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Lipidomics reveal aryl hydrocarbon receptor (Ahr)-regulated lipid metabolic pathway in alpha-naphthyl isothiocyanate (ANIT)-induced intrahepatic cholestasis

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Abstract

1. Ultra-performance liquid chromatography coupled with electrospray ionization quadrupole mass spectrometry (UPLC-ESI-QTOF MS)-based lipidomics was employed to elucidate new mechanism of alpha-naphthyl isothiocyanate (ANIT)-induced intrahepatic cholestasis in mice.
2. Multiple lipid components significantly increased in ANIT-induced intrahepatic cholestasis, including PC 16:0, 20:4, PC 16:0, 22:6, PC 16:0, 18:2, LPC 18:2, PC 18:2, LPC 18:1, PC 18:1, 14:0, SM 18:1, 16:0, oleoylcarnitine and palmitoylcarnitine. This alteration of lipid profile was induced by the changed expression of genes choline kinase (Chk) a, sphingomyelin phosphodiesterase (SMPD) and stearoylcoenzyme A desaturase 1 (SCD1).
3. Knockout of aryl hydrocarbon receptor (Ahr) in mice can significantly reverse ANIT-induced intrahepatic cholestasis, as indicated by lowered ALT, AST and ALP activity,

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Disclosure statement

No potential conflict of interest was reported by the authors.

and liver histology. Aryl hydro-carbon receptor knockout significantly reversed ANIT-induced lipid metabolism alteration through regulating the expression of Chka.

4. In conclusion, this study demonstrated ANIT-induced lipid metabolism disruption might be the potential pathogenesis of ANIT-induced intrahepatic cholestasis in mice.

Keywords

Intrahepatic cholestasis; alpha-naphthyl isothiocyanate (ANIT); lipidomics; aryl hydrocarbon receptor (Ahr)

Introduction

Lipids, conventionally regarded as major constituents of cell membrane, have been demonstrated to play a key role as the regulator towards a wide range of biological process (Lam & Shui, 2013). More and more evidences have demonstrated the important physiology function of lipid metabolic networks, and a complete snapshot of lipid profile is an important prerequisite to versatile functions of lipids. Lipidomics, a lipid-targeted metabolomics approach, can give a complete lipids profile through comprehensive analysis of lipids in biological system. Lipidomics research will provide insights for new mechanisms of diseases, toxicity, and discovery of new therapeutic targets (Murphy & Nicolaou, 2013). Transcriptome can profile the genes actively expressed at any given time through using high-throughput techniques based on DNA microarray technology (Garrido et al., 2013). The lipid metabolic pathway can be clearly elucidated through the combination of lipidomics (evaluating lipid metabolic profile) and transcriptomics (evaluating lipid metabolic genes).

Alpha-naphthyl isothiocyanate (ANIT) is a hepatotoxicant used in rodents to model human intrahepatic cholestasis. ANIT-induced liver damage has been regarded to be a useful model to study the drug-induced cholestasis, because some drugs (e.g. erythromycin estolate, chlorpromazine, etc.)-induced liver damage and cholestasis in human can be well mimicked by the exposure of ANIT to rodents (Kossor et al., 1993). Drug toxicity mechanism can be well understood through deeply elucidating ANIT-induced liver toxicity in rodents. Multifaceted mechanisms have been indicated to contribute ANIT-induced liver damage, including metabolic factors and inflammation factors (Dietrich et al., 2001; Faiola et al., 2010; Kodali et al., 2006).

The disturbance of ANIT treatment towards the compositions of lipids and lipoproteins has been reported in the previous literatures (Chisholm & Dolphin, 1996). The levels of cholesterol and phospholipids (PLs) significantly increase after 48-h exposure of ANIT (100 mg/kg), and the increased expression of apolipoproteins A-I and E is associated with the increased lipids. Additionally, the accumulation of these lipoproteins can be diminished in human apolipoprotein A-I transgenic rats (Chisholm et al., 2000). To date, the comprehensive view towards lipid components has not been rigorously examined.

Aryl hydrocarbon receptor (Ahr), a basic-helix-loop-helix (bHLH) Per-Arnt-Sim transcription factor, has been well known for its key role in xenobiotic-induced toxicity and

carcinogenesis (Barouki et al., 2007). Ahr has been regarded as a key player in different liver toxicity model. The Ahr-null mice can exert resistant to a variety of TCDD-induced liver toxicity (Matsubara et al., 2012). Ahr activation can significantly enhance the liver damage induced in bile duct-ligated mice (Ozeki et al., 2011). To date, the Ahr's role in ANIT-induced liver damage has just been investigated in see-through medaka which is a widely used fish model, and the results indicated that Ahr agonist partially protected against acute hepatobiliary alteration in this model (Volz et al., 2008). However, the role of Ahr for ANIT toxicity remains unclear in rodents which are the common and suitable models for investigation of disease, toxicity, and drug therapy for human.

The present study aims to investigate the alteration of lipid metabolic pathways affected by ANIT-induced liver toxicity using the combination of lipidomics and transcriptomics. Additionally, the insensitivity of Ahr-null mice towards ANIT-induced liver toxicity was demonstrated through reversing this kind of alteration.

Materials and methods

Chemicals and reagents

1-Naphthyl isothiocyanate (N4525–10G), oleoylcarnitine and palmitoylcarnitine were purchased from Sigma-Aldrich (St. Louis, MO). The standards of PC16:0, 20:4, PC16:0, 22:6, PC16:0, 18:2, LPC18:2, PC18:2, SM18:1, 16:0, PC18:1, 14:0 were purchased from Avanti Polar Lipids (Alabaster, AL).

Animals and treatment

The Ahr^{-/-} mice and corresponding wild type mice have been described in the previous literatures (Matsubara et al., 2012). All animal experiments were carried out in accordance with the Institute of Laboratory Animal Resources guidelines and approved by the National Cancer Institute Animal Care and Use Committee. Groups of 6–8-week old mice were administered with oral gavage of a single dose of ANIT (75 mg/kg) dissolved in corn oil. The mice were sacrificed by CO₂ asphyxiation 48 h after ANIT administration according to the previous literatures (Fang et al., 2017; Wang et al., 2017). The blood samples were collected in BD Microtainer™ serum separator tubes (Franklin Lakes, NJ) by retro-orbital bleeding, and serum was obtained through centrifugation for 15 min at 8000×g. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) catalytic activities, total cholesterol (TC), and PL in serum were determined using commercial assay kit (Catachem, Bridgeport, CT) according to the manufacturer's instructions. Liver was collected and divided into two parts. One part was fixed in 10% PBS-buffered formalin for histological analysis, and the other was frozen in liquid nitrogen for mRNA and protein expression analysis.

Liver histopathology analysis

The histopathology analysis was carried out as previously described (Patterson et al., 2012). Tissue blocks were embedded in paraffin and cut into 4-mm-thick sections. The sections were stained by the hematoxylin and eosin method.

UPLC-ESI-QTOFMS-based lipidomics analysis of serum

Serum lipids were extracted as previously described (Li et al., 2013). In brief, serum samples (volume = 25 μ L) were mixed with four-volume chloroform/methanol (v/v = 2:1). After centrifugation at 18,000 $\times$ g for 20 min, the lower organic phase was taken and evaporated under an N₂ stream. Then, the lipid extract was dissolved in 25 μ L chloroform/methanol (1:1) and diluted to a final solution with 10-volume isopropanol/acetonitrile/water (50/25/25). The aliquots (10 μ L) were used to undergo UPLC-ESI-QTOFMS analysis under the positive mode. The separation of serum lipid components was performed using an Acquity UPLC BEH C18 column (1.7 μ m, 2.1 50 mm, Waters Corp., Milford, MA). The mobile phase was comprised of 0.1% aqueous formic acid (A) and acetonitrile containing 0.1% formic acid. The following gradient elution was used: 100% A for 5 min, increased to 100% B over the next 7.5 min and returned to 100% A in last 2 min. Nitrogen was used as both cone gas (50 L h⁻¹) and desolvation gas (600 L h⁻¹). Source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. The capillary voltage and cone voltage were 3000 and 20 V, respectively.

Multivariate data analysis and lipid components identification

MassLynx software (Waters Corp., Milford, MA) was used to acquire the chromatogram and mass spectrometric data in centroid format. After the generation of a multivariate data matrix, the data set was exported into SIMCA-P + 12.0 (Umetrics, Kinnelon, NJ) for further analysis, as previously described (Fang et al., 2013). The ions with high contribution scores in the S plot were identified through searching in the metabolomics database (Human Metabolome Database (HMDB)), and some ions were furtherly identified with commercially obtained standards.

RNA analysis

The present study contained RNA microarray analysis and quantitative polymerase chain reaction (qPCR) analysis. For microarray analysis, dye-coupled complementary DNAs (cDNAs) were purified with a MiniElute PCR purification kit (Qiagen, Hilden, Germany) and hybridized to an Agilent 44K mouse 60-mer oligo microarray (Agilent Technologies, Santa Clara, CA). Genespring GX software (Agilent Technologies, Santa Clara, CA) was used to process and analyze the data. Compared with control samples, the genes with >2-fold alteration was regarded to be significantly changed genes. For qPCR study, TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract the total liver RNA, and cDNA generated from 1 μ g RNA with a SuperScript IITM Reverse Transcriptase kit and random oligonucleotides (Invitrogen, Grand Island, NY). Quantitative PCR (qPCR) was performed using SYBR green PCR master mix and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Messenger RNA quantitation was performed using the comparative cycle threshold (CT) method, and results were normalized to mouse 18S RNA.

Data analysis

The experimental data were given as mean $\pm$ SEM. Statistical analysis was carried out using GraphPad Prism 5.0 (San Diego, CA). Comparisons between two groups were performed

using a two-tailed unpaired Student's *t* test or Mann–Whitney's *U* test. Multiple groups were compared using a two-way ANOVA.

Results

The hepatic toxicity behavior of ANIT

After treated with ANIT (75 mg/kg) for 48 h, the strong liver toxicity occurred. The serum in the ANIT-treated mice showed more yellow color than vehicle-treated mice (Figure 1(A)). Histological analysis of liver sections in ANIT-treated group presented large areas of necrosis of the biliary epithelium of interlobular bile ducts, hepatocytic injury and necrosis, and inflammatory cell infiltration (Figure 1(B), Supplemental Figure 1). The activity of ALT ($p < 0.01$), AST ($p < 0.001$) and ALP ($p < 0.05$) was significantly increased in ANIT-treated mice (Figure 1(C)). The level of PL in serum increased by 3.7-fold ($p < 0.001$), and the level of TC in serum increased by 2.06-fold ($p < 0.001$) (Figure 1(D)).

ANIT treatment induced the alteration of lipidomics profile

UPLC-ESI-QTOFMS analysis coupled with MDA was utilized to profile the serum lipids. Unsupervised principle components analysis (PCA) of the serum from the ANIT-treated and untreated mice revealed the well separation, as shown in Figure 2(A). Furthermore, scatter loading plot was used to search the major ions contributing to this separation, and the 20 topped ions are listed in Table 1. Through the mass-based searches in metabolomics databases (Figure 2(B,C)), these components were proposed to be PC, LPC, SM components or fatty acid–carnitine conjugates. Some lipid components were furtherly confirmed through the comparison of the retention time and MS/MS fragmentation patterns between the standard and the serum samples (Supplemental Figure 2A–K). These lipid components were identified to be PC 16:0, 20:4, PC 16:0, 22:6, PC 16:0, 18:2, LPC 18:2, PC 18:2, LPC 18:0, LPC 18:1, PC 18:1, 14:0, SM 18:1, 16:0, oleoylcarnitine and palmitoylcarnitine. Furthermore, the time-dependent alteration of these lipid components was investigated, and the results are given in Figure 3. For PC 16:0, 20:4 and PC 16:0, 22:6, the significant difference was observed at 10 h after treatment of ANIT ($p < 0.01$), and its level in serum continued elevation until the termination of this experiment (48 h). For LPC 18:2, oleoylcarnitine, palmitoylcarnitine, PC 16:0, 18:2 and PC 18:2, the significant difference was detected at 3 h after treatment of ANIT, and the trend continued until the end of this experiment. The increased trend of PC 18:1, 16:0 in ANIT-treated group appeared between 20 h and 48 h of ANIT treatment. The significant elevation of LPC 18:1 and SM 18:1, 16:0 only occurred at 40 h and 48 h after the treatment of ANIT.

ANIT treatment affects gene expression involved in lipid metabolism

The expression of the genes related with the metabolic homeostasis of these lipid components was investigated. The relevant enzymes involved in the metabolic conversion of these lipid components are shown in Figure 4. Microarray results showed that the genes in many metabolic pathway were affected after treatment with ANIT, and the genes related with the metabolic pathways of PC, LPC, SM and fatty acid–carnitine conjugates are given in Figure 4(A), and the corresponding changes are shown in Figure 4(B) with the heat map. Furthermore, Q-PCR was used to confirm the microarray results. After ANIT treatment, the

expression of SMPD3 was significantly increased by 11.4-fold ($p < 0.05$). However, no marketed difference was observed for the gene expression of SMPD1, SMPD2 and SMPD4 (Figure 5(A)). ANIT treatment did not alter the expression of PLD1 and PLD2 (Figure 5(B)). As shown in Figure 5(C), the treatment of ANIT markedly increased the expression of Chka (8.2-fold, $p < 0.05$), and significantly inhibited the mRNA level of stearoyl-coenzyme A desaturase 1 (SCD1) ($p < 0.05$). The RNA expression of Pcyt1a, Pcyt1b, Pcyt2 and Chpt1 was not influenced by ANIT treatment. The results shown in Figure 5(D) also indicated no effect of ANIT treatment towards the expression of Lpcat1–4. ANIT treatment induces the expression of representative target genes of Ahr, Cyp1b1 and Cyp1a1 (Supplemental Figure 3). We also directly demonstrated the activation of AhR by ANIT using XRE luciferase experiment (Supplemental Figure 4).

Ahr-null mice are insensitive to ANIT-induced liver injury and lipid profile alteration

After the treatment of ANIT for 48 h, the serum color in *Ahr-null* mice showed normal color, which is with the serum color in vehicle-treated *Ahr-null* mice. However, the same treatment time with the same dose of ANIT induced more yellow color than the serum in control group (Figure 6(A)). The histology alteration induced by ANIT in WT mice can be reversed by the knockout by Ahr (Figure 6(B) and Supplemental Figure 5). Additionally, the elevated activity of ALT, AST and ALP, and the elevation of serum level of PL and TC in ANIT-treated WT mice did not appear in ANIT-treated *Ahr-null* mice (Figure 6(C,D)). The influence of Ahr knockout towards the lipid profile alteration was furtherly investigated. The *Ahr-null* mice can significantly prevent the elevation of all lipid components induced by ANIT treatment (Figure 7), including PC16:0, 20:4 ($p < 0.001$), PC18:2 ($p < 0.001$), PC16:0, 22:6 ($p < 0.001$), PC 16:0, 18:2 ($p < 0.001$), PC 18:1, 16:0 ($p < 0.001$), LPC 18:2 ($p < 0.001$), LPC 18:1 ($p < 0.001$), SM 18:1, 16:0 ($p < 0.01$), oleoylcarnitine ($p < 0.001$) and palmitoylcarnitine ($p < 0.001$). For gene expression, ANIT treatment cannot increase the expression of Chka in *Ahr-null* mice. However, like ANIT-treated WT mice, ANIT treatment in *Ahr-null* mice also induced the expression of SMPD3, and inhibited the expression of SCD1 (Figure 8).

Discussion

Lipids homeostasis plays a key role in maintaining the healthy body. The disruption of lipid homeostasis was observed under various diseases and toxicity conditions of xenobiotics (Meikle & Christopher, 2011). Liver is the major organ for lipid metabolism, so, the liver damage can always induce the metabolic disruption of lipids. Global view of lipids profile through metabolomics or lipidomics can accelerate our understanding of mechanism of disease or toxicity, and the corresponding technologies have been widely employed to elucidate the liver injury mechanism induced by diseases or xenobiotics. Fernando et al. used nuclear magnetic resonance (NMR)-based lipidomics method to establish biomarker signatures after long-term exposure of rat liver to ethanol which can reflect the progression and severity of alcoholic liver disease (Fernando et al., 2011). In nonalcoholic steatohepatitis (NASH) induced by administration of methionine- and choline-deficient (MCD) diet, the levels of lipid components LPC 16:0, LPC 18:0 and LPC 18:1 in serum significantly decreased due to the increased hepatic inflammation signaling (Tanaka et al., 2012). The

alteration of lipid profile has also been observed in cholestasis models. For example, lithocholic acid (LCA)-induced cholestasis can significantly disrupt the homeostasis of PL and sphingolipid, as marketed by decreased LPC 16:0, LPC 18:0, LPC 18:1 and LPC 18:2 (Matsubara et al., 2011).

The alteration of lipid profile induced by ANIT treatment was investigated in the present study, and many lipid components changed, including PC 16:0, 20:4, PC 16:0, 22:6, PC 16:0, 18:2, LPC 18:2, PC 18:2, LPC 18:1, PC 18:1, 14:0, SM 18:1, 16:0, oleoylcarnitine and palmitoylcarnitine. Furthermore, the relevant molecular mechanisms resulting in the alteration of these lipid components were investigated. Choline kinase (Chk) a, a key enzyme catalyzing the first reaction in the choline pathway for biosynthesis of PC (Bansal et al., 2012), was detected to increase in ANIT-induced liver injury, which can explain why the level of many PC components increased in ANIT-treated mice. The elevated level of PC furtherly resulted in the increase of LPC, SM and fatty acid–carnitine conjugate through the biotransformation of PC components towards these components. The alteration of lipid components was furtherly complicated by the altered expression of Smpd3 and Scd1. Sphingomyelin phosphodiesterase (SMPD), a hydrolase enzyme converting SM to PC components (Hannun, 1994), can furtherly increase the levels of PC through biotransformation of SM to PC. Stearoyl-CoA desaturase 1 (SCD1), an endoplasmic reticulum (ER) enzyme is responsible for biosynthesis of oleic acid (18:1) and palmitoleic acid (16:1) from stearic acid (18:0) and palmitic acid (16:0), respectively. The inhibition of SCD1 has been reported to increase the level of saturated LPC and decrease the level of unsaturated LPC (Chen et al., 2008). Therefore, the alteration of lipid components in ANIT-treated mice will be the final results affected by the combined change of all these genes.

Different with previous fish model in which the agonist of Ahr can partially protect against acute hepatobiliary alteration induced by ANIT, the Ahr-KO mice exhibited strong resistance towards ANIT-induced liver injury. Additionally, the altered lipid profile in ANIT-treated mice was significantly reversed in Ahr-KO mice, and the reason might be that Ahr-KO mice prevented the ANIT-induced expression of Chka. Due to the embryonic lethal property for loss of Chka (Wu et al., 2008), we tried to create mice with a conditional Chka mice. However, we failed to get this mice model. It should be noted Ahr KO mice have lower levels of Chka, implying that ANIT regulates Chka expression through Ahr. Indeed, Moffat et al. have reported that Chka mRNA was up-regulated in rats but was significantly downregulated in Ahr-null mice (Moffat et al., 2010). This implies that Ahr KO rats might respond very differently with Ahr KO mice. The regulation of Ahr-KO towards ANIT-induced lipids profile alteration might be a potential protection mechanism of Ahr knockout towards ANIT-induced toxicity. For example, LPC components have been widely investigated as the pro-inflammation factors which can activate some immune cells such as monocytes and neutrophils (Liu-Wu et al., 1998; Nishioka et al., 1998). Therefore, the decrease of LPC components in ANIT-treated Ahr-KO mice might prevent the ANIT-induced liver damage through weakening the inflammation reaction. Additionally, a recent study for the role of SM in intestinal epithelial cells showed that SM treatment increased the apoptosis of intestinal epithelial cells (Leucht et al., 2014), indicating the decreased SM level might be beneficial for prevention of cell apoptosis. The increased level of long-chain

fatty acid–carnitine conjugates have been observed in many hepatotoxicity models induced by many compounds, such as acetaminophen (Chen et al., 2009).

In conclusion, as shown in Figure 9, ANIT treatment strongly altered the lipid profile as demonstrated by lipidomics. Ahr knockout can reverse the lipid profile through reversing the alteration of Chka gene expression. Further deeper mechanistic studies should be investigated in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

ANIT-induced liver toxicity in C57BL/6 wildtype mice. (A) The representative serums from control and ANIT-treated mice. (B) H&E staining of livers from control and ANIT-treated mice. (C) Serum ALT, AST and ALP enzyme levels from control and ANIT-treated mice. Four to five mice in each group, and the values were given as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (D) Phospholipids (PL) and total cholesterol (TC) level in the serum from control and ANIT-treated mice. Four to five mice in each group, and the values were given as mean $\pm$ SEM. $p < 0.01$; *** $p < 0.001$.

Figure 2. Metabolomics analysis of serum in control and ANIT-treated C57BL/6 wildtype mice. (A) PCA scores scatter plot for serum of control and ANIT-treated mice at two days. (B) PCA loading plot to find differentiated lipid components contributing the separation between control and ANIT-treated C57BL/6 wildtype mice. P1, P2 and P3 were representative lipid components. (C) Heat map to compare the top 10 changed lipid components between control and ANIT treated wild-type mice.

Figure 3.

Time-course alteration of the top 10 changed lipid components in control and ANIT-treated mice. Each point represents the mean value of five mice.

Figure 4. (A) Description figure to show the genes related with the metabolic pathways of PC, LPC, SM and fatty acid–carnitine conjugates; (B) heat map to show the alteration of the genes with the metabolic pathways of PC, LPC, SM and fatty acid–carnitine conjugates.

Figure 5.

Q-PCR determination of the genes related with the metabolic pathways of PC, LPC, SM and fatty acid-carnitine conjugates. N.S.: not significant. * $p < 0.05$.

Figure 6.

Ahr knockout protected ANIT-induced liver toxicity. (A) The representative serums from control C57BL/6 wildtype mice, ANIT-treated C57BL/6 wildtype mice, control Ahr-null mice and ANIT-treated Ahr-null mice. (B) H&E staining of livers from control C57BL/6 wildtype mice, ANIT-treated C57BL/6 wildtype mice, control Ahr-null mice and ANIT-treated Ahr-null mice. (C) Serum ALT, AST and ALP enzyme levels from control C57BL/6 wildtype mice, ANIT-treated C57BL/6 wildtype mice, control Ahr-null mice and ANIT-treated Ahr-null mice. Four to five mice in each group, and the values were given as mean $\pm$ SEM. N.S.: not significant; * $p < 0.05$; *** $p < 0.001$. (D) Phospholipids (PL) and total

cholesterol (TC) level in the serum from control C57BL/6 wildtype mice, ANIT-treated C57BL/6 wildtype mice, control Ahr-null mice and ANIT-treated Ahr-null mice. Four to five mice in each group, and the values were given as mean $\pm$ SEM. N.S.: not significant; *** $p < 0.001$.

Figure 7.

Ahr knockout reversed ANIT-induced elevation of level of PC, LPC, SM and fatty acid-carnitine conjugates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 8.

Ahr knockout reversed ANIT-induced alteration of Chka but not SMPD3 and SCD1. N.S.: not significant; * $p < 0.05$; ** $p < 0.01$.

Figure 9.

The conclusion of the results in this study. ANIT treatment alters the lipidomic profile through altering the expression Chka, SMPD3 and SCD1. Ahr knockout reversed ANIT-induced alteration of Chka, and then altered the lipidomic profile.

Table 1.

List of the major lipid biomarkers to contribute the separation of control group and ANIT-treated group.

Lipid marker	RT (min)	m/z	Identity
P1	9.49	782.571	PC 16:0, 20:4
P2	8.85	806.571	PC 16:0, 22:6
P3	9.85	758.573	PC 16:0, 18:2
P4	1.22	520.34	LPC 18:2
P5	8.07	782.571	PC 18:2
P6	7.74	780.555	PC components
P7	2.17	524.371	LPC 18:0
P8	7.50	756.554	PC components
P9	8.39	756.554	PC components
P10	1.60	522.356	LPC18:1
P11	7.78	806.572	PC components
P12	9.14	732.553	PC18:1, 14:0
P13	7.69	756.554	PC components
P14	8.39	703.576	SM 18:1,16:0
P15	9.18	832.586	PC components
P16	7.89	369.352	N.D.
P17	9.58	764.520	N.D.
P18	1.54	426.357	Oleoylcarnitine
P19	1.45	400.341	Palmitoylcarnitine
P20	7.50	778.539	PC components