

Plasma Lipidome Acts as Diagnostic Marker and Predictor for Cyclosporin Response in Patients with Aplastic Anemia

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Abstract

The lipid metabolomic profile has been well defined in the pathogenesis and differential diagnosis in patients with different myeloid diseases. We assumed that the serum lipid metabolites could also help the diagnosis and prognostic prediction of aplastic anemia (AA). In this study, serum lipid profiles were explored in AA patients before and after cyclosporin (CsA) treatment. Meanwhile, hypocellular myelodysplastic syndrome (h-MDS) patients and the healthy volunteers were compared as controls. 15 AA patients, 11 h-MDS patients and 20 age and sex matched health controls were enrolled. All the AA patients were diagnosed to be non-severe aplastic anemia with transfusion dependency and were treated by CsA 3-5mg/kg/d for at least 6 months. AA patients had decreased arachidonic acid pathway metabolites and retinol metabolism-related metabolites as compared with h-MDS and the health ($P < 0.05$), whereas h-MDS patients had increased metabolism of proline and threonine and abnormal sphingolipid metabolism compared with AA patients and the normal controls. After 6-month of CsA treatment, serum arachidonic acid, PGE₂, PGJ₂, 15(S)-HETE, leukotriene B₄ and Protectin D1 decreased significantly. Patients who had response to CsA had higher levels of baseline protectin D1 ($p = 0.011$), leukotriene B₄ ($p = 0.011$), 15(S)-HETE ($p = 0.004$) and all-trans-retinal ($p = 0.000$) than those who had no response.

Introduction

Aplastic anemia (AA) is a bone marrow failure disease characterized by reduced bone marrow hematopoietic cell proliferation and peripheral blood cytopenia. The pathogenesis is complex and a differential diagnosis with hypocellular myelodysplastic syndrome (h-MDS) can be difficult. Furthermore, although immunosuppressive treatment has led to remissions in 50–70% of AA patients (1), approximately 15–20% of them develop secondary MDS/acute myeloid leukemia by the 10-year follow-up (2). Therefore, it is important to identify diagnostic and prognostic biomarkers.

In recent years, metabolomics, especially lipidomics, has increasing great attention as a promising tool for biomarker discovery. It had been used in the early diagnosis, progression and treatment for cancer and other diseases. With regard to hematological diseases, the lipid metabolomic profile was found to be different in the different myeloid diseases (3). Studies found that the serum sphingomyelin species containing saturated odd chain fatty acids (OCFAs) in the side chain were lower in leukemia and MDS patients compared to normal serum. Moreover, the fatty acid metabolism was deregulated in patients with leukemia and serum free fatty acids like C14:0 may indicate the progression of pre-leukemia diseased to leukemia(4). However, few studies focused on the lipid profiles of AA yet.

Therefore, we aimed to apply lipidomics to AA differential diagnosis and treatment response. In this study, serum lipid profiles were explored in AA patients before and after treatment. Meanwhile, h-MDS patients and the healthy volunteers were compared as controls.

Methods

Patients and methods

Patients newly diagnosed with AA at the Peking Union Medical College Hospital from March 2019 to December 2019 were enrolled in this study. The modified Camitta criteria were used to diagnose and define the severity the AA (5). Those with congenital AA, paroxysmal nocturnal hemoglobinuria (PNH), granulocyte clone size $\geq 50\%$, and secondary AA were excluded. All the enrolled AA patients were treated with CsA 3–5 mg/kg/day and followed up regularly, at least every 2 months for more than 6 months. At the same time, patients with newly diagnosed low-risk h-MDS were used as controls. The diagnosis of MDS was in accordance with WHO 2016 criteria (6). The h-MDS was defined based on bone marrow biopsies when the bone marrow cellularity was $<30\%$ in patients younger than 70 years or $<20\%$ in those older than 70 years. The classification and risk stratification were also defined according to the 2016 WHO criteria. For all the patients, those with a history of hyperlipidemia, diabetes, obesity (body mass index $> 28 \text{ kg/m}^2$) or complications with other malignant diseases at diagnosis were excluded.

Peripheral fasting serum levels of the AA patients were measured at the time of diagnosis and 6 months post CsA treatment. Peripheral fasting serum samples of the patients with h-MDS were measured at diagnosis and those from age and sex matched healthy volunteers were used as controls. All the serum aliquots were stored at -20°C .

Analyses using lipid extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Each 100 μL of the serum sample was mixed with 750 μL of methanol and incubated with 2.5 ml of methyl tert-butyl ether at room temperature in a shaker for 1 hour. Then, 625 μL of MS-grade water was added for the phase separation. After 10 min of incubation at room temperature, the mixture was centrifuged at $1,000 \times g$ for 10 min. The upper (organic) phase was dried and dissolved in 100 μL of isopropanol and then analyzed using LC-MS/MS. The samples for QC were prepared following the same protocol as mentioned previously. All purified compounds of QC were $\geq 95\%$ purity based on analytical HPLC.

The lipidomic profiles were obtained using a Vanquish UHPLC system (Thermo Fisher, Germany) coupled with an Orbitrap Q ExactiveTM HF mass spectrometer (Thermo Fisher, Germany). The chromatographic separation was carried out at 40°C using a Thermo Accucore C30 column ($150 \times 2.1 \text{ mm}$, $2.6 \mu\text{m}$) with a 20-min linear gradient at a flow rate of 0.35 mL/min . The mobile phase buffer A was acetonitrile/water (6/4) with 10 mM ammonium acetate and 0.1% formic acid, whereas buffer B was acetonitrile/isopropanol (1/9) with 10 mM ammonium acetate and 0.1% formic acid. The solvent gradient was set as follows: 30% B, initial, 30% B, 2 min, 43% B, 5 min, 55% B, 5.1 min, 70% B, 11 min, 99% B, 16 min, 30% B, 18.1 min. The Q ExactiveTM HF mass spectrometer was operated in positive [negative] polarity mode with the following parameters: sheath gas: 20 arbitrary units, sweep gas: 1 arbitrary unit, auxiliary gas rate: 5 [7], spray voltage: 3 kV, capillary temperature: 350°C , heater temperature: 400°C , S-Lens RF level: 50, resolving power (full scan): 120000, scan range: 114–1700 m/z, automatic gain control target: 1×10^6 , resolving power (MS^2): 30000 (Top20), normalized collision energy: 25, 30 [20,24,28], injection

time: 100 ms, isolation window: 1m/z, automatic gain control target (MS^2): $1e5$, and dynamic exclusion: 15s.

Data handling and statistical analysis

The raw data generated from the UHPLC-MS/MS were processed using the Compound Discoverer 3.01 (CD3.1, Thermo Fisher) to perform the peak alignment, peak picking, and quantitation of each metabolite. The peak intensities were normalized to the total spectral intensity to predict the molecular formula. The peaks were then matched with the Lipidmaps, Lipidblast and HMDB database to obtain accurate qualitative and relative quantitative results. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed using the metaX software. A univariate analysis (t-test) was performed to calculate the statistical significance (P -value). The metabolites with a $VIP > 1$, P -value < 0.05 , and fold change (FC) > 1.2 or < 0.833 were considered as differential metabolites. Volcano plots were used to filter the metabolites of interest based on the $\text{Log}_2(\text{FC})$ and $-\text{log}_{10}(P\text{-value})$ of metabolites. Clustering heatmaps were plotted using the statistical software R (R version R-3.4.3) and MetaboAnalyst 5.0 was used for the pathway analysis.

Quantitative data were compared using a two-tailed t test with the assumption of equal variance, while categorical data were compared using the chi-square test. The comparisons between the different groups were calculated using a log-rank test. A two-sided P value of < 0.05 was considered as statistically significant. The statistical tests were performed using the SPSS 21.0 statistical software.

Results

Baseline characteristics for patients with AA and different controls

15 AA patients, 11 h-MDS patients and 20 age and sex matched healthy controls were enrolled in this study. The detailed baseline characteristics were shown in Table 1.

All the AA patients were non-severe and transfusion dependent. They were six females and nine males with a median age of 44 (18–61) years. Before treatment, the average hemoglobin (HGB) level was 94 ± 25 g/L, the average absolute neutrophil count (ANC) was $1.31 \pm 0.58 \times 10^9/\text{L}$, and the platelet count was $48 \pm 61 \times 10^9/\text{L}$. The average reticulocyte count was $62 \pm 20 \times 10^9/\text{L}$. Seven patients had elevated ferritin levels, with the average value 659 ± 511 ug/L. Only one patient had +8 abnormal chromosomes and none of them had PNH clones. AA patients had normal liver function but two of them had mild kidney dysfunction (Chronic Kidney Disease Stage I–II) at the time of the diagnosis. The median duration of the CsA treatment was 9 months (6~15 months) and the CsA concentration was maintained at 100~200 ng/mL during the treatment period. The median follow-up time was 12 months (9~15 months). After the 6-month CsA treatment, 13.3% (2/15) achieved a complete response (CR), 44.4% (8/15) achieved a partial response (PR), and the overall response rate (ORR) was 66.7%. There was no difference in the baseline characteristics between patients with or without response. No PNH clone expansion or death occurred during the study period.

For h-MDS patients, they were five females and six males with a median age of 55 (44~73) years. The age was significantly higher in patients with h-MDS than AA ($P=0.009$). Five were MDS with single-lineage dysplasia, three were MDS with multilineage dysplasia, and three were MDS-Excess Blasts 1 (MDS-RAEB1), according to 2016 WHO criteria. The International Prognostic Scoring System (IPSS) was 0 in three patients, 0.5–1 in six patients and 1.5–2 in two MDS patients. Six MDS patients had cytopenia of more than one lineage. The absolute platelet count was higher than that in AA patients ($P=0.037$). Among the 11 MDS patients, one had three chromosome abnormalities, four had two abnormalities and the others had no chromosome abnormalities. None of them had abnormal chromosome 7. There was no significant difference in the ANCs, HGBs, reticulocytes, ferritin level and other biochemical indices between the AA and h-MDS patients at baseline. Nine IPSS low- and intermediate-1 (Low/Int-1) risk MDS patients were treated with CsA and androgen, while 2 intermediate-2 (Int-2) risk patients were treated with thalidomide and androgen. The median follow-up time was 12 months (9~15 months). Two MDS-RAEB1 patients experienced transformation into acute myelocytic leukemia (AML) at the end of our follow-up. No deaths occurred during the follow-up.

Table 1: The baseline characteristics of AA patients, MDS patients and the control

Basic characteristics	AA	MDS	Healthy control	P-value (AA vs. MDS)
Sex, N (%)				0.485
Female	6(40%)	5(45%)	8(40%)	
Male	9(60%)	6(55%)	12(60%)	
Age, years, median (range)	44(18-61)	55(44-73)	47(18-69)	0.009
Baseline blood cell count				
ANC, $\times 10^9/L$, mean \pm SD	1.31 \pm 0.58	1.36 \pm 0.91	3.12 \pm 1.22	0.875
HGB, g/L, mean \pm SD	94 \pm 25	83 \pm 26	135 \pm 20	0.302
PLT, $\times 10^9/L$, mean \pm SD	48 \pm 61	104 \pm 64	185 \pm 82	0.037
Ret#, $\times 10^9/L$, mean \pm SD	62 \pm 20	59 \pm 22	-	0.813
ALT, U/L, mean \pm SD	19 \pm 17	22 \pm 17	15 \pm 20	0.623
Cr, umol/L, mean \pm SD	70 \pm 26	84 \pm 22	62 \pm 20	0.160
Fer, ug/L, mean \pm SD	659 \pm 511	583 \pm 492	-	0.715
PNH, N(%)	0(0%)	0(0%)	-	-
Abnormal chromosome, N(%)	1(7%)	5(45%)	-	0.04

Abbreviations: ANC: absolute neutrophil count, HGB: hemoglobin, PLT: platelet, Ret: reticulocyte, ALT: alanine transaminase, Cr: creatinine, Fer: ferritin, PNH: paroxysmal nocturnal hemoglobinuria.

Baseline lipid metabolism profile for AA patients

The PCA score plot from the discovery set showed different trends between the AA patients and the healthy controls. The PLS-DA model further confirmed the differences between the two groups. The cross-validation plot indicated that this model was non-overfitting (Fig. 1). The differential metabolites, defined by a VIP > 1, P -value < 0.05, and ≥ 2 or ≤ 0.5 , are listed in Table S1. Compared with the healthy controls, 106 lipid molecules were upregulated and 208 were downregulated in ESI+ mode, while 71 molecules were upregulated and 118 were downregulated in ESI- mode for patients with AA. The KEGG pathway analysis indicated that these lipid metabolites were involved mainly in the metabolism of arachidonic acid, retinol, glycerophospholipids, aminoacyl-tRNA biosynthesis and the biosynthesis of unsaturated fatty acids. The volcano plot and heatmap was then plotted to display the significant difference (Fig. 1). Phosphatidylcholine (0.17 FC), arachidonic acid (0.4 FC), prostaglandin G2 (0.6 FC), prostaglandin E2 (0.66 FC), prostaglandin J2 (0.31 FC), leukotriene B4 (0.31 FC) and 15(S)-HETE (0.39 FC) in the arachidonic acid pathway were significantly downregulated in AA patients compared with normal controls ($P < 0.05$). All-trans-retinal (0.48 FC), dehydroretinaldehyde (0.29 FC) and tretinoin (0.47 FC) in retinol metabolism were also lower in AA patients ($P < 0.05$).

To analyze the differences between AA and h-MDS patients, PCA, PLS-DA and cross validation was first explored to confirm the reliability (Fig. 2). Compared to h-MDS patients, AA patients had 42 lipid molecules upregulated and 165 downregulated in the ESI+ mode, while 40 were upregulated and 76 downregulated in the ESI- mode. The differences were then visualized using volcano plots and heatmaps (Fig. 2). The differentially expressed molecules are listed in Table S2. According to the pathway analysis, the changes focused on the metabolism of arachidonic acid, retinol, glycerophospholipids, and sphingolipid metabolism. Compared with patients with h-MDS, patients with AA showed decreased arachidonic acid pathway metabolites, including phosphatidylcholine (0.16 FC), arachidonic acid (0.18 FC), prostaglandin G2 (0.6 FC), prostaglandin E2 (0.59 FC), prostaglandin J2 (0.16 FC), leukotriene B4 (0.23 FC), 15(S)-HETE (0.13 FC) and the retinol metabolism-related metabolites ($P < 0.05$). These differences were unique for AA and h-MDS because there were no differences in the above pathways between the patients with h-MDS and the healthy controls. On the other hand, h-MDS patients showed increased metabolism of proline (1.91 FC) and threonine (1.76 FC) and abnormal sphingolipid metabolism including increased ceramide (d18:1/16:0) (1.34 FC), sphingosine-1-phosphocholine (1.52 FC) and decreased SM (d18:0/20:2(11Z,14Z)) (0.47 FC) compared with the normal controls, which was normal in AA patients as well.

Change of lipid profiles after 6-month CsA treatment in patients with AA

The lipid profiles of patients with AA were measured again after the 6-month of standard CsA treatment and compared with the baseline values. The PCA and reliability tests were performed, and the results are shown in Fig 3. After the 6-month CsA treatment, 105 lipid molecules increased and 9 decreased in the ESI+ mode, while 41 increased and 14 decreased in the ESI- mode (Table S3). The KEGG pathway analysis revealed that the main changed metabolites were involved in the metabolism of arachidonic

acid and retinol. In details, leukotriene B4 (0.49 FC) and 15(S)-HETE (0.49 FC), which were involved in arachidonic acid metabolism, and all-trans-retinal (0.4 FC) and protectin D1 (0.42 FC), which were involved in retinol metabolism, decreased after the 6-month of CsA treatment.

Molecules at baseline were also compared between the ten patients who achieved at least a partial response (PR+CR) and five patients who did not achieve a response (NR) after CsA treatment. Patients who achieved PR+CR had significant higher levels of baseline protectin D1 ($p=0.011$), leukotriene B4 ($p=0.011$), 15(S)-HETE ($p=0.004$) and all-trans-retinal ($p=0.000$) than those who had no response (Fig. 4). Further analysis revealed that the levels of protectin D1 at baseline was significantly higher in CR patients than in PR patients ($p<0.05$), while other molecules such as leukotriene B4, 15(S)-HETE, and all-trans-retinal tended to be higher in patients with CR than those with PR, but not significant. The baseline lipid molecules were also correlated with the rapidity of the CsA response: patients who achieved CR+PR within a 3-month period tended to have higher baseline expression of the above four metabolites than those who achieved CR+PR after 3~6 months of CsA treatment, although the differences were not significant.

Discussion

There is a long history of research on lipid profiles and lipid metabolism in hematological diseases. In the 1960s, the lipid composition of normal and abnormal leukocytes was reported, the total lipid content, especially cholesterol was found higher in normal mature leukocytes than in immature cells of the same morphological series (7). Recently, studies on the lipid metabolism showed a greater increase in membrane lipid peroxidation in AA patients than in normal controls (8, 9), which may be one of the pathogenesis for bone marrow failure (10). With the development of mass spectrometry, the lipidomic analysis of peripheral blood was used to investigate the role of lipid on the pathophysiology of hematological diseases. In 2014, Adriana et al. reported discriminative lipid profiles in patients with AML, MDS, and myeloproliferative neoplasms using untargeted shotgun LC-MS/MS analyses (3). Although studies on AML and MDS have illustrated the inner correlations of the two entities to some extent, very few studies have focused on the lipid profiles of patients with AA, let alone the comparison of the changes after CsA treatment and the difference between AA and h-MDS. We performed, for the first time, an untargeted shotgun MS/MS analysis in patients with AA and we monitored these patients dynamically after a 6-month of CsA treatment. Meanwhile, the difference between patients with AA and h-MDS and normal controls was compared.

Our data showed that the lipid profiles were different between patients with AA and normal controls. Among the differences, metabolites in the arachidonic acid pathway and retinol metabolism were the most important. Interestingly, these differences also exist between AA and h-MDS. Several studies have demonstrated the role of arachidonic acid and eicosanoids in hematopoietic cell proliferation and differentiation (11), and found that arachidonic acid *de novo* synthesis pathway increased statistically in AML, and in particular, in AML with high bone marrow blasts (12). Arachidonic acid may metabolize through cyclooxygenase, lipoxygenase and cytochrome P450 pathways into a number of metabolites

including prostaglandins, hydroxy-eicosatetraenoic acid (HETE), and leukotrienes. Although there are conflicting reports on their effects depending on the species and on the class of prostaglandins, prostaglandins can help modulate erythropoiesis. PGE1, PGE2, and PGD2 increase the number of erythroid colony-forming units (CFU-E) and erythroid burst-forming units in human bone marrow cells (13). 15-HETE is converted from arachidonic acid by 5-lipoxygenases and is considered to influence the commitment of early immature hematopoietic cells toward the erythroid lineage (14). Leukotriene B4, derived from the normal human bone marrow and from arachidonic acid, can significantly enhance the cloning efficiency of the CFU-GM, as shown by the significant increase during the granulocytic differentiation of myeloid cells (15). In our study, AA patients had lower baseline levels of arachidonic acid and the downstream metabolites, which was in accordance with the previous findings of those molecules on hematopoiesis promotion. Although bone marrow hypo-cellularity is often seen in h-MDS, molecules involved in the above pathway is relatively normal in h-MDS compared with AA, indicating a “real” bone marrow failure status in patients with AA.

MDS may have some different ways of pathogenesis as compared with AA. As shown in our data, the biosynthesis of proline, threonine and the ceramides increased in h-MDS patients compared with normal controls and AA patients, which provided another potential differential diagnosis way for AA and h-MDS. The proline metabolic cycle is found to play an emerging role in cancer cell survival, proliferation and metastasis (16, 17), while threonine metabolism is related to embryonic stem cell self-renewal (18). The increase of proline and threonine biosynthesis in h-MDS patients may indicate abnormal proliferation rather than failure of bone marrow, although the detail mechanisms remain unclear. Sphingomyelin (SM) composes of ceramide and phosphorylcholine and is involved in cancer cell metabolism and drug resistance through bioactive lipids such as ceramide (19). Atsushi et al. (4) found that AML and MDS both had lower levels of the SM species containing saturated OCFAs in the side chain compared with normal controls, but AML patients had even lower levels in ceramides whereas MDS patients had lower proportions of the SM species with saturated (C20 and C22) and mono-unsaturated fatty acids (C18, C20, C22). Other studies showed that the level of ceramides with C16 or C24 fatty acids can promote neutrophil apoptosis (20). In our study, the h-MDS patients showed an increase in ceramides and sphingosine-1-phosphocholine and a decrease in SM (d18:0/20:2(11Z,14Z)), which was in accordance with the above findings. Therefore, we speculated that the changes in h-MDS may cause abnormal proliferation and increased cell apoptosis which result in clone evolution and hypocellular bone marrow. Different lipid profiles between AA and h-MDS correlated with the different pathologic mechanisms between the two diseases and probably can be used as a differential diagnosis and prognosis predictor in the future.

To investigate the potential indicators for CsA response, the lipid molecules were followed up after the CsA treatment. Serum arachidonic acid, PGE2, PGJ2, 15(S)-HETE and leukotriene B4 were found decreased after treatment, which may be explained by the consumption of arachidonic acids during hematopoiesis reconstitution. Meanwhile, this finding suggested that the immune attack, which response for the bone marrow failure in AA, may act through the above pathway, due to the change of the above pathway after CsA treatment. Those with lower baseline values had a worse response, which indicated

that the insufficient storage of arachidonic acids may influence the hematopoiesis recovery. Whether the extra supply of arachidonic acids could promote the hematopoietic cell proliferation and differentiation in AA merits further investigation. Kedar et al. reported that arachidonic acid or docosahexanoic acid fed mice had enhanced hematopoiesis (21), supported our findings. The all-trans retinoic acid (ATRA) was first used in treating the acute promyelocytic leukemia (APL) based on its effects of inducing terminal granulocytic differentiation of the malignant promyelocytes (22). However, evidence has showed that retinoid-mediated regulation played an important role in myelopoiesis (23). It has been shown that the retinoic acid signaling regulates the differentiation of granulocytes and enhances erythropoiesis in adults (24). ATRA or related compounds may play a significant role on enhancing the hematopoietic stem cell self-renewal as well as the production and differentiation of regulatory T cells (25). We found that the patients with AA, especially those with a poor response to CsA, had lower all-trans-retinal, which may provide a new way of treating AA by adding ATRA. In 2020, Tang et al. reported that ATRA inhibited Th17 cell differentiation, promoted regulatory T-cell development, and provided an effective treatment in an immune-mediated mouse model of AA (26).

There were some limitations in our study. The sample size was relatively small for the evaluation of treatment response of AA and the distribution of MDS subtypes. The follow-up time was also too short for clonal evolution and disease progression. Patients should be followed up for a longer time and those who progressed to MDS/AML should be included and their lipid profiles should be analyzed in future study. Despite these limitations, we found that the lipid profiles of patients with AA showed significant differences between healthy controls and those with h-MDS. Metabolites in arachidonic acid pathway and retinol metabolism were lower in the AA patients, whereas the proline and threonine biosynthesis and ceramides were higher in h-MDS compared with AA. The baseline levels of leukotriene B4, 15(S)-HETE, all-trans-retinal, and protectin D1 in AA patients may predict the CsA response at 6 months.

Declarations

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Competing Interests

We declare no conflict of competing interests.

Author contributions

All authors contributed to the study conception and design. Miao Chen, Yali Du and Chen Yang helped to collect clinical data and provide critical suggestions. Material preparation and data analysis were

performed by Jing Ruan and Bing Han. The first draft of the manuscript was written by Jing Ruan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

*** Compliance with ethical standards.**

The study was conducted in accordance with the Declaration of Helsinki.

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*** Ethical approval.**

The study protocol was approved by the Ethics Committee of Peking Union Medical College Hospital.

*** Informed consent.**

Informed consent was obtained from all the patients.

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Figures

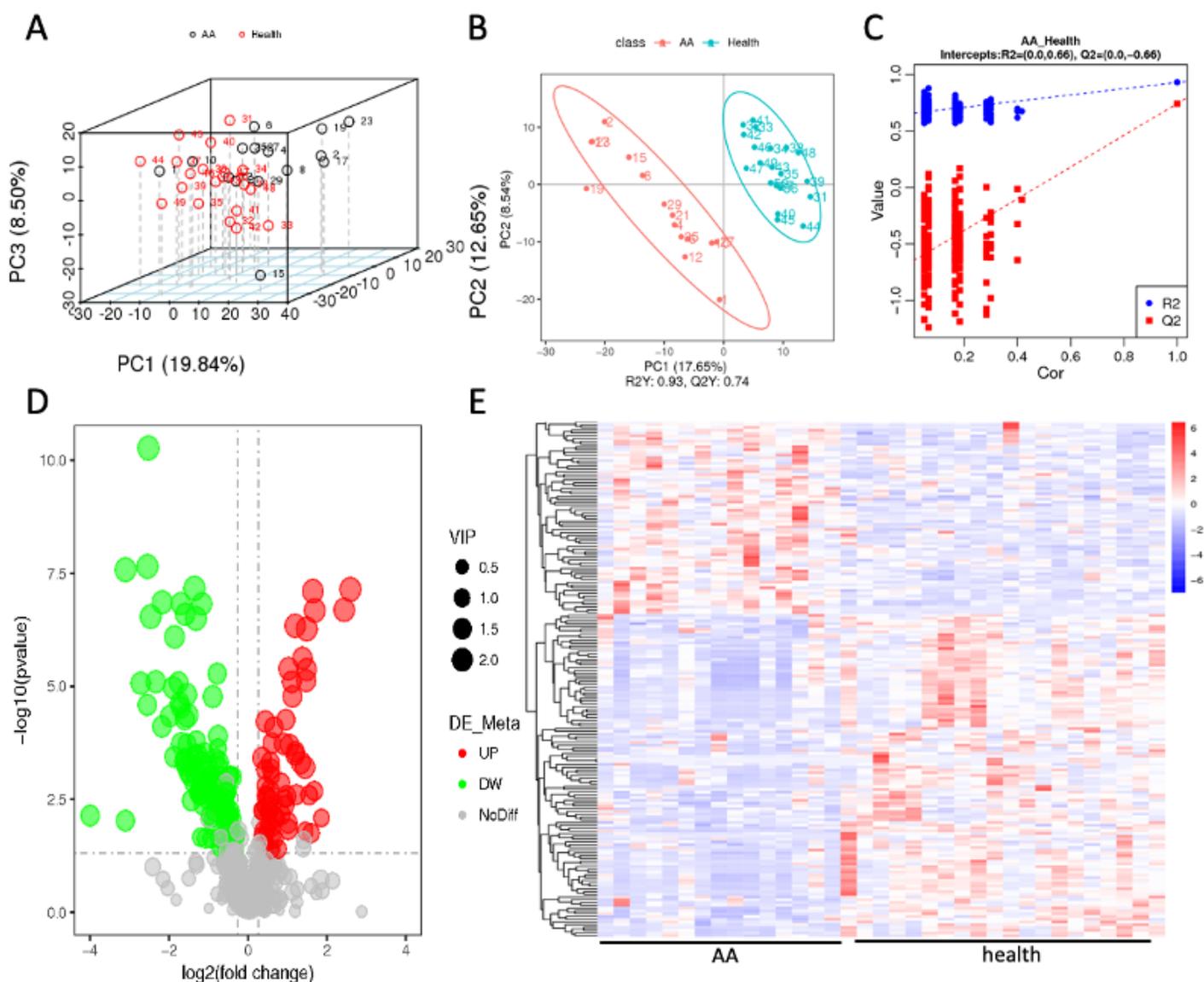


Figure 1

The differential analysis of lipid profiles between AA patients and the healthy controls. A. PCA score plots of the discovery set samples showed a separated trend between AA patients and the healthy controls. (AA

patients: black circle, healthy controls: red circle) B. PLS-DA score plots of the discovery set samples. C. Cross-validation plot with a permutation test repeated 200 times. The intercepts of $R^2 = (0.0, 0.66)$ and $Q^2 = (0.0, -0.66)$ illustrated the PLS-DA model was not over-fitting. D. The volcano plot displayed the differences of lipid profiles intuitively. E. Heatmap of the differentially expressed lipid molecules between AA patients and the healthy controls.

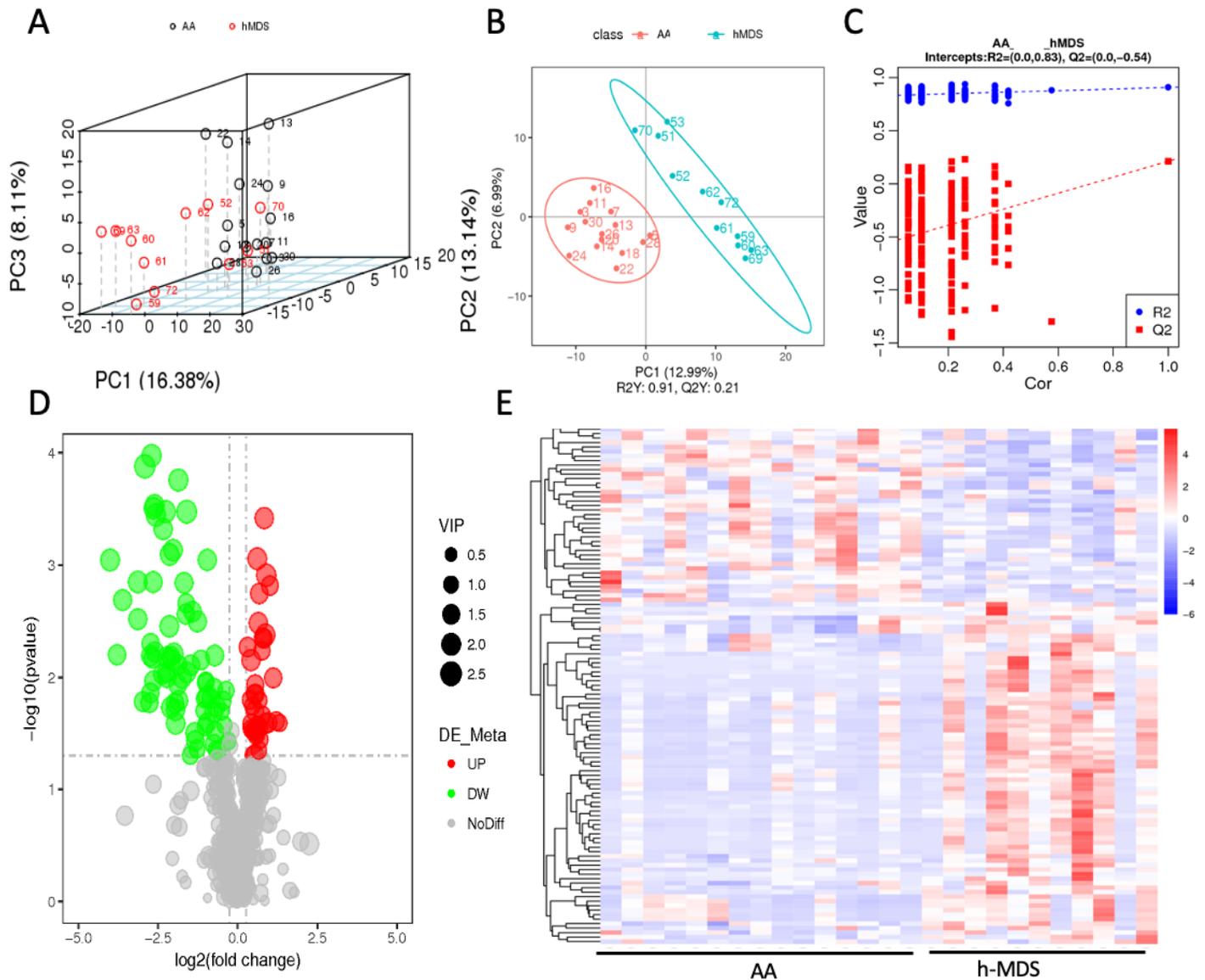


Figure 2

The differential analysis of lipid profiles between AA patients and h-MDS. A. PCA score plots of the discovery set samples showed a separated trend between AA and h-MDS patients. (AA patients: black circle, h-MDS patients: red circle) B. PLS-DA score plots of the discovery set samples. C. Cross-validation plot with a permutation test repeated 200 times. The intercepts of $R^2 = (0.0, 0.83)$ and $Q^2 = (0.0, -0.54)$ illustrated the PLS-DA model was not over-fitting. D. The volcano plot displayed the differences of lipid profiles intuitively. E. Heatmap of the differentially expressed lipid molecules between AA and h-MDS patients.

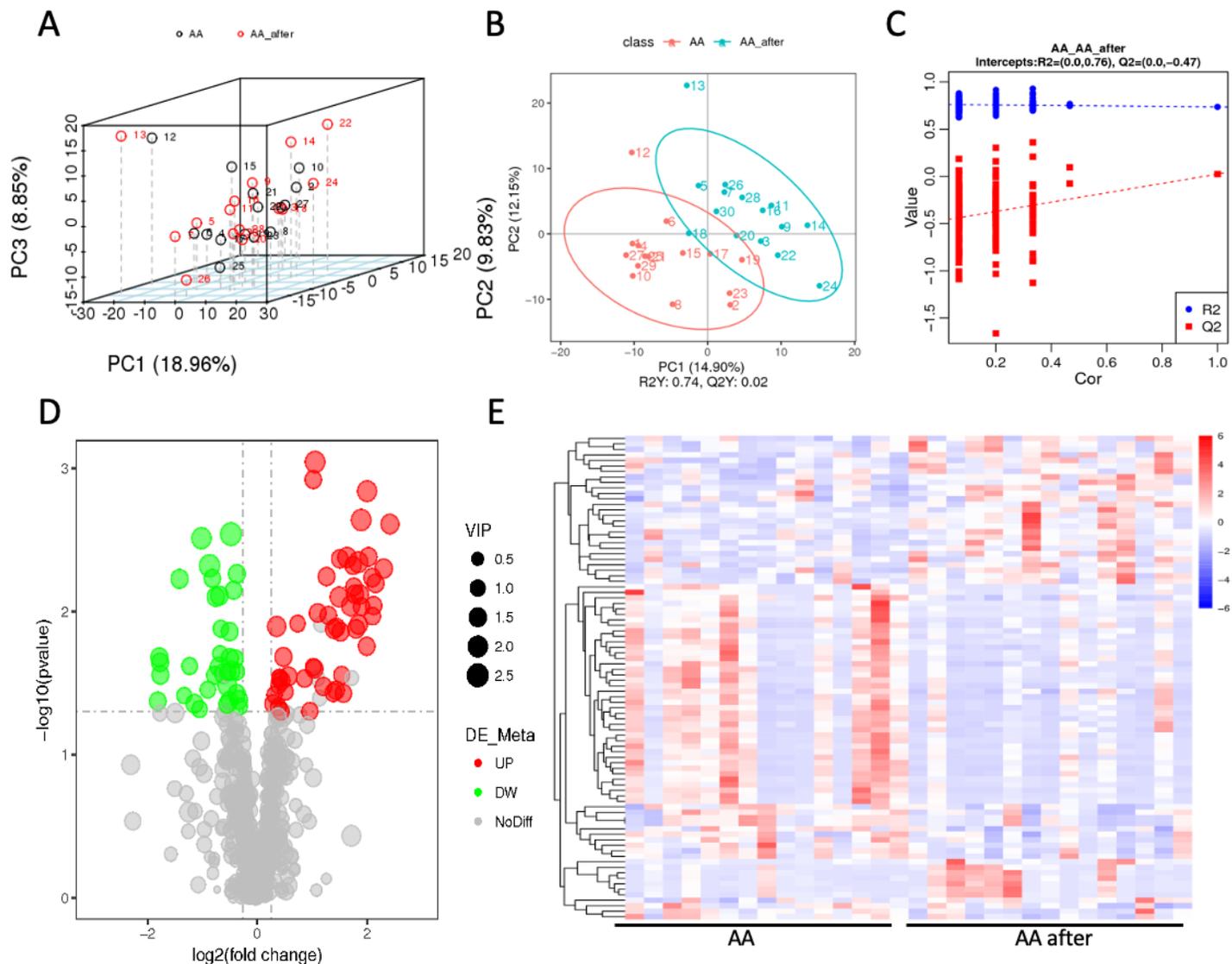


Figure 3

The changes of lipids after CsA treatment. A. PCA score plots of the discovery set samples showed a separated trend for AA patients after treatment. (AA patients: black circle, AA patients after treatment: red circle) B. PLS-DA score plots of the discovery set samples. C. Cross-validation plot with a permutation test repeated 200 times. The intercepts of $R^2 = (0.0, 0.76)$ and $Q^2 = (0.0, -0.47)$ illustrated the PLS-DA model was not over-fitting. D. The volcano plot displayed the differences of lipid profiles after CsA treatment intuitively. E. Heatmap of the differentially expressed lipid molecules before and after CsA treatment for AA patients.

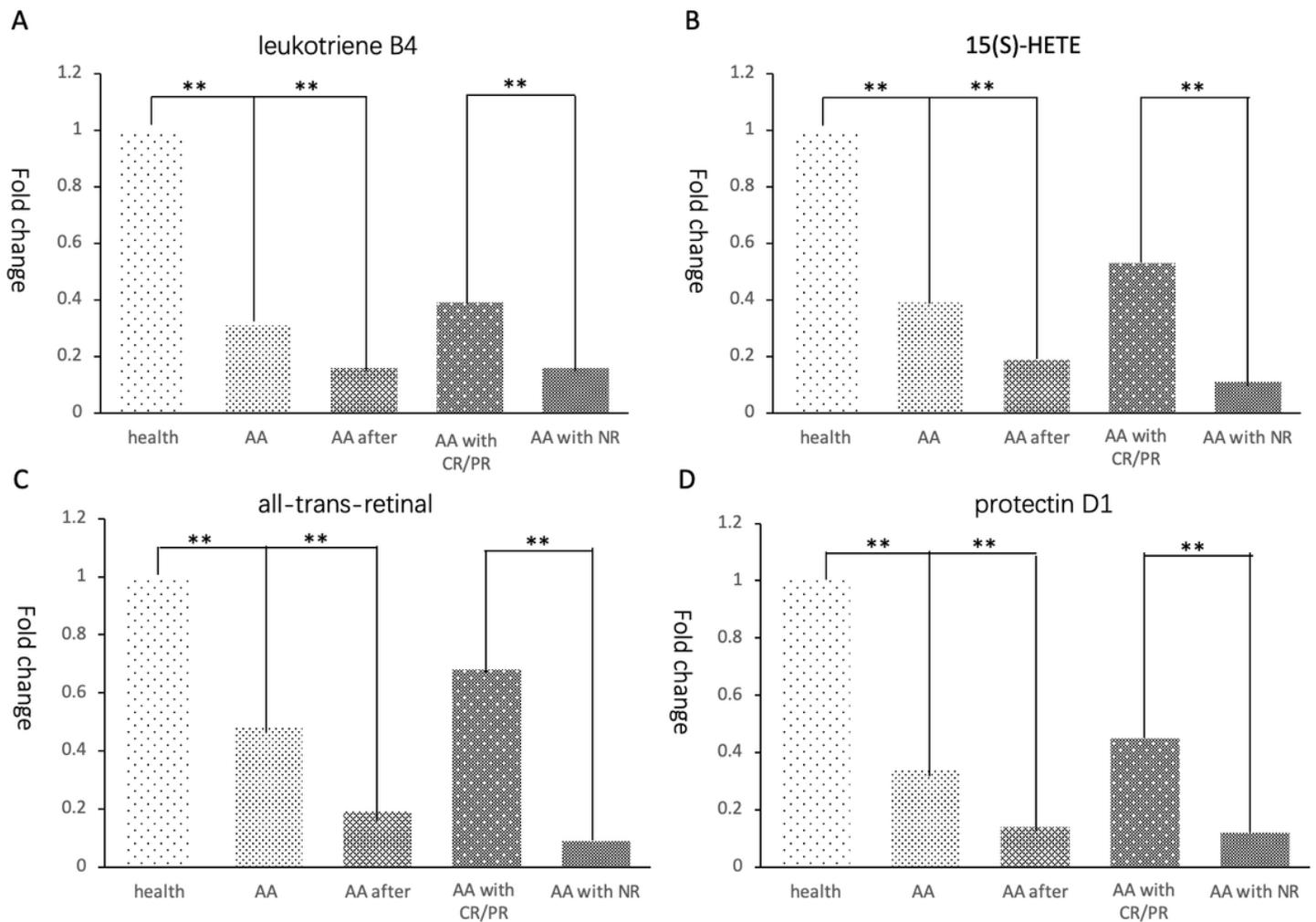


Figure 4

Different expressions of leukotriene B4, 15(S)-HETE, all-trans-retinal and protectin D1 in the health and AA patients. The expression of leukotriene B4, 15(s)-HETE, all-trans-retinal and protectin D1 were significantly lower in AA patients than the health controls. Their expressions were further decreased after the 6-month of CsA treatment. Further analysis indicated that AA patients who had response to CsA had significant higher baseline levels of these molecules than those who had no response.