あり，ゲノム，エピゲノム，トランスクリプトーム，プロテオームという他のオミックスの最下流にあって，疾患や副作用症状などのフェノタイプ（形質）を表現し得ると考えられている．遺伝的要因のほかに環境的要因を反映し得るため，体内の内在性代謝物はバイオマーカーとして有用と考えられる．これを網羅的に測定するメタボローム解析は，ここ数年で多くの解析がなされているが，一方で，1）商業的に確立した測定系は少ない，2）生体内代謝物には親水性が高い核酸，糖リン酸等から，疎水性の高いトリグリセリドやリン脂質等まで，その性質は多様であるため，1つの解析系ですべての代謝物を測定できない，3）標準品があり絶対定量できる代謝物は限られている，4）酵素反応が進み易いため，厳格な条件での試料採取が必要（標準実験プロトコールと条件記録），などの特徴があり，解析には困難を要する．

筆者らは，厚生労働省傘下のナショナルセンター及び慶應義塾大学等と多層的オミックス解析による疾患バイオマーカー及び創薬標的の探索研究を行っている（Fig. 1）．これは，日本人において罹患率が高い，根治の治療法がない，医療経済学的に問題である等の12疾患（てんかん，肺がん，腎がん，乳がん，肥満症，非アルコール性脂肪性肝炎，大動脈瘤，拡張型心筋症，小児白血病，アレルギー疾患，アルツハイマー病，脊柱管狭窄症）の病変・対照組織や患者・健常者の血液等を，ゲノム，エピゲノム，トランスクリプトーム，プロテオーム，メタボロームの5層のオミックスで測定し，多層的な関連解析を行うことにより，確度の高い疾患関連バイオマーカーや創薬標的の同定を目標としている．また測定データや対象試料の臨床情報を，データベース化して公開することを第2の目標としている．

脂質は，生理活性を有するリゾリン脂質，セラミド，酸化脂肪酸（プロスタグランジン等）などを含み，医薬品の有効性や副作用のバイオマーカーとなり得る．われわれは確立した脂質メタボローム測定系を用いて，ヒト疾患試料に関してバイオマーカーの探索を遂行しているが，まだ結論は得られていない．本稿では既に解析を終了した，拡張型心筋症モデルハムスター及びアルツハイマー病モデルマウスの結果を紹介したい（Fig. 2）．[20,21] 各疾患に特徴的に変動するバイオマーカーは，治療指標等の有効性サロゲートマーカーになり得ると考えられる．

筆者らは国立循環器病研究センター研究所との共同研究で，δ-サルコグリカン欠損による拡張型心筋

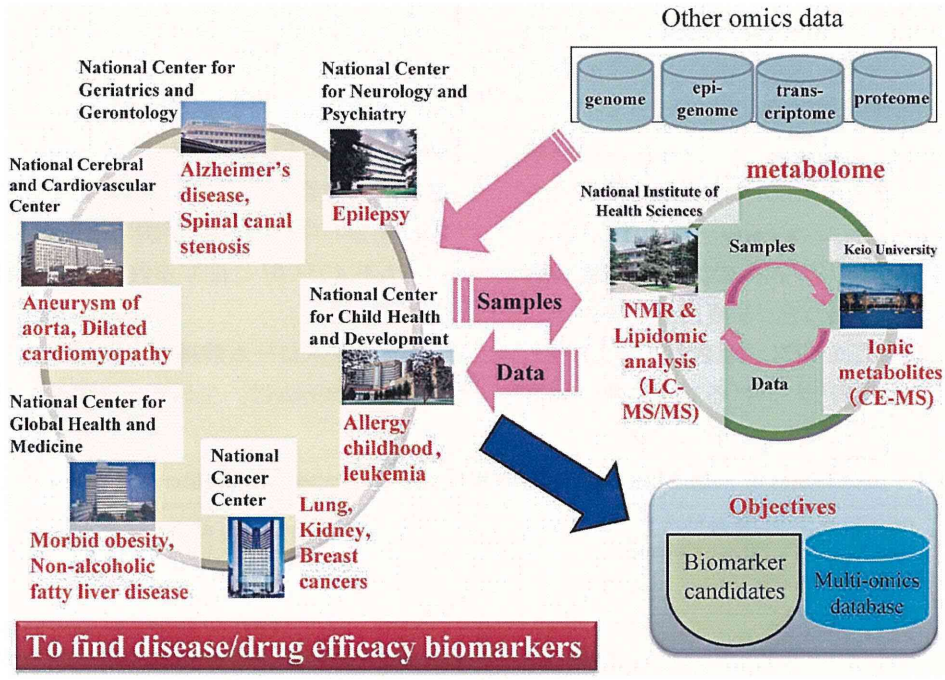

Fig. 1. Objectives and Framework of Multi-omics Research Projects on 12 Diseases

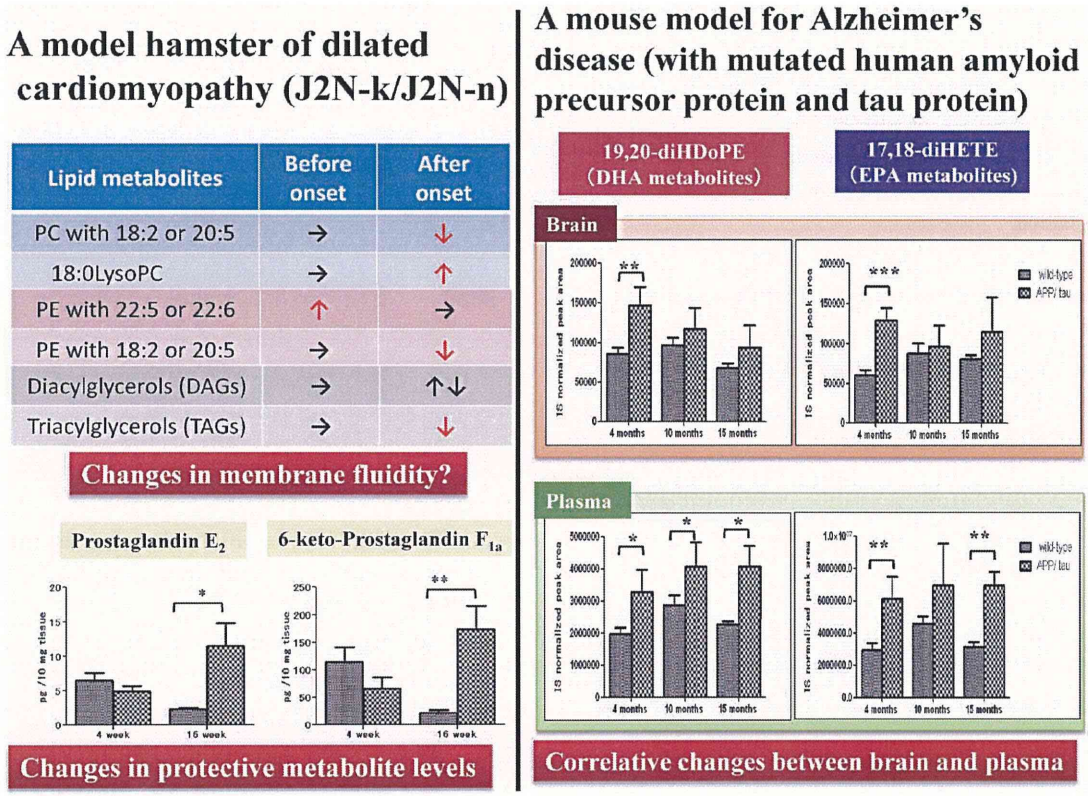

Fig. 2. Main Results from Lipidomic Analysis on Animal Models of Dilated Cardiomyopathy and Alzheimer's Disease

Left: Changes of lipid metabolites on cardiomyocytes from a hamster model of dilated cardiomyopathy. Right: Correlative changes of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) metabolites between the brain and plasma from a mouse model of Alzheimer's disease expressing mutated human amyloid precursor protein (APP) and tau protein. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

症モデルハムスター J2N-k と遺伝的に類似の J2N-n ハムスターの左心室を対象に，発症前（4 週齢）と発症後（16 週齢）についてメタボローム解析を行った．その結果，発症前からのドコサヘキサエン酸を含むホスファチジルエタノールアミン群の増加，発症後のリノール酸やエイコサペンタエン酸を含むホスファチジルコリン群やホスファチジルエタノールアミン群の減少，及び多くのトリグリセリド分子種の減少等を見い出した．さらに発症後において，心臓保護作用を有するプロスタグランジン類の増加が認められた．したがって，発症前からの心筋細胞膜の脂質組成変化，発症後には，脂質メディエーターを含む大幅な脂質変化が明らかとなった．[20] また，ヒト変異型アミロイド前駆タンパク質と変異型タウタンパク質を発現するアルツハイマー病モデルマウスでは，発症前の 4 ヵ月齢において，脳内で 19,20-ジヒドロキシドコサペンタエン酸（19,20-diHDoPE）及び 17,18-ジヒドロキシエイコサテトラエン酸（17,18-diHETE）の有意な増加が認められたが，同様の増加が血漿でも認められ，アルツハイマー病発症前のバイオマーカー候補と考えられた．[21]

5. 薬学研究者がバイオマーカー研究を行うための注意点

バイオマーカーを臨床応用するための一般的なスキームとしては，1) 臨床事象と関連するバイオマーカーの確立，2) 測定法の開発と分析精度等の検証，3) バイオマーカー診断の臨床的有用性の検証及び利用の費用対効果の確認，等が考えられ，いくつかのハードルが存在するものの，基礎研究の成果が臨床応用に結びつけ易いため，アカデミアの研究者が比較的参加し易い分野と言える．一般的な留意点としては，バイオマーカーの探索と同定段階では，1) バイオマーカーを探索する臨床的必要性があるテーマ選択（医師が希望する内容で，ある程度頻度が高いもの），2) 長期間継続可能で，システマティックな試料収集・解析体制（定期的な連絡会開催や専任 CRC の雇用），3) 探索・検証の 2 段階でのバイオ

Table 2. Points to Consider for Researches on Biomarker Identification

1.	Selection of a theme that is required for the clinical setting (with relatively high frequencies)
2.	Clear standards for patient recruitment (standard protocol) and collection of minimum but sufficient clinical information
3.	Establishing a systematic framework for sample collection and data analysis (*e.g.*, periodic meetings, employment of CRC)
4.	Constructing two-step association studies (exploration and validation)
5.	Secure enough research budget
6.	Mutual respect between clinical and basic sides in the research

マーカーの関連性確立（症例数の確保），などが挙げられる（Table 2）．さらに，バイオマーカーの臨床応用段階としては，1）正確度と診断精度が高く，安い診断系の開発，2）少数のマーカーを，なるべくステップの少ない方法で診断する，3）感度，特異度，陽性・陰性的中率が高いこと，などが挙げられる．バイオマーカーの同定段階では，網羅的に探索できるいわゆるオミックス手法がよく用いられるが，複数のオミックス手法を組み合わせることにより，同定する確度が高くなると考えられる．さらに有効性や副作用の評価指標としては，わが国で，若しくは国際的に統一されている基準を用いることが望まれる．また，最終的な臨床的有用性の証明のためには，前向き臨床研究によりバイアスによる影響を小さくすることが重要であり，医療経済学的な有用性の検証を行うことも，保険適用等の関係から望ましい．

6. おわりに

バイオマーカーの同定は比較的容易なものの，その臨床的有用性を確認し，実際に医薬品開発や医療現場で用いるに至るには，多くの分野の協力と社会的認知が必要である．その上でも，他分野の研究者・専門家との協力は不可欠であり，薬学分野のみに留まらず，積極的な交流が望まれる．そのためには，1）医師と対話し得る臨床的知識を有すること，2）薬物動態学，ゲノム，バイオマーカーの病態生化学的関連性，統計学，薬剤疫学などの独自の専門的知識を有すること，3）これら専門的知識に基づいて検討した内容を統合した妥当な臨床研究計画を立案すること，などが望まれる．薬学6年生により臨床的な知識を身につけた薬学研究者が増え，バイオマーカー研究の進展を通じて，わが国の創薬・医療レベルが向上することを期待したい．

謝辞　本稿で紹介した筆者らの報告の一部は，厚生労働省・厚生労働科学研究費補助金や医薬基盤研究所・先駆的医薬品・医療機器研究発掘支援事業の支援を受けてなされたものである．

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

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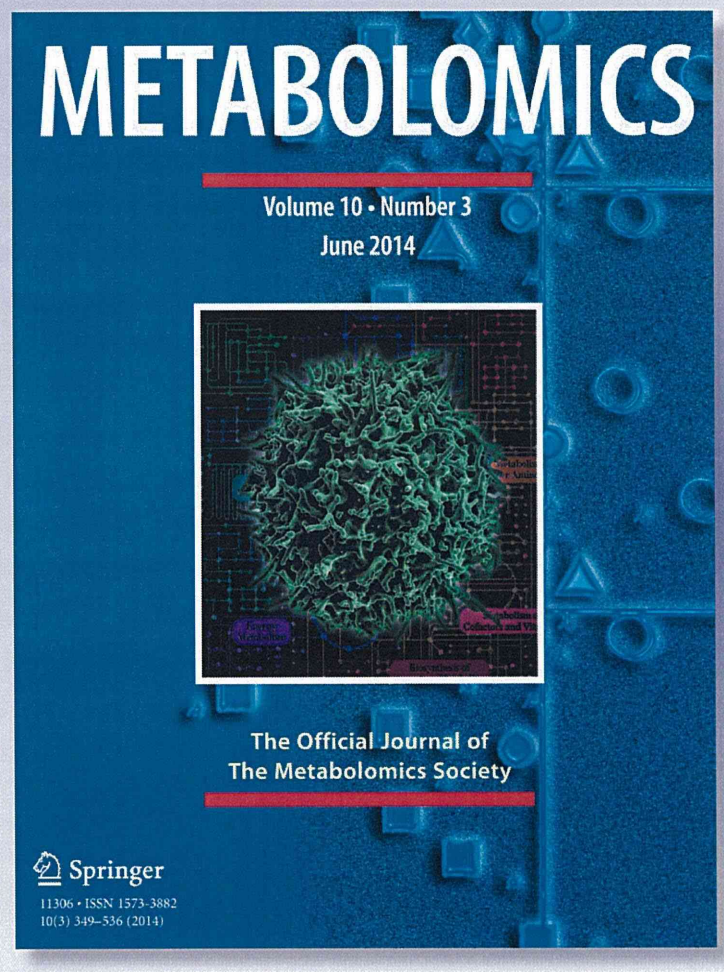
Metabolomics
An Official Journal of the Metabolomics Society

ISSN 1573-3882
Volume 10
Number 3

Metabolomics (2014) 10:402-413
DOI 10.1007/s11306-013-0591-0



Springer

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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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Received: 30 July 2013 / Accepted: 3 October 2013 / Published online: 22 October 2013

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).

Electronic supplementary material The online version of this article (doi:10.1007/s11306-013-0591-0) contains supplementary material, which is available to authorized users.

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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

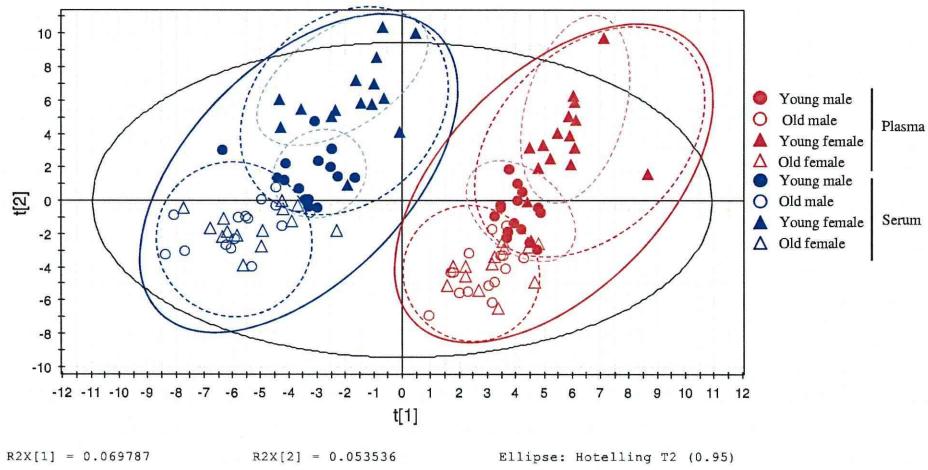

Fig. 1 OPLS-DA model of overall metabolic profiles. Data obtained from human plasma (*red*) and serum (*blue*) samples of young males (*closed circle*), old males (*open circle*), young females (*close triangle*), and old females (*open triangle*) were analyzed. The goodness-of-fit parameter R2 and the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