Plasma and Serum from Nonfasting Men and Women Differ in Their Lipidomic Profiles

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Biomarkers will play important roles in disease diagnosis, drug development, and the proper use of drugs. Blood is considered the best biofluid for biomarker research because it is easy to access and a wealth of data are available. However, previous studies revealed that several ionic metabolites showed different levels (including presence or absence) in plasma and serum. Thus, attention should be paid to selecting the best biofluid for biomarker exploration. Many lipid molecules have biological significance and thus would be candidate biomarkers. However, no comprehensive study revealing differences in lipid metabolite levels between plasma and serum has been undertaken. Furthermore, gender differences have not been reported. To clarify the difference in the levels of lipid metabolites between human plasma and serum from both genders, we performed lipid metabolomic analysis using liquid chromatography-mass spectrometry-based systems for phospholipids (PLs), lysoPLs, sphingomyelins, ceramides and oxidative fatty acids. Our results revealed that most of the lipid metabolites were present at similar levels in plasma and serum and in males and females. However, several oxidative fatty acid metabolites showed differences. Of the metabolites related to clotting processes, three showed higher levels in serum than in plasma, and three were detected only in serum. Furthermore, four metabolites were present at different levels between males and females, and two were detected only in males. Thus, attention should be paid to the selection of plasma or serum when utilizing these lipid metabolites as biomarkers.

Key words lipid metabolite; plasma; serum; gender; level difference; biomarker

Biomarkers are expected to play important roles in disease diagnosis, drug development, and the proper use of drugs. The information will guide decisions regarding the selection of patient subpopulations and optimal dose, benefit–risk assessment, and regulatory approvals, as surrogate markers for clinical end points.[1)] Many useful genomic biomarkers have already been found and used to predict drug responses by stratifying patient populations, such as *UGT1A1* variations for irinotecan therapy and K-ras mutations for anti-epidermal growth factor receptor (EGFR) antibody therapy.[2,3)] However, differences in drug responses cannot be satisfactorily predicted only by genomic biomarkers. Metabolomics, analyzing the comprehensive profile of small molecule metabolites found in biological specimens, is expected to lead to novel diagnostic markers for disease status and drug–responses, including adverse reactions.[4)]

Blood is an appropriate biofluid for biomarker research because it is easy to access and there is a wealth of background data. Serum and plasma are two distinct biofluids separated from blood after phlebotomy. Recently, several studies focused on the differences in metabolite concentrations between plasma and serum. Thus, choosing between plasma and serum for biomarker exploration must be done carefully. For example, glucose concentrations were reported to be lower in plasma than in serum, possibly as a result of fluid shift from erythrocytes to plasma caused by anticoagulants.[5)] Liu *et al.* reported that 36 metabolites, mainly bearing ionic features, differed between plasma (ethylenediaminetetraacetic acid (EDTA) was the anticoagulant) and serum obtained from healthy fasting volunteers: 29 showed higher levels in serum and seven higher in plasma.[6)] Most of them were amino acids and glucose derivatives involved in energy production and the urea cycle. Untargeted metabolomics revealed that 19 (mainly ionic) metabolites were detected in either plasma (heparin was the anticoagulant) or serum from small-cell lung cancer patients.[7)] Thus, plasma and serum have different metabolite profiles. When seeking biomarkers with applications to clinical research, choosing between plasma and serum should be determined by the nature of the molecules to be measured.

Lipid metabolites such as lysophospholipids (lysoPLs), ceramides (Cers) and eicosanoids are important extracellular signaling molecules through specific receptor interactions or unknown mechanisms. Therefore, they were expected to become candidate biomarkers for early diagnosis of disease, drug therapy, and pathology of various diseases. For instance, several lysophospholipids (especially lysophosphatidic acid) and sphingosine-1-phosphate in plasma were reported to be potential diagnostic and prognostic biomarkers of ovarian cancers.[8)] In murine plasma, it was suggested that 20-hydroxyeicosatetraenoic acid (20-HETE), an arachidonic acid-metabolite, was a mediator of rofecoxib-induced facilitation of platelet aggregation, and thus offers a possible explanation for the adverse cardiovascular events associated with its administration.[9)]

Therefore, lipid molecules in blood are promising targets for the discovery of candidate biomarkers. However, previous studies have primarily focused on the levels of ionic metabolites in plasma and serum. In contrast, comprehensive studies of lipid metabolite levels have not been conducted. Furthermore, gender differences in lipid metabolite levels have not been reported in plasma and serum. To clarify possible difference in the levels of lipid metabolites between human plasma and serum from both genders and to utilize this information for future biomarker discovery studies, we conducted lipid

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metabolomic analysis using liquid chromatography-mass spectrometry (MS)-based systems. We focused on phospholipids (PLs), lysoPLs, sphingomyelins (SMs), ceramides (Cers), and oxidative fatty acids (oxFA) as target molecules.

MATERIALS AND METHODS

Subjects Venous blood was collected from 10 nonfasting Caucasian volunteers after antecubital venipuncture into 5 mL Vacutainer Serum Separator Tubes with clot activator (Becton Dickinson) and 4.5 mL Vacutainer Plasma Separator Tubes containing K_2EDTA for plasma separation (Becton Dickinson). Participants consisted of 5 males, aged 27 to 33 years old (median 32 years old) and 5 females, aged 26 to 33 years old (median 32 years old). Blood collections from all of the donors were performed in the morning, prior to noon. We could not obtain further personal information on participants such as body weight, height, the individual dietary constituents, and postprandial period except for their gender and ages. According to manufacturer's instructions the samples were centrifuged, and serum and plasma were separated within 2 h of blood collections, immediately frozen, and stored at −80°C for up to one month. The samples were prepared by PromedDX (Norton, MA, U.S.A.) after obtaining informed consent from all participants, and shipped on dry ice. This study was approved by the ethics committee of the National Institute of Health Sciences.

Lipid Metabolite Extraction Serum or plasma (100 μL) samples diluted with methanol were transferred into glass tubes, and a mixture of internal standards (ISs) was added. Internal standards consisted of the following: 1,2-dipalmitoyl D6-3-*sn* glycerophosphatydylcholine (20 nmol/100 μL serum or plasma, Larodan, Malmo, Sweden), deuterated prostaglandin E2 (PGE2-d4, five ng, Cayman Chemical, Ann Arbor, MI, U.S.A.) and deuterated leukotriene B4 (LTB4-d4, 5 ng, Cayman Chemical) were used. Then, chloroform, methanol and 20 mM potassium phosphate (Kp_i) buffer were added to achieve a volume ratio of buffer–methanol–chloroform= 0.8 : 2 : 1, and it was mixed vigorously for 5 min. Phase separation was achieved by adding 1 mL of both chloroform and 20 mM Kp_i buffer. After vortexing, the mixture was centrifuged at 1000×***g*** for 10 min. The upper aqueous layer was collected, and the lower organic layer was re-extracted by adding an equal volume of aqueous solution consisting of 100 mM potassium chloride (KCl)–methanol–chloroform=48 : 47 : 3. The organic layer was then collected, dried under a gentle stream of nitrogen, dissolved in 1 mL chloroform–methanol (1 : 1), and stored at −90°C until use (BD sample). To distinguish alkenylacyl and alkyl phospholipid species with the same exact mass, a small aliquot of each BD sample was acid-hydrolyzed using 0.5 N HCl as described previously (BD-HCl sample).[10] BD samples and BD-HCl samples, which contained PLs and sphingolipids, were measured using reverse-phase liquid chromatography/electrospray ionization-mass spectrometry (RPLC/TOF MS). The detailed analytical methods and data processing are described in the supplemental information.

Samples of the aqueous layer were subjected to solid extraction to obtain oxidative fatty acids (oxFAs). Briefly, samples were diluted 10-fold using water adjusted to pH 3.0 with 1 N HCl, and then applied to preconditioned Oasis SPE cartridges (60 mg, Waters, Milford, MA, U.S.A.). After washing the column with 3 mL of Milli-Q water followed by 3 mL of hexane, the samples were then eluted with 3 mL methyl formate (MF). MF fractions were dried under nitrogen, dissolved in 1 mL chloroform–methanol (1 : 1), and stored at −90°C until use (MF sample). MF samples were measured using RPLC-triple quadrupole mass spectrometric multiple reaction monitoring. Their detailed analytical methods and the data processing are described in the supplementary information.

Statistical Analysis Data were analyzed statistically using the Wilcoxon (matched-pairs) signed-rank test for the comparison of each metabolite level between serum and plasma (from the same subjects), and the Mann–Whitney *U*-test for the levels between males and females. The statistical analysis was performed with Prism ver. 5.0 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). p values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Glycerophospholipids and Sphingolipids PLs, lysoPLs, SMs and Cers were measured using RPLC/TOF-MS. A total of 72 metabolites were identified consisting of 27 phosphatidylcholines (PCs), 6 ether-type PCs, 7 lysoPCs, 5 phosphatidylethanolamines (PE), 3 plasmalogen PEs, 2 lysoPEs, 6 phosphatidylinositol (PI), 14 SMs, and 2 Cers. Supplementary Table 1 summarizes relatively quantified data of IS-normalized peak heights of each metabolite.

There were no significant differences in the 72 lipid levels between plasma and serum in either gender. On the other hand, female serum contained significantly higher levels of 4 metabolites than did male serum: 36 : 2 PE (fold-change of median level in females relative to that in males was 1.8-fold, p=0.032), 36 : 2 SM (1.3-fold, p=0.032), 40 : 2 SM (1.5-fold, p=0.016) and 42 : 1 Cer (1.6-fold, p=0.008). These results suggested that glycerophospholipid and sphingolipid levels are generally similar between the plasma and serum and between males and females, although several metabolites showed significant but less than 2-fold differences between genders.

One study showed that lysophosphatidylinositols (LPIs) were more abundant in plasma than in serum, possibly because their consumption was prevented in plasma by inhibition of the blood clotting cascade that activates thrombin and

Table 1. Oxidative Fatty Acid Metabolites with Different Levels in Plasmas and Sera or in Genders

Metabolites with higher levels in serum than in plasma (more than 10-fold depending on the individual)		
Thromboxane B2	12-HHT	12-HETE
Metabolites detected only in serum (plasma levels were below the detection limit)		
12-HEPE	14-HDoHE	20-Hydroxy leukotriene B4
Metabolites with different levels between males and females (Fig. 1)		
11,12-diHETrE	14,15-diHETrE	17,18-diHETE
20-hydroxy leukotriene B4		
Metabolites detected only in males (females levels were below the detection limit)		
16-HETE	18-HETE	

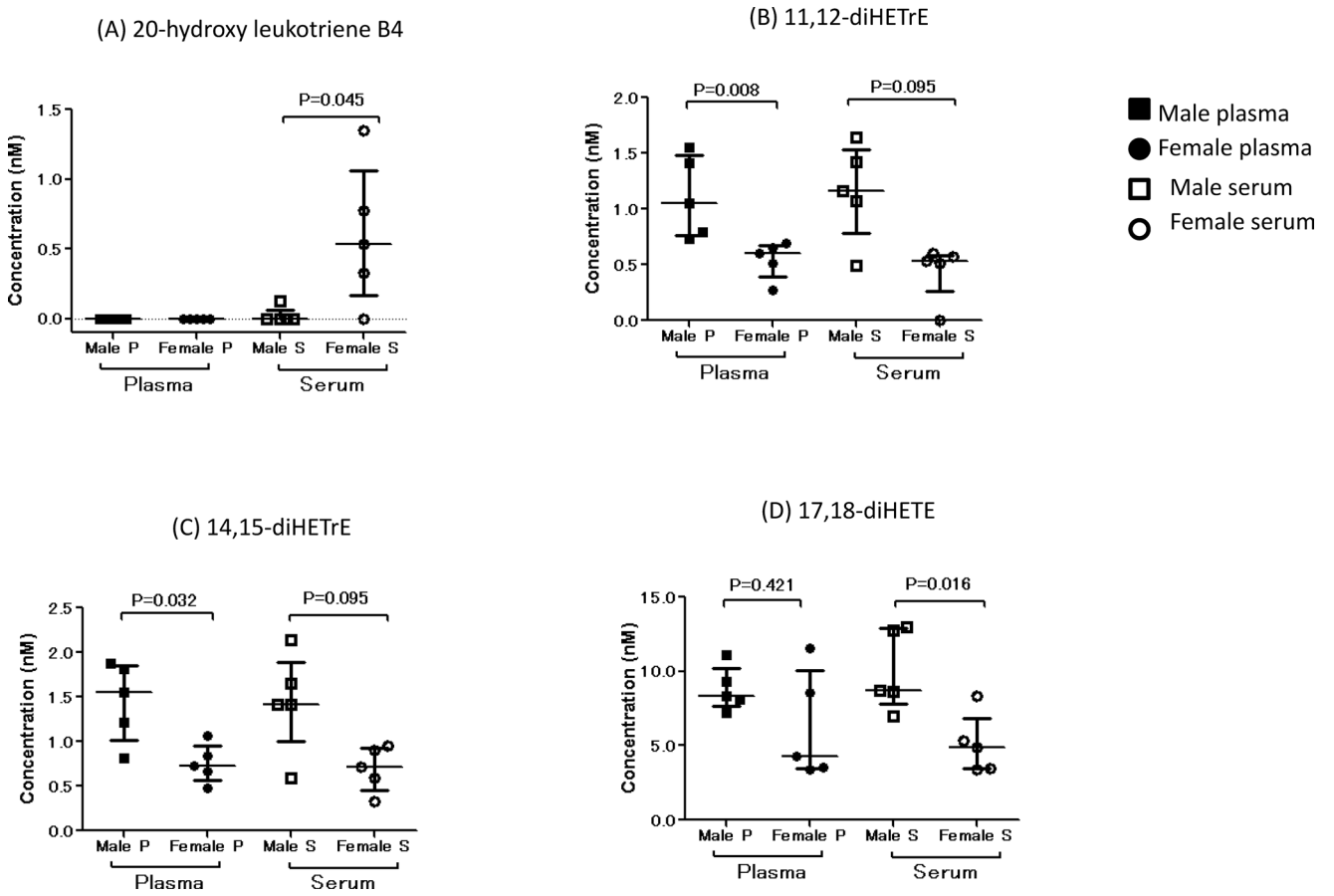

Fig. 1. Oxidative Fatty Acid Metabolites with Levels Significantly Different between Males and Females

Oxidative fatty acid metabolites were quantified in plasmas (n=5) and sera (n=5) of both genders, and compared between males and females for all matrices by Mann–Whitney U-test. The graph shows the median with interquartile range. Symbols: closed square, male plasma; closed circle, female plasma; open square, male serum; open circle, female serum.

other proteases.[11] However, we could not detect LPI because they were too hydrophilic to retain in our RPLC system.

Oxidative Fatty Acids Free polyunsaturated fatty acids and their oxidized metabolites were quantified using standard chemicals, and 30 detected species are listed in supplemental Table 2. Statistical analysis was performed for the levels in plasmas and sera, and we found that no metabolite was significantly different. However, some serum samples contained much higher (more than 10-fold) levels of three metabolites than did the corresponding plasma derived from same individuals: thromboxane B2 (TXB2) (maximally 156-fold), 12-hydroxyheptadecatrienoic acid (12-HHT, maximally 149-fold) and 12-HETE (5 to 208-fold). These variations resulted in wide inter-individual differences between serum and plasma concentration ratios. In addition, several oxFAs were detected only in serum. Two metabolites 12-hydroxyeicosapentaenoic acid (12-HEPE), and 14-hydroxydocosahexaenoic acid (14-HDoHE) were detected only in serum from both genders, while in plasma, they were below the quantification limits shown in supplemental Table 3. Moreover, 20-hydroxy LTB4 was mostly detected only in female serum (Fig. 1).

The essential difference between plasma and serum is that serum is collected after clotting, whereas plasma is collected without clotting in the presence of an anticoagulant such as ethylenediamine tetraacetic acid (EDTA) or heparin. During blood coagulation, arachidonic acid is released primarily from membrane phospholipids in platelets by phospholipase A2. The oxidative fatty acids that were increased (or only detected in serum) compared with plasma from the same blood donor were likely involved in the clotting process. The 12-HHT was co-generated in the process of TXA2 (a potent activator of platelet aggregation) generation by thromboxane synthase.[12] TXB2 is the stable metabolite of TXA2. Thrombin-activated platelets produce proinflammatory factor LTB4 *via* the 5-lipoxigenase (5-LOX) pathway and is further metabolized into the 20-hydroxy form,[13] Activation of 12-LOX in platelets could produce 12-HETE from arachidonic acid,[14] and 12-HEPE from eicosapentaenoic acid,[15] and 14-HDoHE from docosahexaenoic acid.[16] The 12-LOX products of PUFA such as 12-HETE, 12-HEPE and 14-HDoHE function as inhibitors of platelet aggregation.[17] Thus, a higher abundance of these oxFAs in serum than in plasma is likely due to the clotting process.

Next we compared the oxFAs levels between males and females. As described in the above section, 20-hydroxy LTB4 levels were significantly higher in female serum than in male serum (p=0.045). Furthermore, the levels of 11,12-dihydroxyeicosatrienoic acid (11,12-diHETrE, 0.6-fold in plasma, p=0.008), 14,15-diHETrE (0.5-fold in plasma, p=0.032) and 17,18-dihydroxyeicosatrienoic acid (17,18-diHETE, 0.6-fold in serum, p=0.016) were higher in males than in females in both plasma and serum, though the statistical significance was dependent on the blood matrices (Fig. 1). The metabolites 16-HETE and 18-HETE were detected only in some males,

while their levels in females were below the detection limits irrespective of blood matrices. The reason for these differences is currently unknown. Further studies are needed to provide a mechanistic explanation for gender differences in these metabolites.

In conclusion, current lipidomic study has revealed that most of the lipid metabolites are present at similar levels in human plasma and serum and between males and females, although several oxidative fatty acid metabolites showed different levels between both of them. The levels of 3 metabolites (TXB2, 12-HHT, 12-HETE) were more than 10-fold higher in some sera than their corresponding plasmas from the same individuals although statistical significance was not obtained due to wide inter-individual differences. Three metabolites (12-HEPE, 14-HDoHE and 20-hydroxy LTB4) were detected only in serum compared with plasma. Above 6 metabolites (TXB2, 12-HHT, 12-HETE, 12-HEPE, 14-HDoHE and 20-hydroxy LTB4) were reported to be related to the clotting process (platelet aggregation and its inhibition). Furthermore, 4 metabolites (11,12-diHETrE, 14,15-diHETrE, 17,18-diHETE, 20-hydroxy LTB4) were present at significantly different levels between males and females, and 2 (16-HETE, and 18-HETE) were detected only in males. The limitation of this study is that small numbers of subjects in each gender category might decrease the power for the detection of metabolites with wide inter-individual difference in their levels. Furthermore, the present study did not consider the individual dietary variations which may affect lipid metabolite levels in blood.[18]

Biomarkers should reflect normal biologic processes in a body (health or disease state or drug responses). Therefore, the metabolites that involved in platelet aggregation such as TXB2, 12-HHT, 12-HETE need to be measured using plasma because the clotting process caused large increases in these metabolite levels, resulting in no reflection to their real levels in the blood. As for hydrophilic metabolites such as amino acids and glucose derivatives, however, serum was recommended as an appropriate biofluid for biomarker exploration because these compounds were biochemically metabolized *in vitro* more quickly in plasma (maybe due to active metabolism in the blood cells) than in sera after blood collection.[6] Thus, the choice of which biofluids as a sample in clinical situation depends on the nature of the molecules to be measured. The present study showed that attention should be paid in selecting plasma or serum and for utilizing lipid metabolites as biomarkers. Although further studies clearly needed using larger sample sizes (such as thousands of subjects), our results provide basal information useful for future exploration and selection of biomarkers for disease diagnosis and therapeutic intervention.

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