Plasma metabolomics indicates potential biomarkers and metabolic pathways of melasma

Xiaoli Zhang  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Yi CHEN  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Hedan YANG  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Hui Ding  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Pingping CAI  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Yiping GE  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Huiying ZHENG  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Xiaojie SUN  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Yin YANG  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Xinyu LI  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Tong LIN  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College

Tong LIN  
(ddlin@hotmail.com)  
Hospital for Skin Disease, Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College
Abstract

Introduction

Melasma is a common and chronic pigmentary disorder that has negatively impacted patients’ quality of life. The pathogenesis of melasma is complicated. Metabolomics may contribute to understanding the pathogenesis and identifying intervention strategies for melasma.

Objectives

To analyze changes in plasma metabolites of female melasma patients and search for disease markers and potential therapeutic targets.

Methods

Plasma samples from 20 female patients with melasma and 21 age-matched healthy female controls were collected for untargeted metabolomics. Ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) was used to analyze the plasma metabolites. Significantly differential metabolites in patients with melasma were identified by metabolic pathways and receiver operating characteristic curves, and correlation analysis was conducted with modified Melasma Area and Severity Index (mMASI) and oxidative stress level.

Results

Compared with healthy subjects, melasma patients showed significant changes in 125 plasma metabolites, including amino acids, lipids, and carbohydrate-related metabolites. KEGG pathway analysis indicated that tryptophan metabolism and biosynthesis of phenylalanine, tyrosine, and tryptophan pathways may be the main pathways related to melasma pathogenesis. Some metabolites can be considered as biomarkers significantly associated with melasma by ROC analysis and correlation analysis.

Conclusions

This study identified significant changes in plasma metabolites in melasma patients using UPLC-MS-based metabolomics, which may provide new insights into the pathogenesis of melasma and explore new therapeutic methods.

1 Introduction

Melasma is a chronic pigmentary disorder characterized by symmetrical light to dark brown macules and patches on the face. Melasma is predominant in women, with at least 90% of affected patients being women in some populations (Hexsel et al., 2014). The condition affects up to 30% of childbearing women in certain populations, leading to a negative impact on the life quality of affected individuals (Passeron &
Picardo, 2018a). However, the treatment of melasma is still incredibly difficult (McKesey Tovar-Garza & Pandya, 2020; Neagu et al., 2022).

Melasma is a complex disease influenced by genetic susceptibility, ultraviolet radiation, hormone imbalance, vascularization, oxidative stress, impaired barrier function and so on (Artzi et al., 2021; Choubey et al., 2017; Kim, Kim, Lee, & Kang, 2007). The transcription analysis of melasma lesions and adjacent non-lesional skin identified more than 300 differentially regulated genes, highlighting the complexity of melasma (Chung et al., 2014; Kang et al., 2011). Normal metabolic environment is indispensable for maintaining physiological homeostasis (Muthubharathigowripriya & Balamurugan, 2021). The dysregulation of metabolites in vivo is also an important potential factor influencing the pathogenesis of melasma. Metabolomics is a powerful tool that can help identify potential biomarkers and therapeutic targets for many diseases (Cui Lu & Lee, 2018; Jacob, Lopata, Dasouki, & Abdel, 2019).

In this study, we investigated the potential role of metabolites in melasma pathogenesis using untargeted metabolomics performed by UPLC-MS. Plasma metabolite changes were compared in melasma patients and healthy controls to identify dysregulated metabolites that could contribute to melasma pathogenesis.

2 Methods

2.1 Subject selection

This study was approved by the Medical Ethics Committee of Hospital for Skin Disease (Institute of Dermatology), Chinese Academy of Medical Science (Approval number: (2022) Immediate Approval No.009). A total of 20 female melasma patients and 21 age-matched, healthy female volunteers with Fitzpatrick skin types III-V were involved in this study. All the patients and healthy controls aged 18 to 60 years old. Subjects who are pregnant or lactating, combined with other systemic diseases, or oral administration of drugs that may affect this study were excluded. Each participant provided written informed consent and blood samples. The mMASI score is used to assess the severity of melasma.

2.2 Sample collection

Fasting peripheral venous blood (5 mL) was collected with an EDTA anticoagulant tube. The blood samples were centrifuged at 3000rpm for 10 min at 4°C within 2 hours, then the supernatant were obtained and stored at -80°C until further analysis.

2.3 Sample preparation

The sample was mixed with 700μL of extractant containing internal standard d3-Leucine (methanol: acetonitrile: water = 4:2:1, v/v/v) for one minute, and then refrigerated at -20°C for two hours. After centrifuged for 15 min at 25000 g, 4°C, 600 μL of the supernatant was transferred to a split Eppendorf tube and dried with a drying machine. The residue was then reconstituted with 180 ul pure water and methanol (1:1 v/v) and centrifuged again. The supernatant was transferred to autosampler vials for
UPLC-MS analysis. Take 20 µL of the supernatant of each sample and mix into quality control (QC) sample.

### 2.4 UPLC-MS analysis

In this experiment, ACQUITY UPLC system (Waters Ltd., Elstree, U.K.) in series with XevoG2-XS Q Tof (Waters, Manchester, UK) was utilized for the separation and detection of metabolites. Chromatographic conditions: Chromatographic separation was performed on a Waters ACQUITY UPLC BEH C18 column (1.8 µm, 2.1 mm × 100 mm, Waters Ltd., Elstree, U.K), and the column temperature was maintained at 45°C. The mobile phase included 0.1% formic acid (A) and acetonitrile (B) in the positive mode, while in the negative mode, it contained 10 mM ammonium formate (A) and acetonitrile (B). The gradient conditions were as follows: 0–1 min, 2% B; 1–9 min, 2%-98% B; 9–12 min, 98% B; 12-12.1 min, 98% B to 2% B; and 12.1-15 min, 2% B. The flow rate was 0.35 mL/min and the injection volume was 5 µL.

Mass spectrometry conditions: Waters XevoG2-XS Q Tof was used to perform primary and secondary mass spectrometry data acquisition. The full scan range was 70–1050 m/z with a resolution of 120000 and the automatic gain control target for MS acquisitions was set to 3e6 with a maximum ion injection time of 100 ms. Top 3 precursors were selected for subsequent MS fragmentation with a maximum ion injection time of 50 ms and resolution of 30000, the AGC was 1e5. The stepped normalized collision energy was set to 20, 40 and 60 eV. ESI parameters were setting as: sheath gas, 40 psi; auxiliary gas, 10 psi; positive-ion mode spray voltage, 3.80 kV; negative-ion mode spray voltage, 3.20 kV; capillary temperature, 320°C; auxiliary gas heater temperature, 350°C.

### 2.5 Data processing and analysis

A data matrix containing information such as metabolite peak area, retention time (RT) and identification results were obtained after importing the off-line data of mass spectrometry into Progenesis QI (Waters) software and analyzing the mass spectrometry data in combination with HMDB database (https://hmdb.ca/) and chemspider online database (http://www.chemspider.com/). All metabolites were normalized by log2 conversion before analysis. To analyze metabolic profiles and identify metabolic differences, principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminate analysis (OPLS-DA) were performed(Di Guida et al., 2016). MetaboAnalyst (https://www.metaboanalyst.ca) was used for the identification of metabolic pathways, receiver operating characteristic curves (ROC curve), differential metabolite hierarchy clustering, differential metabolite correlation heat map. R Studio and related R package were used to draw volcano map.

### 2.6 Determination of oxidative stress parameters

The changes of oxidative stress indicators in female melasma patients and the control group were observed. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were detected by enzyme-linked immunosorbsent assay according to the kits’ instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd., China).
2.7 Statistical analysis

The data were represented as mean ± SD. The significance of differences between groups was assessed by a one-way ANOVA with Dunnett’s multiple comparisons or a two-tailed unpaired Student’s t-test using GraphPad Prism 9.0 (La Jolla, CA, USA). Statistical significance was defined as a p-value < 0.05.

3 Results

3.1 Characteristics of clinical data

Table 1 lists the clinical characteristics of the subjects, including age, disease duration, race/ethnicity, and mMASI. The melasma patients had an average age of 42.55 ± 6.09 years with the disease duration of 7.35 ± 5.40 and the mMASI score of 8.25 ± 3.53, while the control group had an average age of 39.05 ± 7.98 years (Table 1).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Melasma patients</th>
<th>Healthy control subjects</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>20</td>
<td>21</td>
<td>N/A</td>
</tr>
<tr>
<td>Age, y</td>
<td>42.55 ± 6.09</td>
<td>39.05 ± 7.98</td>
<td>0.307</td>
</tr>
<tr>
<td>Disease duration, y</td>
<td>7.35 ± 5.40</td>
<td>0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Female</td>
<td>N/A</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td>Chinese</td>
<td>Chinese</td>
<td>N/A</td>
</tr>
<tr>
<td>mMASI score</td>
<td>8.25 ± 3.53</td>
<td>N/A</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

N/A, not available; mMASI, modified Melasma Area and Severity Index.

3.2 Stability analysis of metabolomics methodology and metabolites overall analysis

The result of good base peak chromatograms overlapping of all QC samples, little fluctuation of retention time and peak response intensity showed that the instrument is in good condition and the signal is stable in the whole process of sample detection and analysis (Fig. 1). By establishing PCA model between melasma group and control group, it was observed that samples from the same group were generally clustered within the 95% confidence interval and samples from different groups were well distinguished, indicating significant differences in metabolites between female melasma patients and female healthy subjects (Fig. 2a, Fig. 2b). To maximize the differences of the metabolic profiles, PLS-DA and OPLS-DA were performed (PLS-DA analysis was shown in Fig. S1). The evaluation parameters R2Y and Q2 of
model are obtained after 200 response permutation tests in positive mode ($R^2_Y = 0.963$, $Q^2 = 0.949$) and negative mode ($R^2_Y = 0.351$, $Q^2 = 0.102$), indicating good explanatory ability (Fig. 2c, Fig. 2d).

### 3.3 Differential metabolites identification

According to the screening criteria: 1) variable important for the projection (VIP) of OPLS-DA model $\geq 1$, 2) p-value $< 0.05$, 125 differential metabolites were obtained, of which 74 were up-regulated and 51 were down-regulated. To visualize the variation in metabolites between the two groups, hierarchical clustering heat maps and volcano maps were plotted (Fig. 3).

### 3.4 Enrichment analysis of metabolite set and metabolic pathways

The main metabolite differences between melasma patients and healthy controls were amino acids, lipids, and carbohydrate-related metabolites (Fig. 4a). Pathway analysis shows the two metabolic pathways, tryptophan metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis was notably affected (Fig. 4b).

### 3.5 ROC curve analysis of potential biomarkers

ROC curve analysis was performed using biologically meaningful metabolites to screen potential biomarkers. ROC curve analysis showed that the area under curve (AUC) of some metabolites were 1, such as (5R)-5-hydroxyhexanoic acid, 2-indolecarboxylic acid, urocanic acid, S-allylcysteine, 12-Keto-leukotriene B4, prostaglandin F1a, 2-arachidonylglycerol (2-AG), butyric acid, S-methylmethionine, O-adipoylcarnitine, butyrylcarnitine, neuromedin B (1–3), aminoacidic acid. Additionally, the AUC of 6-hydroxymelatonin, L-phenylalanine, L-tryptophan, isobutyryl-L-carnitine were greater than 0.85, indicating that these metabolites may act as biomarkers of melasma (shown in Fig. 5a). Therefore, these metabolites were identified as main differential metabolites based on pathway analysis and ROC analysis. The boxplot represents the changes in the relative expression levels of main metabolites in the melasma group compared with the control subjects, among which 9 metabolites are up-regulated and 8 metabolites are down-regulated (Fig. 5b, Table S1).

### 3.6 Oxidative stress level and correlation analysis

In addition, the superoxide dismutase activity was found to be decreased in patients with melasma. Malondialdehyde (MDA) content was slightly increased, but no significant difference was found compared with the control group (Fig. 5c, Fig. 5d). The levels of the main metabolites were significantly correlated with the mMASI score of melasma and slightly correlated with MDA levels (Fig. 5e).

### 4 Discussion
Melasma is a complex disease that involves various pathological changes mainly mediated by hormonal influences and ultraviolet light, and is associated with aging, inflammation, oxidative stress, etc. (Filoni-Mariano & Cameli, 2019; Kwon, Na, Choi, & Park, 2019; Passeron & Picardo, 2018b; YangZeng & Lu, 2022). Recent advances in metabolomics have provided new opportunities to study disease mechanisms and identify potential intervention strategies (Jacob et al., 2019; Johnson Ivanisevic & Siuzdak, 2016). In this study, we used UPLC-MS to identify 125 differential metabolites in plasma between melasma patients and healthy controls.

The differential metabolites between melasma patients and healthy controls were primarily amino acids, lipids, and carbohydrate-related metabolites, which were directly related to basic metabolism and demonstrated that melasma patients had abnormal metabolic alterations. Enrichment analysis of the KEGG pathway suggested that tryptophan metabolism and biosynthesis of phenylalanine, tyrosine and tryptophan were mainly affected in melasma. These two metabolic pathways are the primary ones involved in pigment synthesis and are essential for sustaining an organism's normal physiological function (Lambrus et al., 2015; Valko-Rokytovska et al., 2019; Wen et al., 2022). Tryptophan and phenylalanine are regarded as necessary amino acids and the precursors of melanin production (Chakraborty & Chakraborty, 1993; Chakraborty Roy & Chakraborty, 1996; Fitzpatrick & Miyamoto, 1957; Schallreuter & Wood, 1999). The decrease of tryptophan and phenylalanine in plasma of patients with melasma may be responsible for the increased metabolism of these two amino acids into downstream substances to produce melanin.

Some kinds of lipids, such as 2-Arachidonylglycerol, Prostaglandin F1a, 12-Keto-leukotriene B4, were significantly increased in female patients with melasma. O-adipoylcarnitine, butyrylcarnitine and isobutyryl-L-carnitine were significantly down-regulated in patients with melasma. 2-Arachidonylglycerol is one of the most abundant endocannabinoids with the ability to bind to and activate type 1 and type 2 cannabinoid receptors (CB1 and CB2) (Chen Zhang & Chen, 2011). The action of 2-AG in vivo may be caused by activation of cannabinoid receptors, or mediated via 2-AG conversion to arachidonic acid, or oxidation of 2-AG by inflammatory enzymes such as cyclooxygenase or lipoxygenase (Biernacki & Skrzydlewska, 2016). Prostaglandin F1a and 12-Keto-leukotriene B4 are both arachidonic acid metabolites (Chiurchiu Leuti & Maccarrone, 2018). Melasma with increased 2-AG levels may also have elevated arachidonic acid levels, which would further promote inflammation (Rahaman & Ganguly, 2021). Additionally, melanin synthesis can be stimulated by proper concentration of 2-AG (Pucci et al., 2012).

In addition, urocanic acid was significantly elevated in the melasma patients, which may be connected to the increase of ultraviolet radiation (Zhu et al., 2018). In primary human keratinocytes, cis-UCA has been demonstrated to up-regulate many oxidative stress-related genes and can cause human keratinocytes to produce ROS (Kaneko et al., 2008). Cis-UCA significantly increases prostaglandin E2 production by upregulating cyclooxygenase 2, the rate-limiting enzyme in prostaglandin biosynthesis. This further increases melanogenesis, leading to or aggravating melasma (Kaneko et al., 2009; Kaneko et al., 2011; Kim, Shin, Kim, Hann, & Oh, 2012). Carnitine and acylcarnitine molecules may alter the metabolic status of mitochondria, and the decline of O-adipoylcarnitine, butyrylcarnitine, isobutyryl-L-carnitine results in
mitochondrial metabolic abnormalities, which are associated with aging (Zammit, Ramsay, Bonomini, & Arduini, 2009).

Patients with melasma were shown to have altered levels of certain metabolites known to have antioxidant properties, such as S-allylcysteine, 6-hydroxymelatonin, L-tryptophan, and L-phenylalanine (Feng et al., 2017; Janjetovic et al., 2017; Kim, Lee, Chang, Chun, & Kim, 2006; XuLiu & Fu, 2018). Among them, S-allylcysteine was up-regulated in patients with melasma, and 6-hydroxymelatonin, L-tryptophan, and L-phenylalanine were down-regulated. Oxidative stress may play a role in the development of melasma (Bellei & Picardo, 2020; Choubey et al., 2017; Sarkar, Devadasan, Choubey, & Goswami, 2020; Seckin et al., 2014). Elevated stress levels in melasma patients may be related to the alterations of antioxidant metabolites levels. In addition, we observed that SOD activities were significantly decreased in patients with melasma, which was consistent with previous studies (Sarkar et al., 2020). MDA contents in melasma patients were slightly increased, but no significant difference was found compared with the control group.

The main metabolites, including (5R)-5-hydroxyhexanoic acid, 2-indolecarboxylic acid, urocanic acid, S-allylcysteine, 12-Keto-leukotriene B4, prostaglandin F1a, 2-arachidonylglycerol, butyric acid, S-methylmethionine, O-adipoylcarnitine, butyrylcarnitine, neuromedin B (1–3), aminoadipic acid, 6-hydroxymelatonin, L-phenylalanine, L-tryptophan, isobutyryl-L-carnitine are expected to be used as disease markers and therapeutic targets for melasma by ROC analysis and correlation analysis. This study's sample size is small, however larger samples can be used in future research to corroborate the changes and applications of these metabolites in melasma.

Furthermore, our study discussed the changes of plasma metabolites in female patients with melasma for the first time. The metabolic status of female melasma patients was significantly changed, and it is believed that the metabolic pathways of tryptophan metabolism, phenylalanine, tyrosine, and tryptophan production may be related to the onset of melasma. These metabolic pathways are expected to be therapeutic targets. More studies are being conducted to fully elucidate the mechanism underlying changes in plasma metabolites in patients with melasma.

5 Conclusions

This study utilized UPLC-MS to identify differential metabolites in plasma between melasma patients and healthy controls. Tryptophan metabolism and biosynthesis of phenylalanine, tyrosine, and tryptophan pathways were found to be the main pathways related to melasma pathogenesis. The study also identified several metabolites that could be used as disease markers for melasma. This study sheds light on significant changes in plasma metabolites in patients with melasma and suggests potential therapeutic targets for treating this condition.

Declarations
**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**References**


**Figures**
Figure 1

Stability analysis of metabolomics methodology. Base peak chromatograms overlapping spectrum of quality control (QC) samples in positive mode (a) and negative (b) mode. In each individual panel, the graph above shows the base peak chromatograms of a single QC sample, and the graph above shows the overlapping of the base peak chromatograms of total QC samples.
Figure 2

Metabolomics analysis of plasma samples from melasma patients (Mel) compared with healthy controls (Con). (a-b) Principal component analysis (PCA) score plots of Mel (green) and Con (red) in positive (a) and negative modes (b). (c-d) Orthogonal partial least squares discriminate analysis (OPLS-DA) score plots for Mel (green) and Con (red) in positive (c, \( R^2_Y = 0.963, Q^2 = 0.949 \)) and negative modes (d, \( R^2_Y = 0.351, Q^2 = 0.102 \)).
Figure 3

Hierarchical clustering heat maps and volcanic maps of differential metabolites. (a-b) Hierarchical clustering heat maps of the differential metabolites in Mel and Con groups in positive (a) and negative modes (b). The different colors in the heat map indicate the relative metabolite abundance of metabolites. (c-d) Volcano maps showed increased or decreased plasma metabolites in Mel group versus Con group, with $p<0.05$, variable important for the projection (VIP) >1, in positive mode (c) and negative mode (d).
Figure 4

Enrichment of metabolite set and pathways of differentially expressed metabolites. (a) The KEGG classification of metabolites significantly changed in Mel and Con groups. (b) Main metabolic pathways based on the altered metabolites enrichment.
Figure 5

Receiver operating characteristic curves (ROC) analysis, oxidative stress level and correlation analysis. (a) The ROC of 17 biological metabolites, with AUC greater than 0.85, indicating good predictive ability. (b) Box plot showing significantly altered metabolites in the plasma. The expressions of the metabolites were converted to log2(x + 1) values for better visualization. (c, d) Determination of SOD activity and MDA content in plasma of melasma patients and healthy controls. (e) Correlation analysis of differential metabolite expression level with mMASI, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfiles.docx