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Temporal Metabolic Profiling of Plasma during Endotoxemia in Humans

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Abstract

Endotoxemia induced by the administration of low-dose lipopolysaccharide (LPS) to healthy human volunteers is a well-established experimental protocol and has served as a reproducible platform for investigating the responses to systemic inflammation. Since metabolic composition of a tissue or body fluid is uniquely altered by stimuli and provide information about the dominant regulatory mechanisms at various cellular processes, understanding the global metabolic response to systemic inflammation constitutes a major part in this investigation complementing the studies undertaken so far in both clinical and systems biology fields. This article communicates the first proof-of-principle metabonomic analysis which comprised of global biochemical profiles in human plasma samples from healthy subjects given intravenous endotoxin at 2 ng/kg. Concentrations of a total of 366 plasma biochemicals were determined in archived blood samples collected from 15 endotoxin treated subjects at 5 time points within 24 hour post-treatment and compared with control samples collected from 4 saline treated subjects. Principal component analysis within this dataset determined the 6th hour as a critical time point separating development and recovery phases of the LPS induced metabolic changes. Consensus clustering of the differential metabolites identified two distinct subsets of metabolites which displayed common coherent profiles with opposing directionality. The first group of metabolites, which were mostly associated with pathways related to lipid metabolism, was up-regulated within the first 6 hr and down-regulated by the 24th hr following LPS administration. The second group of metabolites, in contrast, was first down-regulated until the 6th hr, then up-regulated. Metabolites in this group were predominantly amino acids or their derivatives. In sum, non-targeted biochemical profiling and unsupervised multivariate analyses highlighted the prominent roles of lipid and protein metabolism in regulating the response to systemic inflammation while also revealing their dynamics in opposite directions.

Keywords

Human; endotoxin; LPS; metabolomics; systems biology

Introduction

Elective administration of bacterial endotoxin (lipopolysaccharide; LPS) to healthy human subjects has been used as a reproducible experimental procedure providing mechanistic insights into how cells, tissues and organs respond to systemic inflammation. Low doses of LPS transiently alter many physiologic and metabolic processes in a qualitatively similar manner to those observed after acute injury and systemic inflammation (4, 5); thus allowing the analysis of the responses to infectious stress at multiple physiological levels. This model has been extensively employed for the development and assessment of rational clinical therapies to prevent or attenuate systemic inflammatory response syndrome (SIRS) (5). Beyond the advancement of clinical research on systemic inflammation and other relevant pathologies, global transcriptomic studies started to elucidate the intricate regulatory schemes governing the inflammatory response (6, 7) which has been used in the structural foundations of semi-mechanistic models of inflammation (8–10).

Response to endotoxemia is closely associated with alterations in metabolism. Inflammatory processes change the direction of the substrate flow from the periphery towards splanchnic organs while also triggering the release of catabolic signals in order to meet increased energy and substrate demands (11, 12); and hence, considerably altering the levels of plasma metabolites. Individual changes in the major metabolites, such as some lipids, amino acids and glucose, has been previously documented for the case of human endotoxemia (11). However, an untargeted, bioinformatics empowered approach to elucidate the effects of endotoxemia on the plasma metabolite levels is lacking.

Analysis of the complete metabolic response to systemic inflammation is of special interest since metabolic composition of a tissue is uniquely altered in response to stimuli due to collective effects of the regulations at various levels of cellular processes including transcription, translation and signal transduction. Concentrations of metabolites in a sample at a given time, i.e. the “metabolome” (13), can be thought of as the metabolic fingerprint representative of the state of body at that time and provide information on the dominant regulatory mechanisms. The emerging field of metabonomics, combines this unique metabolic information with bioinformatics approaches to provide an integrated temporal picture of the interactions in the system (14, 15). Since the ultimate phenotype is determined by eventual production of metabolites through the complex cellular processes trickling down from transcription, translation and signal transduction, this field offers promise in advancing the knowledge in many clinical conditions. For endotoxemia, understanding the alterations in plasma metabolome is critical; since, metabolite levels impacts the regulation of anti-inflammatory defenses, in turn, through steering critical cellular processes in immune cells (16).

This study constitutes the first attempt of a complete metabonomic analysis describing the alterations in plasma metabolite composition following exposure to LPS. Plasma samples, sequentially collected from healthy human subjects who had been administered LPS or placebo, were subjected to non-targeted biochemical profiling. Temporal profiles of 366 metabolites in 8 different categories at 5 time points within 24 hour post-treatment were obtained. The data was filtered to identify the significant changes in metabolite levels and enhance the information content. Unsupervised multivariate analyses highlighted the prominent roles of lipid and protein metabolism in regulating the response to systemic inflammation while also revealing their opposing dynamics.

Materials and Methods

Human Plasma Samples

Archived blood plasma samples which had been flash frozen were used in this proof-of-principle study. These samples had been collected from 19 healthy subjects, between ages 18–40, who provided written, informed consent under guidelines approved by the Institutional Review Board (IRB) of Robert Wood Johnson Medical School. 15 of the subjects (11 males and 4 females; mean age of 22.7) had been administered National Institutes of Health (NIH) Clinical Center Reference Endotoxin, at a bolus dose of 2 ng/kg body weight as previously described (1–3). 4 control subjects (3 males and 1 female; mean age of 22.2) had been administered placebo (saline). During the protocol, subjects had received a solution of 5% dextrose and 0.45% saline crystalloid. Blood draws had been conducted sequentially at t=1, 2, 6, and 24 hr from both groups, samples had been inventoried and stored at –80°C until the analysis.

Biochemical Profiling of Plasma Samples

Metabolomic analysis was performed by Metabolon (Durham, NC, USA) according to previously published methods (17). Briefly, samples were prepared by using a proprietary series of organic and aqueous extractions to attain the maximum recovery of small molecules while eliminating the protein fractions in plasma. The resulting extracts were subjected to either liquid chromatography (LC) or gas chromatography (GC) followed by mass spectroscopy (MS) analysis. The LC/MS platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The GC column, in which samples derivatized with bistrimethyl-silyl-trifluoroacetamide (BSTFA) were run, was 5% phenyl and the temperature ramp was from 40° to 300° C in a 16 minute period. Following GC, samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization.

A number of additional samples were included for quality assurance and quality control purposes including internal standards such as an extensively characterized large pool of human plasma sample and aliquots of the extraction solvents used, as well as a composite sample prepared by pooling all the samples in the current study and ultra-pure water used as process blank. Instrument variability was determined by calculating the median relative standard deviation (RSD) for all the internal standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all the measured metabolites (i.e., non-instrument standards) in the processed samples. Values for instrument and process variability, which met the acceptance criteria, were 6% and 11%, respectively.

The data extraction of the raw MS data files yielded information that were loaded into a relational database in which the information was examined and appropriate QC limits were imposed. Peaks were identified using Metabolon's proprietary peak integration software, and component parts were stored in a complex data structure. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. The combination of chromatographic properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity. For the samples which took multiple days to analyze, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. The quality control and curation processes were used to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, mis-assignments, and

background noise. The list of all the metabolites identified through this untargeted biochemical analysis are provided as supplementary material in Table S1. Significance of the LPS treatment with respect to placebo for individual metabolites at each time point was determined by ANOVA and calculated p-values were also included in Table S1.

Data analysis

After identification of the metabolites, the complete dataset of 366 metabolites with temporal profiles was rigorously analyzed through multiple steps. These included filtering for differential metabolites, principal component analysis (PCA) and clustering. Imputed and log transformed datasets were investigated to identify the metabolites which show differential temporal profiles between LPS and placebo groups by using software for the extraction and analysis of gene expression (EDGE) (18). The significance threshold for this test was set as q value <0.1 and p value <0.05. Using these differential metabolites, PCA was performed and the averages of first principal component (PC1) for each treatment group were plotted against time. One way ANOVA was performed to evaluate the significance of PC1 variance over time for each treatment group. Then, to compare PC1 values at each time point, Wilcoxon rank sum test is used (with 1% significance level). Finally, the datasets containing differential metabolites were concatenated to form one single matrix, which was then clustered through consensus clustering (19) (with p-value = 0.05) with the goal to identify the subsets of metabolites with coherent temporal profile in LPS and placebo groups. Interpretation of the biological significance these profiles demonstrate were based on the individual metabolite identities and curated metabolic pathways obtained from publicly available Kyoto Encyclopedia of Genes and Genomes (KEGG) (20) and Human Metabolome Database (21) as well as Ingenuity Knowledge Base (22).

Results

This study aimed to identify the major coherent patterns in human plasma metabolome within the 24 hours after systemic LPS exposure. The study design, a flowchart of which is shown in Figure 1, involved two groups of healthy subjects treated either with a bolus dose of 2 ng/kg body weight LPS or placebo (saline) injection at t=0. Both groups received a solution of 5% dextrose and 0.45% saline crystalloid during the protocol. The blood samples were collected from the subjects at 4 time points throughout 24 hr post-treatment and the response was determined via non-targeted biochemical profiling through MS analysis.

Global biochemical profiles obtained by GC-MS and LC-MS/MS platforms represented temporal information on 366 metabolites including amino acids, short peptides, carbohydrates, lipids, nucleotides, cofactors and vitamins, xenobiotics and intermediate products of major energy production pathways. Complete list of these metabolites are provided as supplementary materials along with ANOVA contrasts at each time point in Table S1. Since the main objective of this study was to identify the major dynamics in the plasma metabolome rather than teasing out each shift in the individual metabolites, we opted out to filter the data through an algorithm originally designed for gene microarray experiments. EDGE procedure utilizes an optimal discovery procedure that uses relevant information from all the elements in the dataset in order to test each for differential expression (18). By applying this algorithm to metabolome dataset we first identified metabolites with differential temporal profiles between LPS and placebo groups. 60 out of the 366 metabolites showed differential profiles which met p-value < 0.05 and q-value < 0.1 cut-offs of EDGE software. The utility of this filtering step was also evident from the change in cumulative distributions of data before and after EDGE as shown in Figure 1. While both treatment groups have almost uniform distributions when the complete metabolome dataset is used, LPS treatment group became distinguishable from placebo at certain time points when only the differential metabolites were included in the analysis.

To identify the dominant patterns among the temporal profiles of these differential metabolites, PCA was performed. The averages of the first two principal components (PC1 and PC2) for the two treatment groups were plotted against time and against each other in Figure 2a–c. As shown in the bar chart in Figure 2d, although much of the variance (63%) was captured by the PC1, PC2 had also contributed in explaining the variability between the subjects in two experimental groups. In average, subjects treated with LPS were clearly separated in both PC1 and PC2; while saline treated subjects showed less variation in PC2, but even lesser in PC1. Average PC1 was analyzed as a function of time in a one way between subjects analysis of variance (ANOVA) and results indicated that variation of PC1 over time for LPS groups is significant ($p\text{-value} = 1.38 \times 10^{-37}$) whereas for saline group it is not ($p > 0.01$). Significance analysis of the PC1 at each time point by Wilcoxon rank sum test identified the most significant difference between the two groups at 6 hr ($p\text{-value} = 0.00065$), which separated the development and recovery phases of the LPS induced metabolic changes. As shown in Figure 2a, at 24hr, average PC1 was still significantly different for the two groups, indicating that the recovery is still in progress.

Next, to identify the subsets of metabolites with common coherent profiles, we applied consensus clustering (23) to the metabolites having differential temporal profiles in between LPS and placebo groups. Clustering is an essential tool for the analysis of high-content data based on organization of the signals with similar behavior. Identification of the coherent patterns which intensify and weaken over time allows us to focus on closely associated interactions within the elements of the data. It also facilitates the recognition of temporal relationships between the sub-clusters of elements, which might imply regulatory hierarchy (19). It is worthwhile to note the refinement in the content of the data by comparing the difference between the empirical cumulative distributions of the clustered dataset from distributions in the previous datasets (Figure 1). In the clustered data, the distributions of LPS group became distinctly separated from the placebo group at each time point. Furthermore, in agreement with the PCA, distribution of the 6th hour data for LPS group displays an easily recognized divergence from the rest of the data. Consensus clustering of the differential metabolites further refined the data and returned 37 of the total of 60 differential metabolites, classified into one of the two clusters with opposing temporal directionality as shown in Figure 3. Metabolites in each cluster and their associations with the metabolic pathways are listed in Table 1. The first cluster (16 metabolites) was up-regulated within the first 6 hr; down-regulated by the end of 24th hr and was mostly composed of metabolites from pathways related to lipid metabolism. The second cluster (21 metabolites), in contrast, was down-regulated within the first 6 hr post-LPS; then up-regulated by the 24th hr. Strikingly 14 out of 21 metabolites in this cluster were amino acids or their derivatives and an additional 2 were dipeptides indicating a significant regulatory shift in the protein metabolism.

Discussion

This study identified the coherent changes in temporal patterns of plasma metabolite levels in response to low dose LPS exposure by using untargeted analytical methodology and unsupervised data analysis techniques. Most striking differences between treatment and control groups were observed in amino acid and lipid levels which displayed self-resolving patterns with different directionality forming two distinct clusters. While amino acids and amino acid derivatives were steadily cleared out from plasma; lipids, mostly mono- and poly-unsaturated fatty acids, accumulated within the first 6 hr following LPS administration, after which the direction of the response was reversed for these two distinct patterns indicating recovery.

Among the **first cluster** of metabolites; 13 out of total 16 were lipids, more specifically, essential and non-essential long chain fatty acids (FAs) including 4 omega-3 FAs, *docosahexaenoate (DHA)*, *docosapentaenoate (DPA)*, *eicosapentaenoate (EPA)*, *stearidonate*; and 2 omega-6 FAs, *dihomo-linoleate (eicosatrienoate)* and *docosadienoate*; and a major saturated FA, *stearate*, in addition to 3 pregnenolone derivatives taking part in steroid hormone biosynthesis. Coherent up-regulation pattern observed in these plasma FAs at 6 hr is consistent with the lipolysis, a well-known adaptive response to inflammation (11). The peripheral mobilization of lipid stores in the form of free FAs was initially considered as a result of catecholamine release in response to infection or injury; however increased biosynthesis and decreased oxidation in liver together with increased whole-body lipolysis are results of complex signaling interactions initiated by stress hormones such as catecholamines, as well as produced cytokines and LPS itself, collectively giving rise to accumulation of FAs in plasma. Since toll-like receptor 4 (TLR4) signaling initiated with recognition of LPS on the cell surface is responsible for expression of many cytokines all of which have major downstream effects on metabolism, teasing apart individual direct and indirect effects of each on lipid homeostasis requires further research (24).

More pronounced increase in omega-3 FAs compared to omega-6 FAs may be related to their differential roles in the inflammatory response. These two fatty acid groups have opposing physiological functions: While omega-6 FAs give rise to pro-inflammatory prostaglandin and leukotriene synthesis, omega-3 FAs compete with omega-6 FAs to modulate this response by inducing the production of less inflammatory derivatives (25). Although, speculative at this level of global metabonomic analysis, selective concentration of omega-3 FAs in plasma in the initial 6 hr of response might have contributed to the resolution and recovery in the following hours. Since dietary supplementation of omega-3 FAs are shown to be associated with a moderate quenching effect on inflammation, this speculation based on the observed selective increase of omega-3 FAs might not be far from truth and might have served as an endogenous adaptive mechanism to suppress inflammation (26). Interestingly, although increasing levels of free FAs in plasma has been associated with insulin resistance (27), glucose levels or associated metabolites in clustering analysis did not reflect a significant perturbation in any of the time points. This might have been related to the relatively fast and subtle kinetics of those metabolites.

Elevated *2-hydroxybutyrate* (or α -hydroxybutyrate; AHB) levels usually point towards increased oxidative stress because AHB is a by-product in the pathway leading to glutathione synthesis from methionine. The activity of this pathway (from methionine $\rightarrow$ cystathionine $\rightarrow$ cysteine $\rightarrow$ glutathione as shown in part of Figure 4) varies in response to the demands against elevated cellular oxidative stress (28, 29). Increased oxidative stress shifts the flow of homocysteine away from transmethylation to methionine toward transulfuration to cystathionine, increasing the flow towards glutathione synthesis. Glutathione is one of the most important antioxidant proteins and plays a crucial role in mitigating the oxidative damage of reactive oxygen species, formation of which in liver is potentially triggered by inflammation (30). Therefore, increased AHB levels at 6 hr post-LPS coinciding with plummeting levels of *methionine*, *serine*, *cysteine* and *glycine* at the same time point can be interpreted as an indication of increased activity of hepatic oxidative defense mechanisms to effectively regulate the inflammatory response induced by LPS. Reverse of the first conversion in this pathway (homocysteine $\rightarrow$ methionine) is possible with incorporation of methyl groups to methionine. One source of the methyl groups for this reaction is betaine, which is derived from *choline* (31). Choline is in the second cluster which shows similar kinetics with the opposite direction of the first cluster, consistent with the opposing directionality in the reactions in this pathway.

3-methyl-2-oxobutyrate (or α -ketoisovaleric acid, KIV) is a branched chain keto-acid (BCKA) and a degradation product of valine which is formed in the initial step of branched chain amino acid (BCAA) catabolism. This conversion exclusively takes place in skeletal muscle due to relatively high activity of BCAA aminotransferase and it is an essential part of the BCAA-BCKA cycling between liver and muscle (32, 33). Increase in KIV levels following LPS exposure occurs at the same time where *valine* concentrations are decreased in plasma, indicating an increase in BCAA catabolism to meet the increased metabolic demands of liver, which can utilize KIV for transamination to other BCAAs for incorporation into acute phase proteins, or complete their degradation for energy production.

The accumulation of intermediates in the steroid hormone biosynthesis pathways such as *21-hydroxypregnenolone*, *pregnenolone sulfate*, *pregnanediol* in LPS treated subjects may suggest an increased capacity for steroid biogenesis which is required for the production of hormones to regulate glucose homeostasis and suppress inflammation. Increase in various derivatives of corticosteroid hormones were anticipated considering the primary roles of these hormones in regulation of inflammatory response and metabolism, and also were consistent with earlier studies (11, 27).

The **second cluster** displayed a response pattern almost exactly in the opposite direction of the first cluster. Concentrations of the metabolites in this cluster gradually decreased until 6 hr after LPS administration, preceding a full recovery period in the following 18 hr. 14 out of 21 metabolites within this cluster were amino acids, strongly indicating their primary role in the immediate response to inflammatory insult. These 14 amino acids include 12 proteinogenic amino acids (*asparagine*, *cysteine*, *methionine*, *glycine*, *serine*, *histidine*, *lysine*, *tyrosine*, *tryptophan*, *isoleucine*, *leucine* and *valine*) and 2 core members of urea cycle (*citrulline* and *ornithine*). Presence of members of the urea cycle together with amino acid degradation pathway intermediates (*isobutyryl carnitine* and *deoxycarnitine*) indicates that amino acids are not only used as the building blocks for the acute phase proteins in liver, but also utilized as the substrates for energy production. Compensation for this rapid clearance of amino acids from plasma starts after the 6th hour and is achieved possibly by the breakdown of protein reserves in skeletal muscle. Presence of proteolytic breakdown products (*gamma-glutamylleucine*, *gamma-glutamyltyrosine*) in this cluster might be associated with this process being incomplete.

Taurolithocholic acid 3-sulfate is a product of bile acid sulfation, which is a minor pathway under normal conditions. In the presence of intrahepatic cholestasis, associated with inflammation (12), this reaction escalates, increasing the aqueous solubility and consecutively renal clearance of these compounds (34). Therefore, an increase in the plasma concentration of these sulfated bile acids might indicate a decline in the renal function in response to LPS-induced inflammation. Furthermore, increased *phosphate* levels have also been suggested as a potential risk factor linked to renal failure (35). These two independent markers of declined renal function also being associated with the same temporal pattern, therefore, might be suggestive of an impairment of normal renal function in the LPS treated subjects.

It should be emphasized that, although experimental model of human endotoxemia simulates systemic inflammation fairly well, it can be best described as a TLR4 agonist induced systemic inflammation (5). In this experimental model, the subjects are pre-screened medically to confirm normal general health; therefore care should be taken when extrapolating the implications of the results to clinically more complex conditions, such as sepsis. Another limitation of the study is related to the utilized data filtering and clustering procedures. Although these techniques ensure that subsets of metabolites with coherent temporal profiles are captured; at the same time, they might have masked some subtle

changes which might be significant but not necessarily correspond to the observed dominant patterns. Metabolites which have a quickly resolving perturbation early in the time course, such as lactate (36), can be an example to this limitation. Furthermore, there is limited number of time points in the study and that the last two time points are considerably far from each other. We observe significant changes in the metabolites starting at 6h which mostly resolve by 24h; however it is likely that some metabolites with different kinetics and show perturbations between these two time points might have been overlooked.

In summary, LPS administration in healthy humans significantly alters the homeostasis of lipid and protein metabolism in humans in the first 6hr. Within 24hr post-treatment, metabolite balances are mostly restored. Perturbation observed in the levels of plasma lipids may well be associated with the established lipolytic effect of inflammation, whereas amino acid deficiency observed early in response is likely due to increased hepatic uptake to meet the higher substrate demand for the synthesis of acute phase proteins and anti-oxidant defenses. Increase in some of the markers associated with renal failure later in the time course suggested that kidney function may have been deteriorated in subjects treated with LPS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

AHB	α -hydroxybutyrate or 2-hydroxybutyrate
BCAA	Branched chain amino acid
BCKA	Branched chain keto-acid
BSTFA	bis(trimethylsilyl)-trifluoroacetamide
DHA	Docosahexaenoate
DPA	Docosapentaenoate
EDGE	Extraction and analysis of gene expression
EPA	Eicosapentaenoate
ESI	electrospray ionization
FA	Fatty acid
GC/MS	Gas chromatography – mass spectrometry
KIV	α -ketoisovaleric acid or 3-methyl-2-oxobutyrate
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LIT	linear ion-trap (mass analyzer)
LPS	Lipopolysaccharide, endotoxin
PC1	First principal component
PCA	Principal component analysis

SIRS	Systemic inflammatory response syndrome
TLR4	Toll-like receptor 4

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Figure 1.

Study flowchart illustrating sample acquisition, biochemical profiling through MS, and data analysis steps. Diagrams below each data symbol display empirical cumulative distribution of the corresponding dataset, with the number of elements indicated below the data symbols.

Figure 2. (a) Temporal changes in averaged PC1 for LPS and placebo treated subjects. (b and c) Trajectory averages in PC1-PC2 coordinates (b) and time-PC1-PC2 space (c). (Star sign indicates significance ($p < 0.01$) measured by Wilcoxon rank sum test and error bars indicate standard error of the mean).

Figure 3. Heat map displaying the differential patterns of metabolic response to LPS. Two clusters of plasma metabolites reflect two distinct patterns with opposing temporal directionality. Clustered metabolites and their associations with the metabolic pathways are also listed in Table 1.

Figure 4.

Pathway associations illustrating the conversion of methionine to one of the major anti-oxidants, glutathione. Metabolites captured in the clustering analysis are indicated with a the name of the cluster and a color bar representing up-(red) or down-regulation (green) at 6 and 24 h time points.

Table 1

Distribution and classification of the differential metabolites to the clusters shown in Figure 3.

	BIOCHEMICAL	SUB-PATHWAY	SUPER PATHWAY
Cluster 1	2-hydroxybutyrate (AHB)	Cysteine, methionine, SAM, taurine metabolism	Amino acid
	3-methyl-2-oxobutyrate	Valine, leucine and isoleucine metabolism	
	Docosahexaenoate (DHA; 22:6n3)	Essential fatty acid	Lipid
	Docosapentaenoate (DPA; 22:5n3)		
	Eicosapentaenoate (EPA; 20:5n3)		
	Tetradecanedioate	Fatty acid, dicarboxylate	
	Stearidonate (18:4n3)	Long chain fatty acid	
	Dihomo-linoleate (20:2n6)		
	Docosadienoate (22:2n6)		
	10-nonadecenoate (19:1n9)		
	Eicosenoate (20:1n9 or 11)		
	Stearate (18:0)		
	21-hydroxypregnenolone disulfate	Sterol/Steroid	
	Pregn steroid monosulfate		
	Pregnen-diol disulfate		
	Phenolphthalein beta-D-glucuronide	Detoxification metabolism	Xenobiotics
Cluster 2	Asparagine	Alanine and aspartate metabolism	Amino acid
	Cysteine	Cysteine, methionine, SAM, taurine metabolism	
	Methionine		
	Glycine		
	Serine		
	Histidine	Histidine metabolism	
	Lysine	Lysine metabolism	
	Tyrosine	Phenylalanine & tyrosine metabolism	
	Tryptophan	Tryptophan metabolism	
	Citrulline	Urea cycle; arginine, proline metabolism	
	Ornithine		
	Isobutyrylcarnitine	Valine, leucine and isoleucine metabolism	
	Isoleucine		
	Leucine		
	Valine		
	Phosphate	Oxidative phosphorylation	Energy
	Taurolithocholate 3-sulfate	Bile acid metabolism	Lipid
	Deoxycarnitine	Carnitine metabolism	
	Choline	Glycerolipid metabolism	
	Gamma-glutamylleucine	Gamma-glutamyl	Peptide
Gamma-glutamyltyrosine			