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# Identification and validation of argininosuccinate synthase as a candidate urinary biomarker for major depressive disorder

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ABSTRACT

*Background:* Major depressive disorder (MDD) is a debilitating psychiatric mood disorder. However, no objective laboratory-based test is yet available to aid in the diagnosis of this disorder.
*Methods:* In order to identify urinary protein biomarker candidates for MDD, the differential proteomic analysis of urine samples from first-episode drug-naïve MDD subjects and healthy controls (HC) was carried out by using two-dimensional gel electrophoresis separation followed by MALDI-TOF/TOF-MS/MS identification. Then, the differential expression levels of some candidate proteins were further validated by immunoblot analysis.
*Results:* Through mass spectrometry and database searching, a total of 27 differential proteins were identified, primarily including enzymes, plasma proteins, serpins, and adhesion molecules. Five proteins were selected for subsequent validation by Western blotting. One arginine recycling enzyme – argininosuccinate synthase (ASS1) – was further confirmed to be significantly downregulated in the urine of 30 depressed subjects while remaining unchanged in the plasma. Importantly, receiver–operator curve analyses revealed that ASS1 displayed strong efficacy in distinguishing MDD subjects from HC.
*Conclusion:* The present study provides a range of urinary protein biomarker candidates for MDD, and further demonstrates that ASS1 has a potential for clinical diagnosis of this disorder.


## 1. Introduction

Major depressive disorder (MDD) is a widespread and debilitating psychiatric mood disorder that is associated with increased morbidity and decrease in the quality of life [1,2]. Currently, the etiological and pathophysiological molecular correlates underpinning MDD are still poorly understood. Its diagnosis is symptomatic and subjective, as psychiatrists rely mainly on interview-based physician–patient communication and diagnosis is not supported by objective laboratory-based diagnostic modalities [3,4]. An earlier clinical meta-analysis of 50,371 patients with depression from 41 studies found that the accuracy of symptom-based diagnosis of MDD was only 47% [5]. In light of these factors, the development of empirical laboratory-based diagnostic approaches for MDD is still required.

To address this challenge, proteomics—a non-hypothesis-driven experimental approach that enables the study of protein expression and the detection of potential protein biomarkers in a comprehensive and global manner—is considered to be a powerful tool and has been previously applied in cancer, cardiovascular disease, and psychiatric illness [6–8]. Proteomic analysis of patients with MDD can help establish biomarkers for diagnostic use, identify new treatment targets, and improve our understanding of the disorder.

Because MDD is a neuropsychiatric disorder, sampling of brain tissue or cerebrospinal fluid (CSF) would be most suitable for proteomic analysis in order to identify relevant disease biomarkers. However, these samples are not practically accessible in a clinical setting [7,9–11]. In contrast to brain tissue and CSF, human plasma and urine can be sampled easily without invasive procedures. For example, our previous comparative proteomic analysis of plasma samples from patients with depression and healthy controls (HCs) showed alterations in several proteins that are primarily involved in lipid metabolism and immunoregulation, which may serve as potential biomarker candidates for the diagnosis, prognosis, and disease monitoring of MDD [12]. In addition, by comprehensively profiling the small-molecule alterations in plasma

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and urine samples from subjects with depression by using NMR-based metabolomics, we previously identified several plasma and urinary metabolite biomarker panels for MDD diagnosis [13,14] These two previous studies present promising findings, especially with respect to distinguishing between MDD groups in a blinded manner. Hence, human plasma and urine appear to be promising sources of biomarkers for MDD.

Therefore, in this study, we used urine samples to identify potential urinary protein biomarkers for MDD. The urinary proteomes of subjects with depression and of HCs were comparatively analyzed by two-dimensional gel electrophoresis (2-DE). The proteins with differing levels between groups were identified by matrix-assisted laser desorption ionization–time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS/MS). Some of these proteins were then further studied by immunoblot analysis to identify potential urine diagnostic biomarkers for MDD.

## 2. Methods

### 2.1. Subjects

The Ethics Committee of Chongqing Medical University reviewed and approved the protocol of this study and the procedures employed for sample collection and analysis. All subjects gave their written informed consent after a detailed introduction of the study. All procedures were performed according to the Helsinki Declaration.

MDD and HC subjects were enrolled from the Department of Psychiatry and the Medical Examination Center of the First Affiliated Hospital at Chongqing Medical University, respectively. All candidates were interviewed with the Structured Clinical Interview from the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV). The inclusion criteria for MDD subjects included: (i) diagnosis of MDD according to DSM-IV criteria; (ii) first-episode and treatment-naive; (iii) aged 18–65 years; (iv) score ≥18 on the 17-item Hamilton Rating Scale for Depression (HAM-D); and (v) no significant abnormalities in clinical laboratory tests (e.g., urine examination, liver function tests). The exclusion criteria for MDD subjects included: (i) history of any Axis I psychiatric disorders according to DSM-IV criteria; (ii) pregnancy, nursing, or menstruation for female subjects; (iii) any serious physical illness; (iv) chronic medication; (v) history of substance abuse/dependency; or (vi) history of suicidal ideation. After meeting the aforementioned criteria, the Beck Depression Inventory (BDI) was administrated individually to each MDD subject. HC subjects were healthy male and female volunteers with no known past medical or psychiatric history and no family history of psychiatric disease. A total of 30 adults with MDD and 30 healthy volunteers were enrolled. Each cohort included 15 males and 15 females.

### 2.2. Urine collection and storage

Urine sample collection was performed according to the EuroKup/HKUPP urine protocol and recommendations (http://www.eurokup.org/). For all experiments, second-morning midstream urine was used. Within 1 h of collection, samples were centrifuged at 1000 *g* for 10 min at 4 °C and passed through 0.34 mm Whatman chromatography paper in order to remove cellular debris and nuclei. The supernatant was aliquoted (10 ml aliquots) and then stored at −80 °C until further processing.

For 2-DE analysis, the pooled samples were mixed with 200 μl of rehydration solution (containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM dithiothreitol, and 0.2% bio-lyte (pH 3–10)). Total protein concentration was estimated using the Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA, USA) [15], and a total of 100 μg of protein per gel was loaded. For each gel, 17 cm-long pH 3–10 NL and pH 4–7 L IPG strips were passively rehydrated for 16 h in 350 μl of sample at 25 °C; then, 1 ml of mineral oil was added. Isoelectric focusing (IEF) was performed according to the manufacturer's protocol (Bio-Rad) [16]. In order to eliminate disulfide bonds in the focused proteins in preparation for the second dimension, IPG strips were equilibrated in buffer A (6 M urea, 2% SDS, 0.375 M tris–HCl (pH 8.8), 20% glycerin, and 2% dithiothreitol) for 15 min and in buffer B (6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 20% glycerin, and 2.5% indole-3-acetic acid) for 15 min under gentle agitation. The immobilized pH gradient strips were then rinsed in SDS gel running buffer and embedded in 0.5% w/v agarose on top of 12.5% acrylamide slab gels. Second dimension electrophoresis was performed at room temperature followed by 20 mA/gel until the dye front reached the bottom of the gel. Every gel was run in triplicate.

### 2.3. Analysis and matching of protein spots

Post-electrophoresis, gels were silver-stained [17]. Image scans were performed immediately after dying (Epson 10000XL scanner, Epson Co., Ltd., Beijing, China). Protein expression analysis was performed for each of the three gels in parallel using PDQuest software version 8.0.1 (Bio-Rad Laboratories, Hercules, CA, USA), and spots were aligned by definition of three landmarks and normalized by internal calibration standards. Spots were matched prior to classification of the different groups. Then, the ratio change in protein differential expression was obtained with a Student's *t*-test p-value of less than 0.05 considered to be statistically significant between the two groups.

### 2.4. Protein identification by MALDI-TOF/TOF-MS/MS

Spots displaying at least a 1.5-fold difference in protein expression were excised from the preparative gels [18] and destained with 100 mM $NH_4HCO_3$ in 30% ACN. After removing the destaining buffer, the gel pieces were lyophilized and rehydrated overnight at 37 °C for digestion in 30 μl of 50 mM $NH_4HCO_3$ containing 50 ng trypsin (sequencing grade; Promega, Madison, WI, USA). The peptides were extracted three times with 0.1% TFA in 60% ACN. Extracts were pooled together and lyophilized. The resulting lyophilized tryptic peptides were stored at −80 °C until MALDI-TOF/TOF-MS/MS analysis. A protein-free gel piece was treated as above and used as a control for possible trypsin-derived autoproteolysis products. MALDI-TOF/TOF-MS/MS spectra were obtained using the ABI 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Foster City, CA, USA) operating in a result-dependent acquisition mode. Peptide mass maps were acquired in positive ion reflector mode (20 kV accelerating voltage) with 1000 laser shots per spectrum. Particularly, monoisotopic peak masses were automatically determined within the mass range 800–4000 Da with a signal-to-noise ratio minimum set to 10 and a local noise window width of m/z 250. Up to five of the most intense ions with minimum signal-to-noise ratios of 50 were selected as precursors for MALDI-TOF/TOF-MS/MS acquisition (excluding common trypsin autolysis peaks and matrix ion signals). In MS/MS-positive ion mode, spectra were averaged, collision energy was set to 2 kV, and the default calibration was selected. Monoisotopic peak masses were automatically determined with a signal-to-noise ratio minimum set to 5. The local noise window width was m/z 250.

### 2.5. Database searching

The spectra were searched against the UniProt-Human (UniProt-Human, 141,032 entries, download at Dec 31, 2014) using GPS Explorer, version 3.78 (Applied Biosystems) and MASCOT version 2.1 (Matrix Science) with the following parameter settings: enzyme, trypsin; allowance of up to one missed cleavage; fixed modification, carbamidomethylation (C); variable modification, oxidation (at Met); peptide mass tolerance, 100 ppm; fragment tolerance, ±0.3 Da, and a minimum ion score confidence interval for MS/MS data set to 95%.

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