Metabolomic Signature of Amino Acids in Plasma of Patients with non-segmental Vitiligo

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Abstract

Introduction: Vitiligo is an asymptomatic disorder which results from losing pigments (melanin), and causes skin blemishes or mucous, and impairs beauty.

Objective: The pathogenesis of Vitiligo is complicated, and several possibilities were suggested including self-safety, oxidative stress, neurological, and internal defects of melanocytes. However, it is well-known that the metabolism of pigments plays a significant role in the pathogenicity of the disease. The present study aimed to explore the role of amino acids in this disorder using targeted metabolomics for amino acids in order to find possible biomarkers for dialogizing and evaluating disease severity among patients.

Methodology: The amino acid profile was studied in plasma samples using a liquid chromatography equipped with fluorescent detector. First, 20 amino acids derivatized with o-phthalaldehyde (OPA) and fluorylmethyloxycarbonyl chloride (FMOC) were precisely determined. Next, the concentrations of these 20 amino acids and their corresponding molar ratios were calculated in 37 patients (18 females & 19 males) and 34 healthy individuals (18 females & 16 males). The differential concentrations of amino acids were analyzed to determine the contribution of amino acid in the skin involvement of Vitiligo.

Results: Based on the results, the differential concentrations of eight amino acids, i.e., cysteine, arginine, lysine, ornithine, proline, glutamic acid, histidine, and glycine were observed in addition to some pairwise concentration ratios in this disorder. The ratios of cysteine, glutamic acid, and proline increased significantly in Vitiligo patients. In addition, arginine, lysine, ornithine, glycine, and histidine decreased significantly compared to healthy individuals. Considering the percentage of skin area, we also showed that glutamic acid significantly has a higher amount in patients with less than 25% involvement compared to more than 25%. Finally, the two amino acids, i.e., cysteine and lysine, are considered as promising candidates for diagnosing and developing the disorder with high accuracy (0.96) by using the logistic regression assessment.

Conclusion: The findings are consistent with the previously illustrated mechanism of Vitiligo, such as production deficiency in melanin and an increase in immune activity and oxidative stress. Furthermore, new evidence was provided by using amino acids profile

toward the pathogenicity of the disorder. For instance, we provided molecular evidence for the relationship between vitiligo and anxiety, i.e., glutamate concentration profile change. The disease-specific amino acid fingerprints are valuable multi-biomarkers for diagnosing and promising tools for follow-up in disease progression and treatment process.

Keywords: Vitiligo, plasma, metabolomics, amino acids, liquid chromatography

Introduction

Vitiligo is a common chronic skin disorder in which pigment-producing cells or melanocytes are getting in trouble, resulting in varying patterns and degrees of skin depigmentation. Patients are characterized by losing epidermal melanocytes and progressive depigmentation. In addition, it appears in non-segmental (generalized) or segmental skin involvement, varying patterns, and skin depigmentation degrees [1, 2]. The worldwide prevalence of this disorder is in the range of 0.5% to 2% [3]. However, the main problems accompanied with vitiligo are related to its psychological aspect which is experienced by many patients around the world [4]. The levels of hopelessness, anxiety, depression, and general health of vitiligo patients was also compared to normal controls in several studies [5-8]. Hamidizadeh *et al.* demonstrated that anxiety and hopelessness levels were significantly higher in vitiligo patients than those of healthy controls. Along with social or psychological distress, people with vitiligo may be at the increased risk of sunburn, skin cancer, eye problems such as inflammation of the iris (iritis) and hearing loss [9].

The etiology of vitiligo including the reasons for melanocyte death is still unclear [10]. A complex immune, genetic, environmental, and biochemical causes are behind vitiligo, and the exact molecular mechanisms of its development and progression are considered as a challenging issue [2, 10, 11]. Although several vitiligo susceptibility loci were reported using genome-wide association studies (GWAS), a study on monozygotic twins described the vitiligo concordance rate of 23% and suggested a remarkable environmental contribution to its pathogenesis [10]. Zheleva *et al.* [12] revealed that oxidative stress is a triggering event in the melanocytic destruction, which is probably involved in the enteropathogenesis of vitiligo disease. Oxidative stress biomarkers could be found in the skin and blood of vitiligo patients. Sahoo *et al.* [2] reported that human vitiligo cell line and PIG3V have unique lipid profiles, which are potentially associated with vitiligo activity in skin and blood. These profiles contain some biomarker candidates for determining treatment response and progressing the disease earlier and accurately.

However, the role of other small molecules remains unknown in vitiligo. Using the metabolomics approach to assess global low molecular weight metabolites, we can provide insights into the driving mechanisms of vitiligo in detail and propose more potential

biomarkers [11, 13]. The present focused on establishing whether levels of important substrates such as amino acids as the most important primary metabolites altered in the plasma of vitiligo patients. Thus, these molecules may contribute to the vitiligo phenotype in melanocytes. The present study aimed to evaluate a comprehensive profile of amino acids in the plasma of people with vitiligo compared to healthy people in order to find a fast-determinable potential biomarker, treatment targets, and pathogenic pathways.

Material and methods

Patient samples

The study was undertaken according to the Helsinki Declaration and approved by the ethical review board of the Shahid Beheshti University of Medical Science with written and signed informed consent from the study subjects. Table 1 demonstrates the patients and healthy individuals data. In summary, 37 cases with vitiligo and 33 healthy ones attended to the dermatology clinic of Shohadaye Tajrish Hospital. The vitiligo diagnosis was based on the characteristic loss of skin pigmentation and the examination under Wood's lamp. Plasma (with K2-EDTA) were immediately separated from the peripheral venous blood by centrifugation at 4000 rpm at 4 °c for 20 minutes. The supernatant was isolated, snap-frozen, and stored at −80°C until analysis.

Table 1. Demographics of the study cohort

Information	HCs*	Vitiligo
Male	16	19
Female	18	18
Age, years**	35.8±11.8	35.1±12.1
Duration of the disease (year)	_	10.8±9.5
Illness severity (body surface area involvement %)	_	30.8±20.9
Active disease (having new lesions during last 6 months)	_	23
Positive family history	_	24

* HCs= Healthy controls, **= Means ± SD

Amino acid Analysis

To prepare the samples, they are transferred from -80 ° C refrigerator and placed in the ice to be melted. To 50 µL of sample 20 µL norleucine (500 µM) and 200 µL of methanol (kept at -20°C) were added and all are mixed for five seconds. To completely deproteination, they are kept at -20°C for 2 hours. Next, the samples are centrifuged at 13000 rpm for twelve

minutes at 4 °C at the next stage. The supernatant is wholly transferred to a Heidolph rotary evaporator and dried in a vacuum. These samples could be reserved at 4°C for four weeks. For HPLC analysis, previously dried samples were dissolved in 100 µl of water (containing 0.01% formic acid). According to previously described derivatization strategy to 10µl of each sample, 10µL *o*-phthaldialdehyde (OPA,) (for derivatization of primary amino acids) and 10 µL fluoronylmethyl chloroformate (FMOC-Cl) (for secondary amino acid derivatization) were added and then 20 µL of this sample are injected HPLC system [14, 15]. For the HPLC-DAD method, a Knauer system (WellChrom, Germany) equipping with a K-1001 pump, a K-2800 diode array detector detector, an autosampler S3900 (Midas), a K-5004 analytical degasser, and a 2301Rheodyneinjector with a 20 µL loop was used. HPLC separation was achieved using a Eurospher C18 column (4.6 mm × 250 mm, 5 µm), with a gradient elution program at a flow rate of 1.0 ml min^{-1}. The mobile phase was composed of solution A (acetonitrile + 0.05% trifluoroacetic acid, v/v) and solution B (0.05% aqueous trifluoroacetic acid, v/v). Then, the following gradient was applied: 0–10 min, isocratic gradient 70% B; 10–30 min, linear gradient 70-40% B; 30–40 min, linear 40–20% B; 40–50 min, linear 20-0% B; 50–65 min, linear 0-70% B; 65–75 min, isocratic gradient 70% B. The chromatographic peaks of the sample solution were identified by spiking and comparing their retention times and UV spectra with those of reference standards. Quantitative analysis was carried out by integration of the peak using the standard external method. To detect primary amino acids, the fluorescence detector was set at 337 nm and 470 nm for adsorption and excitation, respectively. Also, detection of the first-type and the second-type amino acids were performed at 262 nm and 338 nm. The accuracy and precision of both derivatization and HPLC technique were performed using analyzing of five individual samples. The intra-day mean coefficient of variation (n=3) and the inter-day mean coefficient of variation (n=3) were within 2% and 7%, respectively.

Statistical methods

For statistical analysis, we used software R version 4.0.0 (https://www.r-project.org/). Before the study, we applied Log2 transformation on the data. The data were analyzed for normal distribution using the Shapiro-Wilk test. Since the data are not normally distributed, we implemented non-parametric methods. We used the Mann Whitney U Test (Wilcoxon

Rank Sum Test) with the BH (Benjamini Hochberg) correction to compare study groups [16]. Also, we examined the sample homogeneity by principal component analysis (PCA).

On the other hand, the importance of random forest features (mean Gini reduction) for the response variable in the difference between the two groups was calculated. Then, the confusion matrix was obtained from the random forest classification result. To evaluate the significantly differentiated amino acids, which we selected as biomarkers for the cause probability or severity of the disease, we used logistic regression and sensitivity analysis by the receiver operating characteristic curve (ROC). Besides, to compare the two groups of patients and healthy individuals, the relationship between disease severity, disease progress, family history, and duration of each amino acid was used to evaluate the Mann Whitney U test with BH correction. This analysis was repeated for the two groups of vitiligo patients with different percentages of skin involvement, i.e., more than 25% and less than 25% of the body skin surface area.

Results

Targeted metabolomic analysis of plasma reveals vitiligo-specific biomarker profiles

We performed targeted quantitative analysis of 22 amino acids for the plasma of patients with vitiligo disorders. The patient and control groups (34 healthy cases and 37 vitiligo cases) were analyzed by the principal component analysis (PCA) with all samples. Based on the results, the samples were well-clustered in two separate groups (Fig. 1). Supplementary Table 1 indicates the absolute concentration of 22 amino acids in both groups.

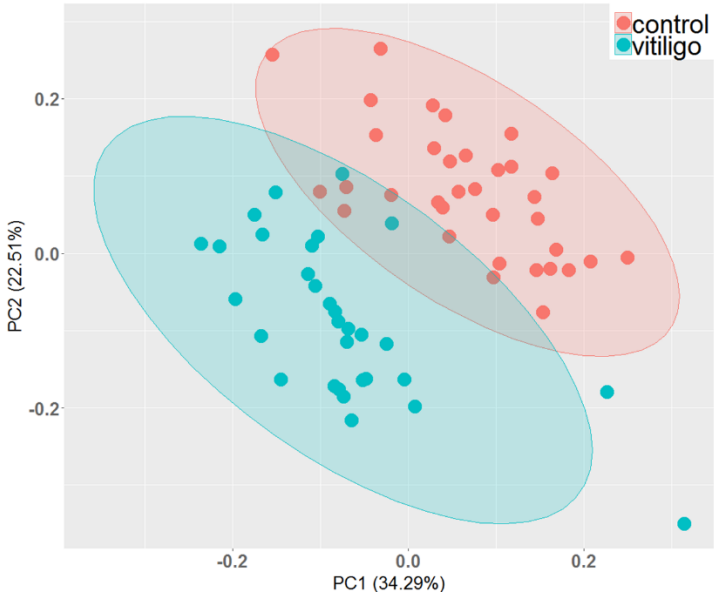


Figure 1. PCA analysis of data obtained by HPLC-FLD. All 22 amino acid concentrations were used to evaluate the sample homogeneity as a quality assessment of this study.

Next, the data were analyzed to find the difference in amino acid concentrations in both groups (Supplementary file 1). Figure 2a demonstrates a volcano graph in which horizontal and vertical axes correspond to log2 fold change of sample concentrations and -log10 adjusted p-values, respectively. As shown, more than 2-fold increase occurred in Cysteine, Proline, and Glutamic acid amounts after using the Wilcox rank test with a p-value less than 0.05. In addition, lysine, arginine, ornithine, histidine, and glycine decreased by half or less

in vitiligo patients (Supplementary file 2). Figure 2b shows the Gini error reduction diagram obtained from the random forest algorithm with a tree number of 500. In this plot, a higher mean decrease in Gini indicates higher amino acid importance. Amino acids are sorted and displayed in this plot based on the Gini index. Cysteine showed the highest Gini index with a high amount in vitiligo samples. Lysine and arginine were in the second and third rank of this plot with a high amount in healthy individuals.

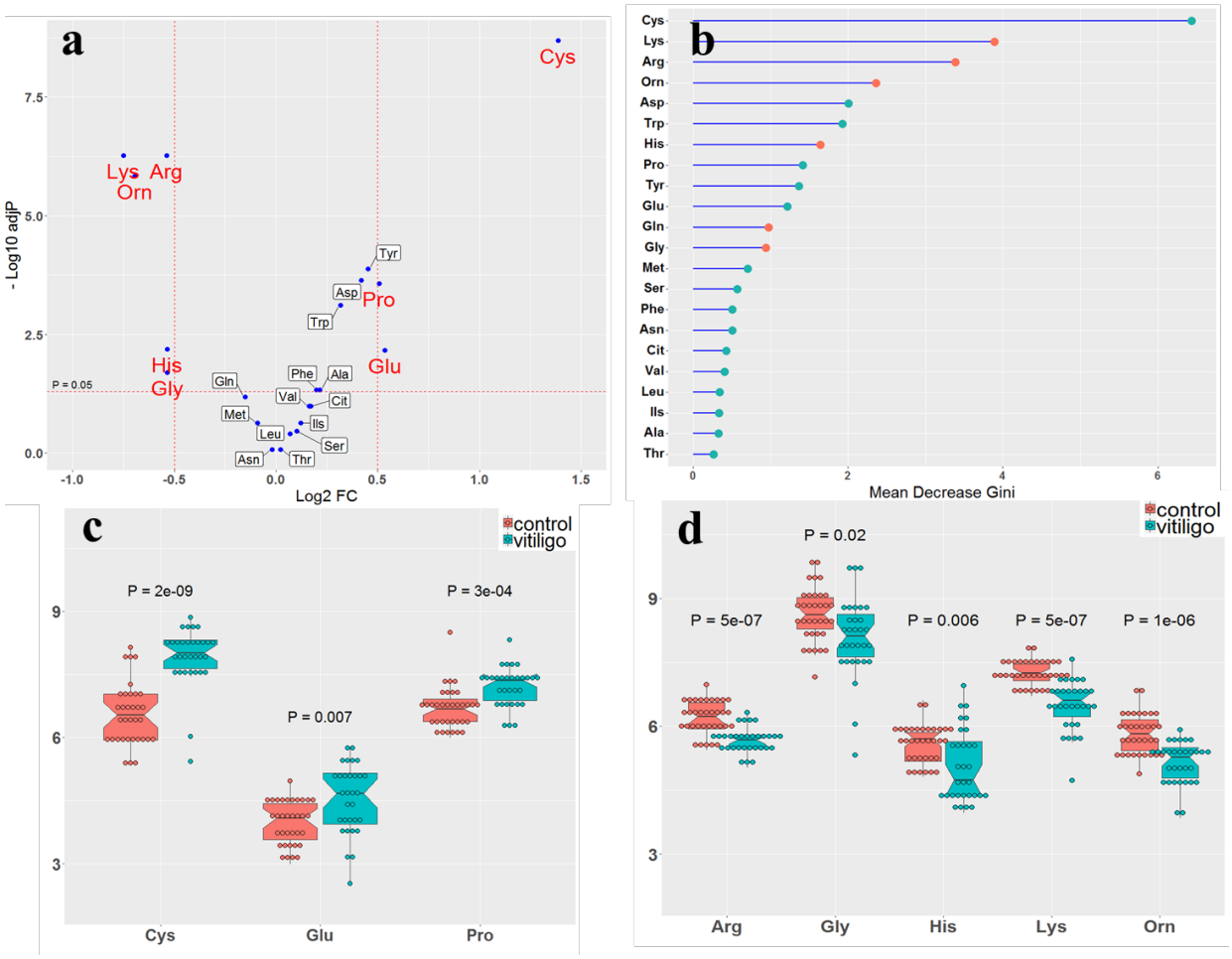
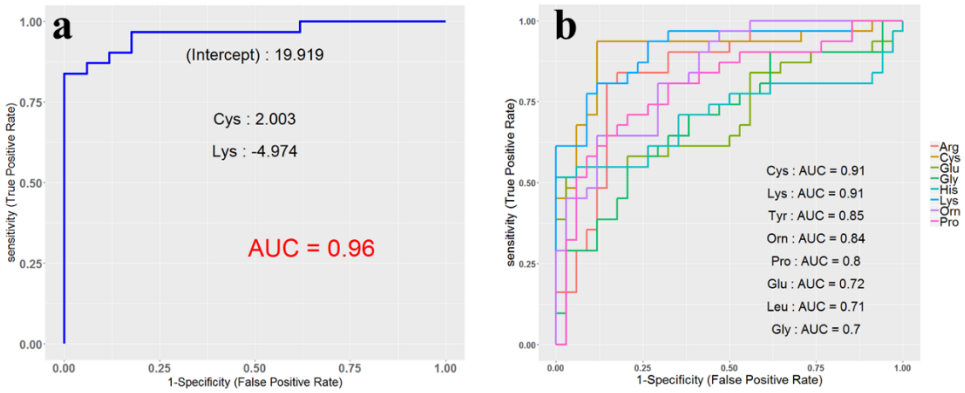


Figure 2. (a) Volcano plot of amino acid plasma concentration, (b) Gini error reduction diagram with tree number of 500, and notched box plot for increased (c) and decreased (d) amino acids. The red and blue dots indicate amino acid values in healthy individuals (control) and vitiligo patients. The adjusted p-values are represented in these plots.

Then, the performance of amino acid was tested as disease biomarkers in a pooled set of vitiligo patients, who were asked which amino acids would demonstrate the best sensitivity and specificity for vitiligo to be distinguished it from healthy controls. Based on receiver

operating characteristic (ROC) curve analysis, the top two significant amino acids with the highest area under the curve (AUC) were cysteine and lysine with 0.91 AUC independently and 0.96 AUC together, called "multi-biomarker" for vitiligo (Figs. 3a & b). A positive/negative coefficient means the role of the amino acids in increasing or decreasing the risk of vitiligo. Next, based on the random forest method, a confusion matrix was developed in which two groups used in the present study were classified with a low amount of classification error (0.032) (Fig. 3c).



OOB estimate of error rate: 1.54%			
	control	vitiligo	class. error
control	34	0	0
vitiligo	1	30	0.032258

Figure 3. (a) ROC curve for Cysteine and Lysine with the highest variations, (b) ROC curve to show the sensitivity and specificity of the eight top amino acids, i.e., Cys, Lys, Tyr, Orn, Pro, Glu, Leu, and Gly independently with the AUC (Area under curve) values (c) confusion matrix based on random forest model.

Glutamic acid as biomarker for Vitiligo progressing

In this section, the role of amino acids in developing vitiligo was evaluated based on skin involvement area. Therefore, the percentage of skin area with vitiligo was used to determine any differential amount of amino acids across a given cut-off. Glutamic acid had a lower amount by using 25% as a cut-off after comparing the patients with more and less than 25% involvement. It means that glutamic acid becomes involved in developing vitiligo while it cannot contribute to vitiligo progress at severe stage. The amount of glutamic acid decreased significantly for the patients with more than 25% involvement, similar to healthy individuals (Figs 4a &b). It is worth noting that no other remarkable changes occurred in the amount of amino acids by considering a serial of cut-offs for skin involvement.

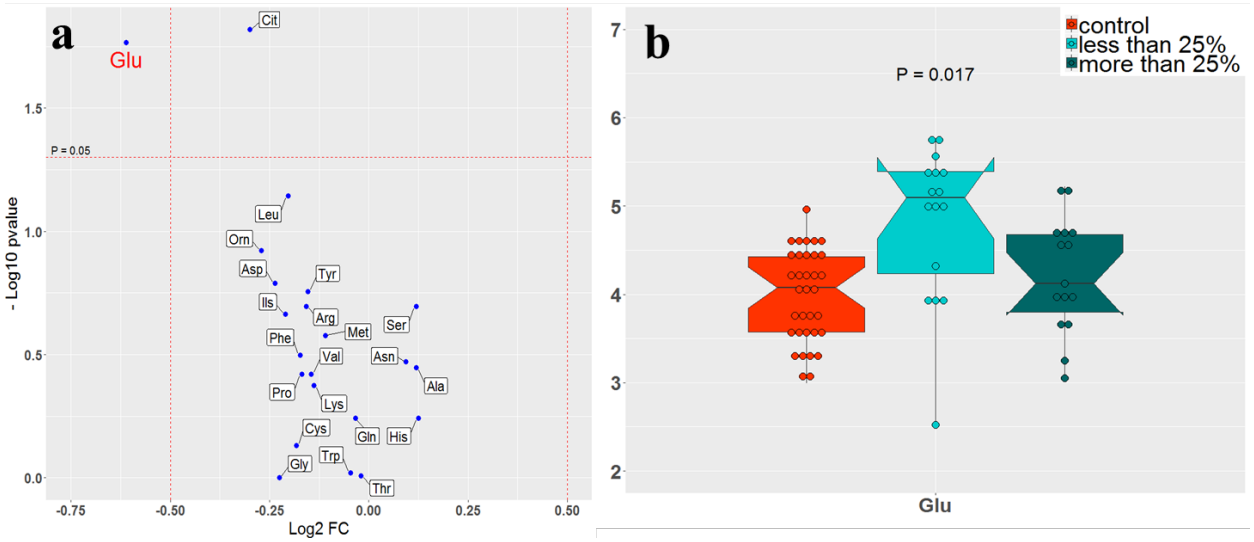
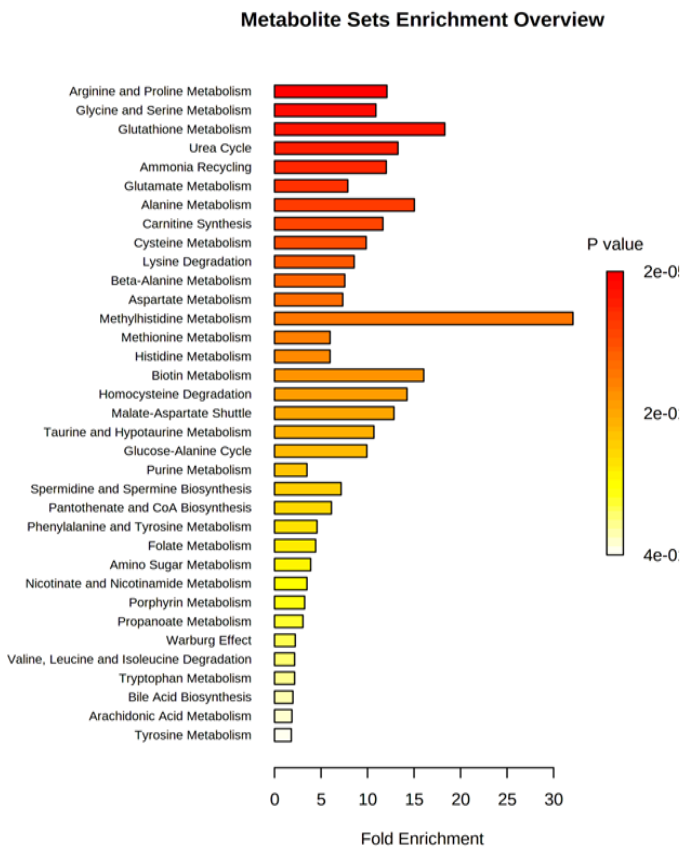


Figure 4. Amino acid amount analysis of patients with more and less than 25% of skin involvement. (a) Volcano diagram shows the amount fold changes of all amino acids with adjusted p-values. (b) The notched box plots indicate the amount of glutamic acid in patients compared to healthy individuals.

Pathway Analysis: Amino acid metabolism remodeled in melanin production

Pathway analysis of plasma amino acids showed several significantly changed pathways common to all vitiligo patients, but not for healthy individuals (Fig. 5). Pathway-associated metabolite and disease-associated metabolite analyses were performed to demonstrate the significantly altered metabolic pathways in Vitiligo cases. Based on the findings, 35 pathways were significantly different between Vitiligo and healthy samples, of which some pathways showed high fold enrichment values. Following metabolites and metabolic terms changed in Vitiligo cases with lower p-values and higher fold enrichment values such as arginine and proline metabolism, glycine and serine metabolism, glutathione metabolism, urea cycle, ammonia recycling, glutamate metabolism, alanine metabolism, carnitine synthesis, cysteine metabolism, lysine degradation, beta-alanine metabolism, aspartate metabolism, and methyl histidine metabolism. After disease-based enrichment analysis, the disease-associated metabolite terms were observed for vitiligo patients compared to healthy controls. Ornithine transcarbamylase deficiency (OTC), Hyperornithinemia with gyrate atrophy (HOGA), Delta-pyrrolidone-5-carboxylate synthase, hyperprolinemia-type II, short bowel syndrome (under arginine -free), 2-hydroxyglutaric acidemia, 3-phosphoglycerate dehydrogenase deficiency dementia, dicarboxylic aminoaciduria, histidinemia, hyperlysinemia I-Family I, phosphoserine aminotransferase deficiency, short-bowel syndrome, and SOTOS syndrome are considered as the significant disease-associated metabolic diseases with the high amount of fold enrichment values.



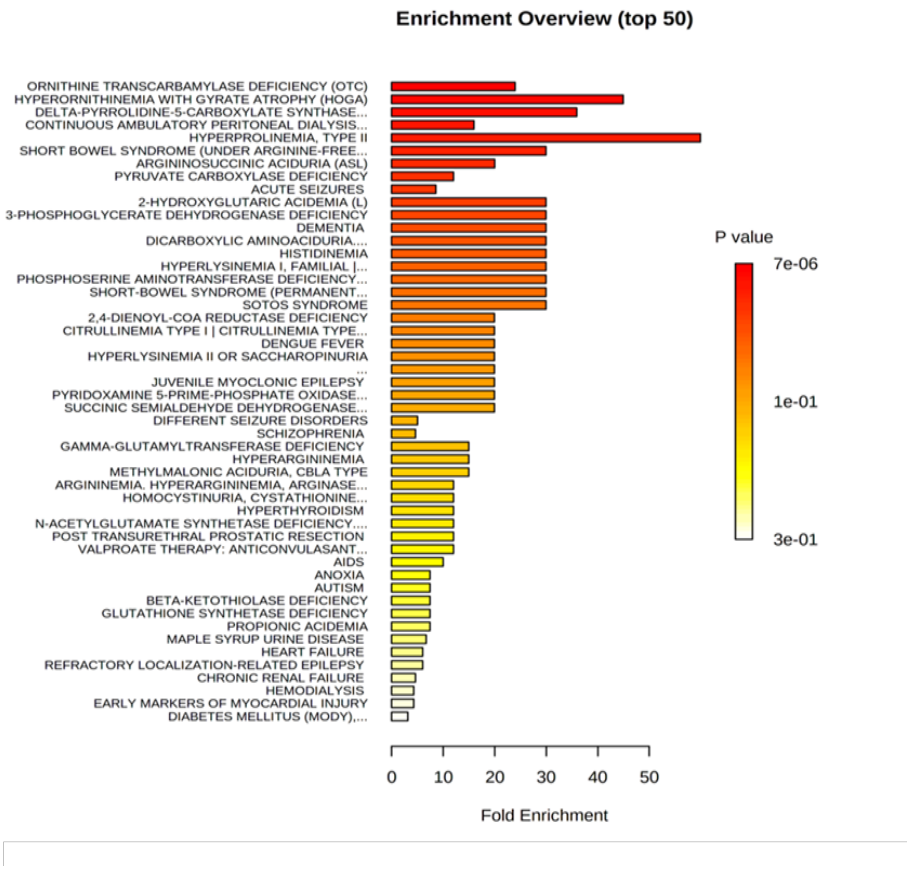


Figure 5. Pathway-associated and disease-associated metabolite analyses. The colored bars are based on P values, and the bar length corresponds to the fold enrichment. The corrected P values are also provided.

Discussion

The present study focused on disease-specific amino acid fingerprints, which were detectable in plasma, vitiligo, as an asymptomatic disorder resulting from losing melanin. Based on the evidence, the disease group showed plasma amino acid fingerprints which were separately clustered from healthy controls, indicating the potential of amino acid fingerprints as multi-biomarkers for diagnosis, disease progression follow-up, and treatment effect. In addition, the patients with skin involvement more than 25% caused similar amino acid changes reflected in plasma by providing more details about the role of amino acids in progressing the disease. Furthermore, the targeted metabolomics approach identified known therapy targets, already in clinical use, and identified new potential targets for treating vitiligo. Thus, it is suggested that targeted metabolomic analysis may be not only valuable for mechanistic studies, but also for metabolic targets in treatment trials. To the best of our knowledge, few studies have investigated the role of some amino acids and the derivative molecules associated with the melanin production in vitiligo such as phenylalanine, tyrosine and glucosamine, trimethylamine, cysteine, homocysteine, and thiol [2, 17]. Based on these observations, there are both conventional and unconventional therapies for vitiligo including (i) amino acids such as L-phenylalanine, (ii) antioxidant agents such as alpha lipoic acid, glutathione (GSH), fluorouracil, levamisole, and melagenine, and (iii) metals such as zinc, minoxidil [18].

However, we first provided the complete profile of free amino acids in plasma to evaluate the changes in the metabolic pathways of vitiligo. In general, amino acids play an essential role in detoxification and immune responses through regulating the activation of T and B lymphocytes, natural killer cells, macrophages, and cellular redox state [19]. We found that arginine and proline metabolism as the most significant pathway enriched based on the altered amino acid profile in vitiligo. In most cell types, arginine, as a precursor of citrulline, regulates the activity of immune system by producing nitric oxide [20]. On the other hand, ornithine is catabolized by proline oxidase in different organs to produce hydrogen peroxide and pyrroline-5-carboxylate (P5C). By converting P5C into proline, a reduction occurs in the ratio of NADP+ to P5C reductase-dependent NADPH [20-25]. Additionally, the proline-P5C cycle regulates the cellular redox state. These products have well-known functions such as

mitochondrial integrity, ion channel activity, cell death, antioxidation, and anti-tumor activity. Additionally, arginine and ornithine decreased and proline increased at the same time in patients. Thus, the findings indicated impaired arginine and proline metabolism, urea cycle, or nitrogen imbalance due to mitochondrial deficiencies in vitiligo. In other words, it is a consequence of a disruption in response to oxidative stress and cell damage. In addition, the amount of glycine in vitiligo was lower than that of healthy controls. Glycine itself is considered as a potent antioxidant scavenging free radical, which is essential for the antioxidative defense of leukocytes. Furthermore, it plays a major role as an anti-inflammatory, immunomodulatory, and cytoprotective agent. Therefore, glycine reduction in the patients strengthens the previously proposed association of developing vitiligo with the oxidative stress response. On the other hand, lysine, which decreases in vitiligo patients, has multiple catabolic pathways such as carnitine biosynthesize. Carnitine and its esters help reduce oxidative stress [26]. Therefore, the reduction of this amino acid highlights the role of oxidative stress in vitiligo.

Additionally, glutamic acid increased in the patient group compared to the healthy control. It means that we can expect the increased activity of the immune system and the cellular redox state. Glutamic acid paves the way for transporting the reducing agents across the mitochondrial membrane and regulating glycolysis and cellular redox state through the malate/aspartate shuttle [27]. In the present study, a high amount of cysteine was observed in vitiligo patients, which is the downstream product of homocysteine. Some studies indicated a high amount of homocysteine and thiols in vitiligo patients, and predicted the cysteine increment in this disease accordingly [10, 28-33].

In addition, the enrichment of tyrosine metabolism was detected in vitiligo patients. Tyrosine is converted into dopaquinone, as a highly intermediary metabolite, which is essential for regulating melanogenesis. Dopaquinone, in a rapid reaction with cysteine, gets involved in the pheomelanin production, which is considered as a common type of melanin pigment found in the hair and skin color [34]. Likewise, we detected a high amount of cysteine levels in the patient, which indicates that the production of pheomelanin pigment is impaired by other factors such as increasing thiol levels. Based on the findings, cysteine and the ratio of cysteine to ornithine increased along with a decrease in the ratio of glycine,

arginine, ornithine, and lysine to cysteine in the patient group (Fig. 2 & Supplementary file XXX). Thus, impaired cysteine metabolism disrupts the production of pigment, increases the activity of immune system, and counteracts the effects of oxidative stress due to the deficiency in the production of antioxidant compounds. Additionally, histidine reduced significantly in the patient group. This amino acid plays a vital role in the skin vulnerability to the UV as an upstream molecule to make urocanic acid. In other words, histidine controls the activity of immune system against the UV radiation from the sun [35-37]; Hence, the decreased level of histidine in vitiligo means a disruption of the skin immune system in this disorder.

In the present study, glutamic acid was considered as a potential biomarker for progressing the disease. In addition, it can help determine 25% involvement of skin as a candidate cut-off to divide patients into the early and late stages. Furthermore, glutamic acid demonstrated a pulse propagation behavior by increasing the spot extent across the body surface. Due to the role of glutamate in regulating the cell cycle [38-40], its altering level in vitiligo can indicate implicated cell metabolism and increased cell death. On the other hand, some studies previously reported the influence of glutamate imbalance through glutamatergic neurotransmission with anxiety and stress [41-43]. Our findings indicated that glutamate also engaged in this prevalent psychiatric disorder in the patients, which has been described in vitiligo frequently [5-8].

Conclusion

Based on the results of the present and previous studies on vitiligo, the melanin production decreased by increasing the amount of cysteine and disrupted oxidative stress based on the glutamic acid and proline enhancement. Moreover, the reduction of arginine, glycine, lysine, histidine, and ornithine can damage melanocytes. Then, they resulted in vitiliginous lesions on the skin surface of patients. This finding demonstrated the importance of these pathways either causing or as a consequence of vitiligo. Thus, examining the proposed biomarkers can contribute to the early diagnosis of at-risk patients. Additionally, considering the changes in glutamic acid levels as biomarkers can help determine the prognosis of the disease. Furthermore, understanding the role of these biomarkers in vitiligo can provide the scientific basis for developing novel therapeutic approaches in this disorder.

In the present study, the separate patients were clustered based on their age- and gender-matched controls in this targeted metabolomic analysis. The results highlight the potential of this approach for mechanistic studies and as biomarkers for disease progression follow-up. Finally, our screen identified targets for treating metabolite, which can confirm the formerly investigated targets and propose novel ones for vitiligo disorder.

Acknowledgments

Supplementary files

Supplementary file 1: The raw data of amino acid concentrations in serum samples for both control and vitiligo groups.

Supplementary file 2: Clustering heat map by Pearson correlation of the vitiligo patients' samples and amino acid profiles. Rows and columns represent the vitiligo and control groups (A) and detected amino acids (B) the serum samples. The color represents correlation coefficients from low (blue) to high (red).

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