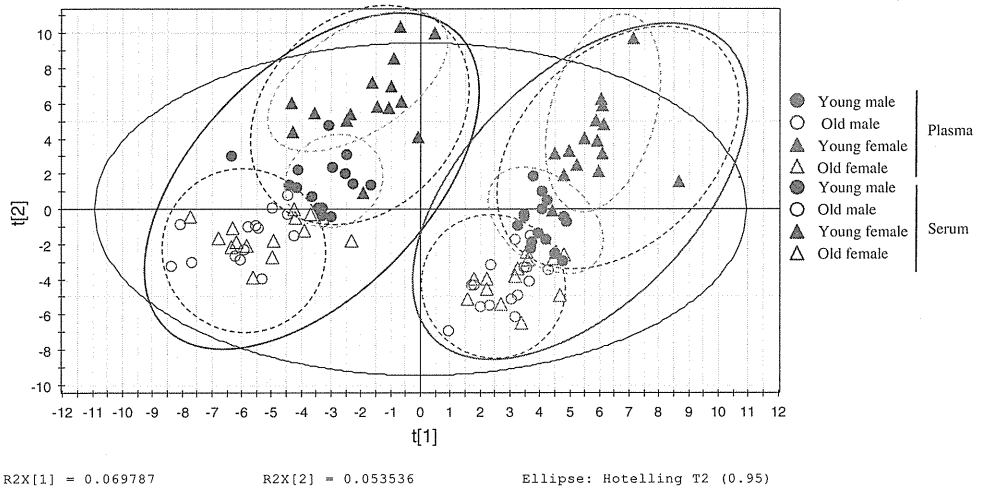

Fig. 1 OPLS-DA model of overall metabolic profiles. Data obtained from human plasma (*red*) and serum (*blue*) samples of young males (*closed circle*), old males (*open circle*), young females (*close triangle*), and old females (*open triangle*) were analyzed. The goodness-of-fit parameter R2 and the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

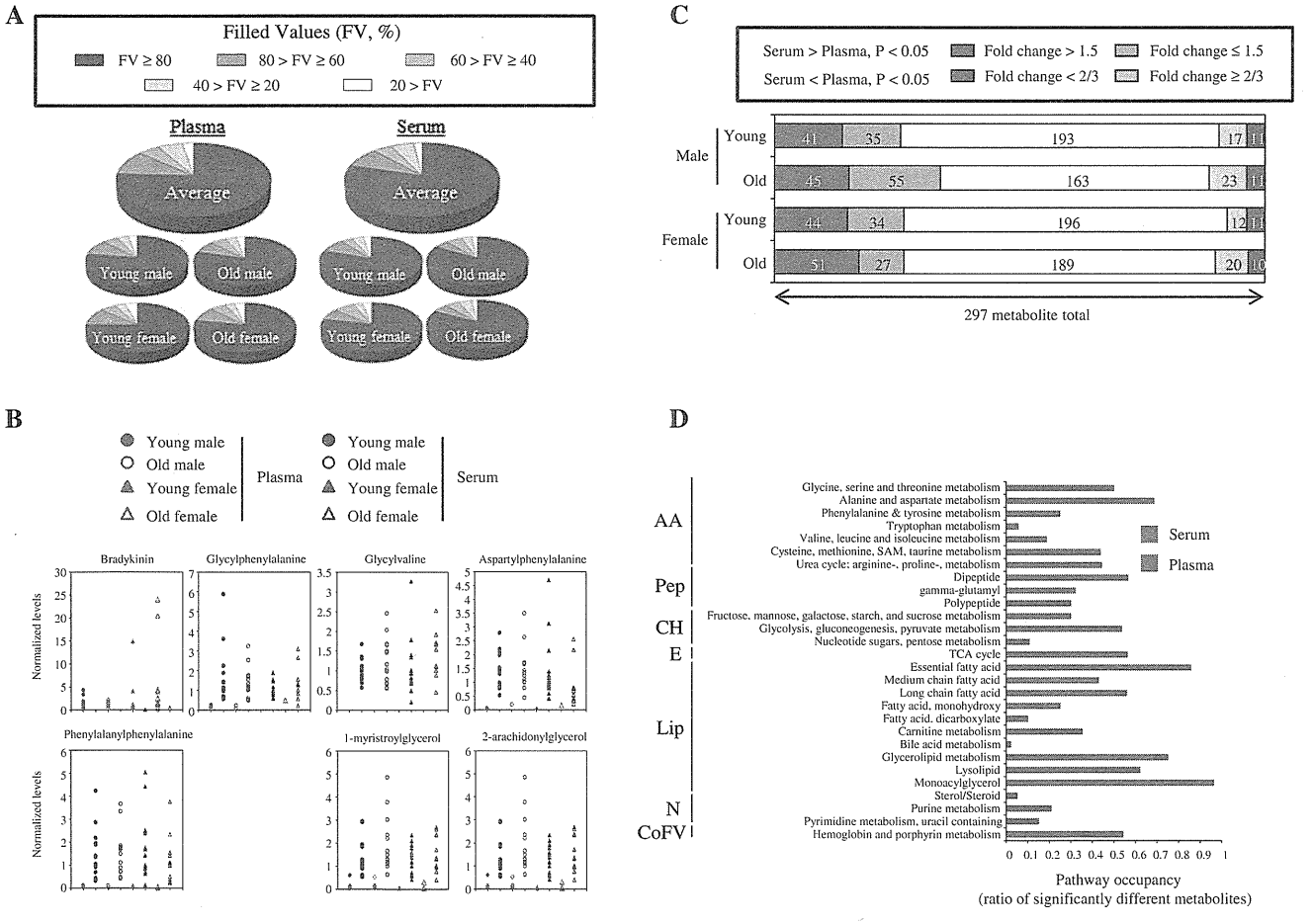

Fig. 2 Differences in the metabolite characteristics between plasma and serum. **a** The distribution of filled values (the percentages of metabolites detected in each subject group). Data obtained from plasma and serum are presented as the average values of all subject backgrounds or each subject background.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

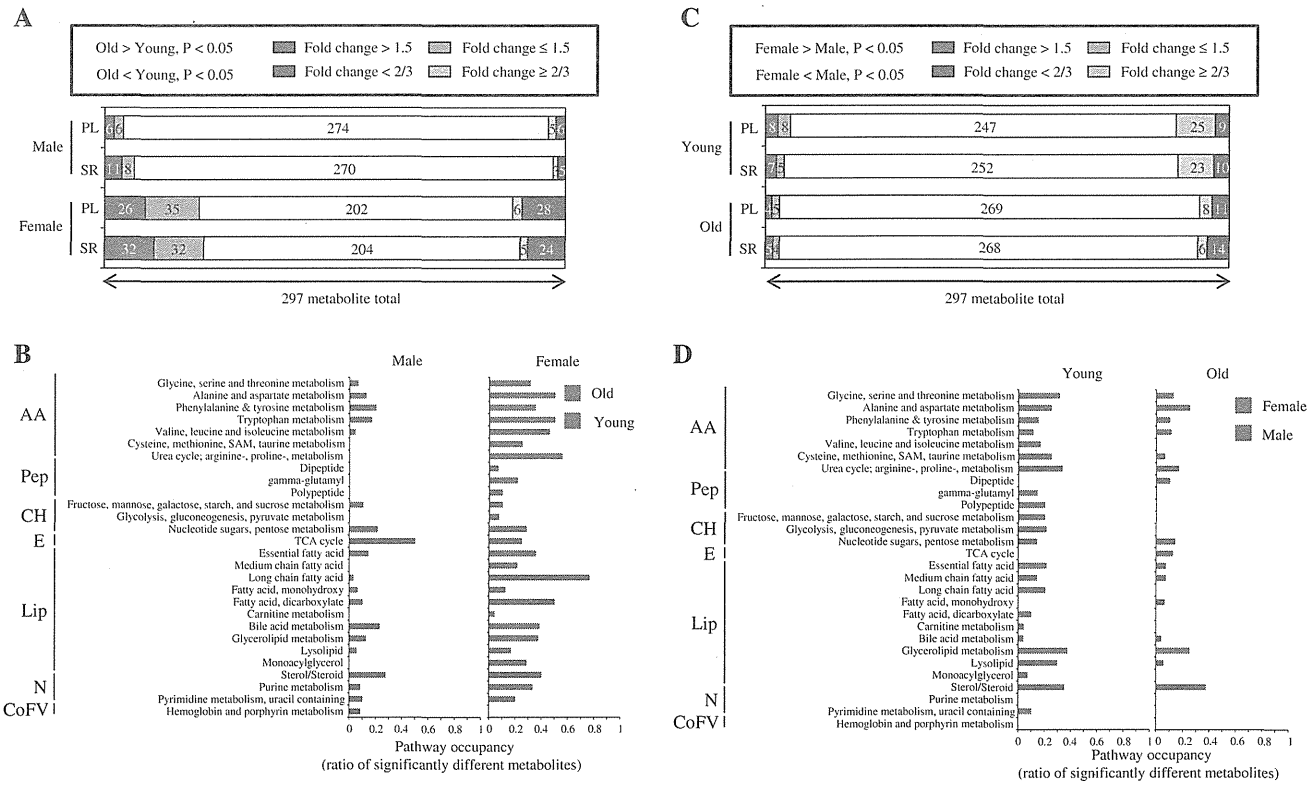

Fig. 3 Differences in the metabolite levels between ages and sexes. **a**, **c** The number of metabolites with statistically significant differences and with at least 50 % changes of the levels between young and old (**a**) or male and female (**c**) subjects. *Values within boxes* indicate the number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

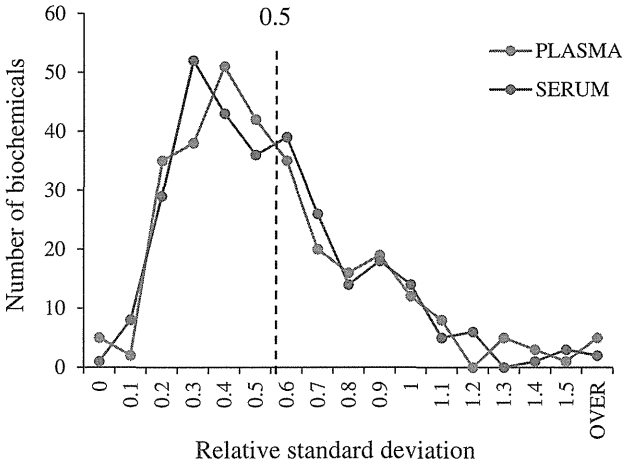

Fig. 4 Inter-individual variations of metabolites in subjects with the same background. Calculated relative standard deviation (RSD) values were rounded to 1 decimal place, and the number of metabolites listed at each RSD value was shown in sum. *Dotted lines* represent RSD values of 0.5 (arbitrary thresholds)

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

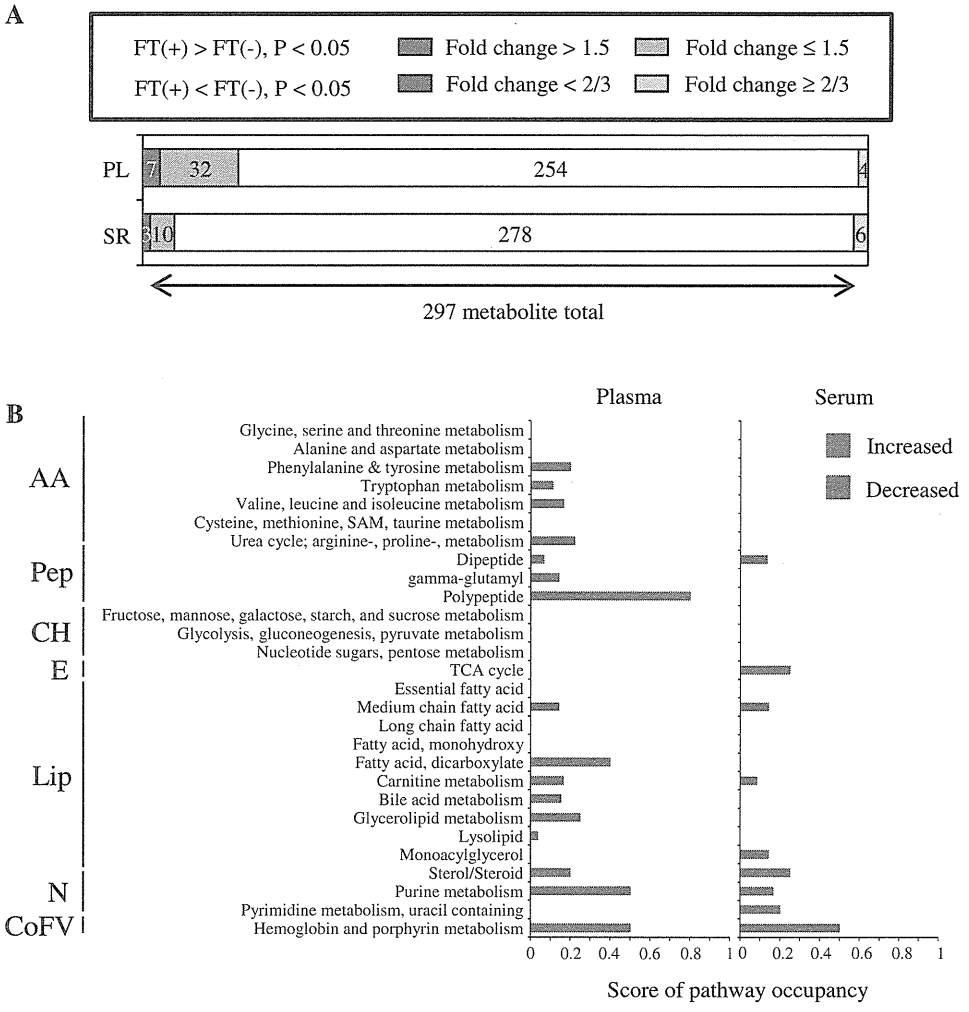

Fig. 5 Effect of freeze–thaw cycles on the metabolite levels. **a** The number of metabolites with statistically significant differences and with at least 50 % changes of the levels between 2 (FT(−)) and 10 (FT(+)) freeze–thaw cycles. *Values within boxes* indicate the number of metabolites. *PL* plasma,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

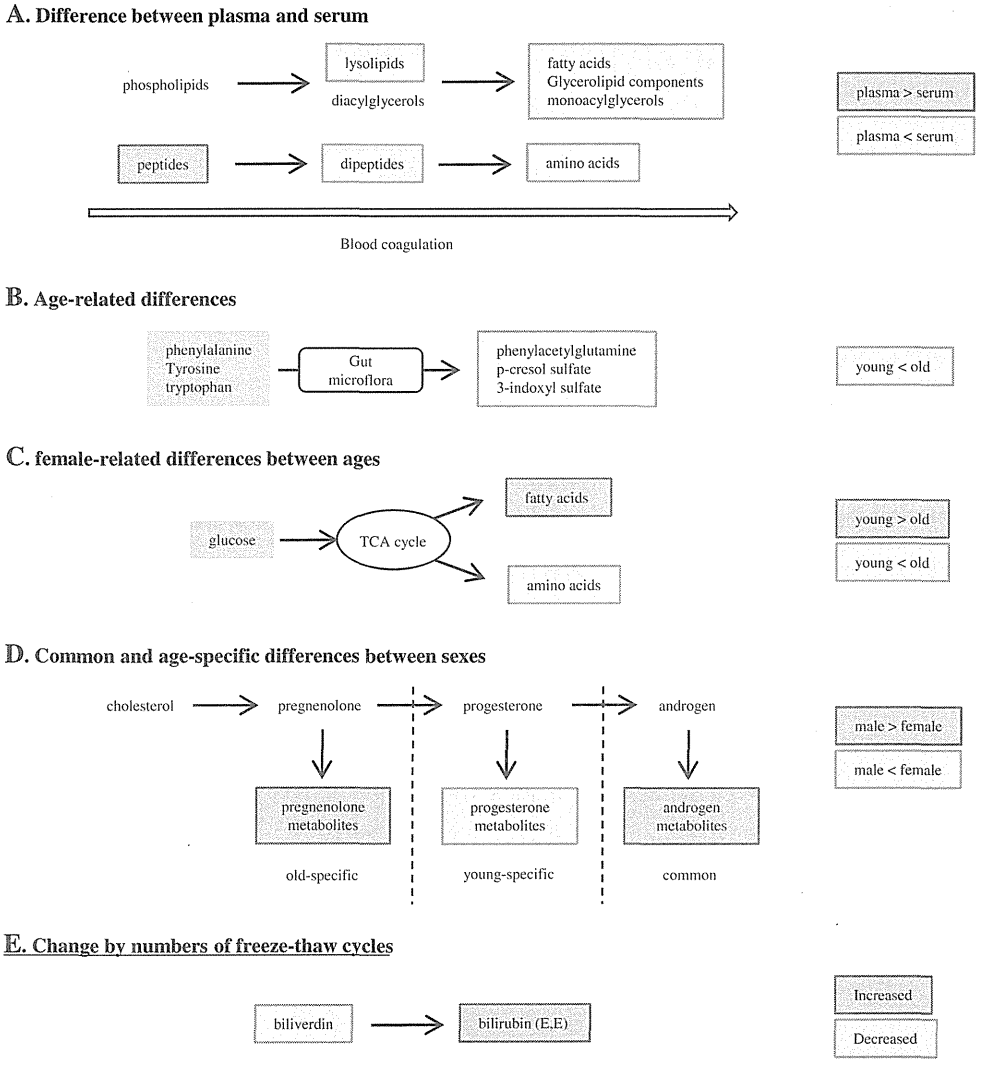

Fig. 6 Highlighted pathways in this study. Highlighted pathways contain specific metabolites showing differences between the subject groups

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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