Comparative proteomic analysis of plasma from major depressive patients: identification of proteins associated with lipid metabolism and immunoregulation

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Abstract

Major depressive disorder is a common neuropsychiatric disorder contributing to several socio-economic burdens including disability and suicide. As the underlying pathophysiology of major depressive disorder remains unclear, no objective test is yet available for aiding diagnosis or monitoring disease progression. To contribute to a better understanding of its pathogenesis, a comparative proteomic study was performed to identify proteins differentially expressed in plasma samples obtained from first-onset, treatment-naive depressed patients as compared to healthy controls. Samples from the two groups were immunodepleted of seven high-abundance proteins, labelled with isobaric tags for relative and absolute quantitation and then analysed by multi-dimensional liquid chromatography-tandem mass spectrometry. The proteomic results were further validated by immunoblotting or enzyme-linked immunoadsorbent assays and analysed with the MetaCore database. The results demonstrate that the functions of the altered proteins are primarily involved in lipid metabolism and immunoregulation. These findings suggest that early perturbation of lipid metabolism and immunoregulation may be involved in the pathophysiology of major depressive disorder.

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Key words: Biomarker, iTRAQ, major depressive disorder, plasma, proteomics.

Introduction

Major depressive disorder (MDD), also known as major depression, is one of the most prevalent psychiatric disorders with a lifetime prevalence rate of 15–17% (Ebmeier et al. 2006). The condition is responsible for a host of socio-economic ills, such as increased health care costs, loss of work productivity, disability and suicide (Simon, 2003). Although several hypotheses have been proposed to explain the aetiology of MDD, the accurate pathogenesis of this mental disorder is still poorly understood. Currently, the diagnosis of MDD is primarily based upon clinical factors and no objective test is available for aiding either diagnosis or prognosis of the illness. Moreover, only about one-third of patients respond to initial antidepressant treatment and it generally takes several weeks of drug therapy before significant mood improvement is observed (Trivedi et al. 2006). These facts indicate that there is a need to improve the understanding of the molecular alterations associated with MDD. This would likely aid in a more accurate earlier diagnosis and more appropriate treatment regimens for this psychiatric disorder through more objective diagnostic and prognostic methodologies.

Hypothesis-free approaches, such as proteomics and genomics, are considered to be powerful tools in discovering novel molecules involved in the
pathophysiology of disease. These approaches have been employed in a wide variety of disorders, including cancer, cardiovascular disease and psychiatric illness (Alaiya et al. 2005; Taurines et al. 2011; van Eyk, 2011). Given post-translational events and the probable inconsistency in translational regulation, proteomics may have some diagnostic advantages over genomics. Although several proteomic studies have been applied to MDD, little attention has been paid to clinically accessible samples from major depressive patients (Martins-de Souza et al. 2010; Taurines et al. 2011).

As MDD is a neuropsychiatric disorder, sampling of brain tissue or cerebrospinal fluid (CSF) would be most suitable for proteomic analysis in order to identify disease biomarkers. However, these samples are not practically accessible in living humans on account of the higher costs and invasiveness of brain biopsy and lumbar puncture. On the other hand, blood samples can be easily obtained with minimal risks. Moreover, approximately 500 ml CSF is absorbed into the blood every day (Hye et al. 2006) and deranged blood–brain barrier permeability has been reported in MDD (Hampel et al. 1997; Niklasson & Ågren, 1984), which implies that protein exchange may occur between the brain and peripheral circulation. Therefore, the peripheral bloodstream may be a promising source for identifying depression-related biomarkers.

In this study, plasma samples from depressed patients and healthy controls were analysed using a quantitative proteomic approach based on isotopic tags for relative and absolute quantitation (iTRAQ) and multi-dimensional liquid chromatography–tandem mass spectrometry (LC-MS/MS). Differentially expressed proteins were further validated by immunoblotting or enzyme-linked immunoadsorbent assays (ELISA) and analysed using the MetaCore database (GeneGo, USA). This proteomic approach may ultimately contribute to a better understanding of the pathophysiology of MDD.

Materials and methods

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.

Subjects and samples

Depressed patients and healthy controls were enrolled from the Department of Psychiatry and the Medical Examination Center of the First Affiliated Hospital of Chongqing Medical University, respectively. All participants were interviewed with the Structured Clinical Interview for DSM-IV. The inclusion criteria for patients included: diagnosis of MDD according to DSM-IV criteria; first-episode and treatment naive; aged 18–50 yr; Hamilton depression score >17 on the 17-item Hamilton Rating Scale for Depression; and no significant abnormalities in clinical laboratory tests (blood and urine examination, liver function tests). The exclusion criteria included: history of any Axis I psychiatric disorders according to DSM-IV criteria; pregnancy, nursing or menstruation for female subjects; any serious physical illnesses, treatment with chronic medication; and/or history of substance abuse/dependency. After passing the aforementioned criteria, the Beck Depression Inventory was then administered individually to each depressed patient. All recruited healthy controls met the aforementioned criteria. Additional exclusion criteria for healthy controls were no current or past diagnosis of MDD and no family history of any psychiatric disorders. Fasting blood samples (approximately 4 ml) were collected into 6 ml EDTA tubes (BD vacutainers catalog no. 367 863; Becton, Dickinson & Co., USA) between 08:00 hours and 10:00 hours and separated for plasma within 1 h of collection. The aliquoted plasmas were then stored at −80 °C until analysis.

Immunodepletion of high abundance plasma proteins

Stored plasma samples were thawed and equal volumes of plasma from 21 depressed patients and 21 healthy controls were pooled. The pooled samples were immunodepleted of seven high abundance plasma proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin and fibrinogen) using a multiple affinity removal column human-7 (4.6 mm ID × 50 mm) (Agilent Technologies, USA). The procedure was performed according to the manufacturer’s instructions. The flow-through fractions (low and medium abundance proteins) were collected and desalted with a homemade C8 trapping column (4.6 mm ID × 10 mm, 5 µm, 300 Å). Then, the desalted samples were concentrated using a SpeedVac (RVT4104; ThermoFisher Scientific Inc., USA) and resuspended with 50 mM phosphate buffer (pH 8.5) containing 8 M urea. The protein concentration was determined by a commercial Bradford Protein Assay Kit (Beytoine, China). To verify the efficiency of immunodepletion, the representative samples of crude plasma, flow-through fraction and eluted fraction were separated on 12% SDS–PAGE gel
and stained with silver as described previously (Yan et al. 2000).

**Protein reduction, alkylation, tryptic digestion and iTRAQ labelling**

All reagents used for iTRAQ labelling were purchased from Applied Biosystems (USA), unless otherwise specified. The proteins (100 μg) in each sample were reduced by the addition of 2 μl 50 mM tri-(2-carboxyethyl) phosphine at 37 °C for 1 h. Thereafter, 1 μl 200 mM methanethiosulfonate was added and incubated at room temperature for 10 min to block the cysteine residues. The samples were diluted with 50 mM phosphate buffer (pH 8.5) in order to reduce the concentration of urea to 0.8 M. Then, trypsin was added [trypsin/protein ratio: 1:50 (w/w)]. After incubation at 37 °C for 12 h, an equal amount of trypsin was added and the digestion continued for another 12 h. Prior to iTRAQ labelling, the digested sample was desalted with a homemade C18 column (4.6 mm ID × 10 mm, 5 μm, 200 Å) to remove urea, salt and other reagents. Then, the purified sample was evaporated to dryness with a SpeedVac (RVT4104; ThermoFisher Scientific Inc.). After resuspension with 0.5 ml triethylammonium bicarbonate (pH 8.5), the samples were labelled with the iTRAQ reagents following the protocol provided by the vendor. Briefly, the required iTRAQ reagents (a vial of reagent 114 and 116, respectively) were thawed at room temperature and spun to drive the solution to the bottom and then 70 μl ethanol was added to solubilize the iTRAQ reagent. Afterwards, the mixture was added to the prepared peptide sample as follows: reagent 114 for the healthy control sample and reagent 116 for the depressed patient sample. The samples were incubated at room temperature for 1 h, and then the labelled samples were mixed at equal ratios.

**Purification of the peptide mixture**

In order to purify the mixed peptides, a homemade cation exchange cartridge (4.6 mm ID × 10 mm, 5 μm, 500 Å) was used. This was performed on a Shimadzu LC-20A HPLC system (Shimadzu, Japan). Buffer A was 10 mM ammonium acetate in 5% acetonitrile (pH 3.0) and buffer B was 1 M sodium chloride in A (pH 3.0). The mixture was eluted with a gradient of 2% B for 10 min, then 80% B for 12 min at a flow rate of 300 μl/min. After each separation, the column was equilibrated in 2% B for 8 min. The collected peptide fraction was desalted and then evaporated to dryness. After that, the peptides were resuspended in 200 μl 0.1% formic acid and stored at −20 °C for further analysis.

**Online 2D LC-chip-MS/MS**

Prior to online 2D chromatographic separation using an Agilent 1200 Series HPLC system, the labelled sample was centrifuged at 15 000 g for 5 min to remove the particulates. The peptides were loaded on to a homemade strong cation exchange (SCX) column (150 μm ID × 3 cm, 5 μm, 500 Å) with an autosampler. The flow rate for the first-dimensional separation was 1 μl/min. The mobile phase for SCX separation consisted of buffer A (10 mM ammonium acetate at pH 3.0) and buffer B (1 M NaCl in 10 mM ammonium acetate at pH 3.0). The eight salt steps were 80, 100, 150, 200, 300, 500 and 1000 mM NaCl (90 min per step), followed by second-dimensional reverse phase (RP) separation. The chip and columns for RP separation were HPLC-chip with a 75 μm × 150 mm analytical column packed with ZORBAX 300SB C18 (5 μm, 300 Å) and a 160 nl enrichment column. The solvents for RP separation were buffer C (0.1% formic acid in water) and buffer D (90% acetonitrile and 0.1% formic acid in water). The elution gradient was conducted as follows: 3% D at the 0 min mark, 8% D at the 3 min mark, 40% D at the 75 min mark, 85% D at the 90 min mark, then 3% D at the 90.1 min mark followed by rebalancing the column for another 8 min. The flow rates were 1 μl/min from the capillary pump to the enrichment column and 0.3 μl/min from the nano pump to the analytical column. The column effluent was analysed by an Agilent 6540 Accurate-Mass Q-TOF MS system. The MS parameters were as follows: positive ion mode; capillary voltage of 1750 V; fragmentor voltage of 175 V; skimmer voltage of 65 V; octopole RF of 750 V; drying gas flow of 31/min at 325 °C. The selected m/z ranges were 300 to 2000 Da in the MS mode (scan rate: 3 s⁻¹) and 50 to 3000 Da in the MS/MS mode (scan rate: 2 s⁻¹). The three most intense ions (with charge states of +2, +3 and ≥3) were subjected to MS/MS analysis. A medium isolation width of 4 amu was used for precursor isolation. Collision energy with a slope of 3.9 and an offset of 2.5 was set for fragmentation.

**Protein identification**

The MS data were exported to a MGF format and searched against the Human IPI database (version 3.71) using the Mascot search engine (version 2.3.2). The peptide and MS/MS tolerances were ±50 ppm and 0.5 Da, respectively. The modifications used were as follows: fixed, iTRAQ 4-plex (N-tern), iTRAQ 4-plex (K) and methylthio (C); variable, iTRAQ 4-plex...
(Y) and oxidation (M). The maximum missed cleavages were two. The significance threshold \( p \) value was set at 0.05. All identified proteins were based on at least two peptides. In addition, a decoy database search strategy was used to check the false discovery rate (FDR) and the FDR for these data was approximately 1.3%. All data were exported into Excel for further manual analysis and interpretation. Relative protein abundances were determined using the ratio of iTRAQ reporter ions in the MS/MS scans.

Immunoblotting analysis
Pooled samples used for iTRAQ analysis and all 42 individual plasma samples (all without depletion) were used for validation. The procedures of electrophoresis, transfer and immunodetection were performed according to our previous study (Yang et al. 2011). The following primary antibodies, including anti-a-1B-glycoprotein (A1BG) mouse monoclonal antibody (diluted 1:2000), anti-afamin (AFM) rabbit polyclonal antibody (diluted 1:2000), anti-apolipoprotein D (Apo-D) mouse monoclonal antibody (diluted 1:1000), anti-ceruloplasmin (Cp) mouse monoclonal antibody (diluted 1:1000), anti-histidine-rich glycoprotein (HRG) mouse polyclonal antibody (diluted 1:1000) and anti-vitamin D-binding protein (VDP) rabbit polyclonal antibody (diluted 1:2000) were used. All primary antibodies were obtained from Abcam (USA) except for anti-A1BG antibody, which was purchased from Santa Cruz Biotechnology (USA). HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG (diluted 1:7500; Biosynthesis Biotechnology, China) were used as secondary antibodies. After immunodetection, the polyvinylidene fluoride (PVDF) membrane was stained with Coomassie Blue and used as the loading control. Each sample was analysed in triplicate.

ELISA assays
Plasma \( \alpha-2 \)-macroglobulin (A2M) and apolipoprotein B-100 (Apo-B100) concentrations in individual samples from depressed patients \((n=21)\) and healthy controls \((n=21)\) were measured using commercial ELISA kits (Catalog No.: CSB-E08959h, CSB-E080100h; Cusabio, China), according to the manufacturer’s instructions. All samples were tested in triplicate and the mean concentrations were calculated. Plasma samples were diluted 1:2000 for the determination of A2M and 1:800 for the determination of Apo-B100, respectively. The coefficients of variation were <5% for A2M and <7% for Apo-B100.

Functional and network analysis using MetaCore
The differentially expressed proteins were mapped into biological networks using the functional analysis tool MetaCore (version 6.6). MetaCore is a proprietary manually curated database including human protein × protein, protein × DNA and protein × compound interactions, as well as metabolic and signalling pathways, which is suited for functional analysis of proteomics data (Ekins et al. 2006). The list of the IPI accession numbers and the corresponding expression ratios were uploaded into the application. The enrichment analysis of biological processes was based on the hypergeometric distribution algorithm and the network building of the dysregulated proteins was generated using the shortest path algorithm.

Results
In the present study, an iTRAQ-LC-MS/MS-based comparative proteomic approach was performed to discover disease-associated molecules in plasma sampled from first-onset, treatment-naive depressed patients. The flow chart of this study is presented in Fig. 1.

Subjects
A total of 42 subjects (21 per group) were enrolled in this study. All subjects are ethnic Han Chinese residing in the Chongqing area. The basic demographic and clinical information of recruited subjects are presented in Table 1. There were no significant differences in gender, age and body mass index between the two groups \((p>0.05)\).

Depletion of high-abundance proteins
To present the efficacy of immunodepletion of high abundance proteins, equal amounts of protein from the crude plasma sample, flow-through fraction and eluted fraction were subjected to one-dimensional electrophoresis and then the gels were stained with silver (Fig. 2). Compared to the crude plasma sample, more protein bands were observed after immunodepletion of high abundance proteins. This observation indicated that immunodepletion is an appropriate approach for the enrichment of low and medium abundance proteins.

Identification of differentially expressed proteins by LC-MS/MS
iTRAQ labelling coupled with 2D LC-MS/MS was conducted to profile the differentially expressed
Subject recruitment

Plasma collection, stores at –80 °C until analysis

High abundance proteins depletion with immunoaffinity chromatography

Salt removal, concentration, reduction, alkylation & trypsinization

iTRAQ labelling

Combine peptides, peptides clean-up

2D-LC-chip-MS/MS

MS data analysis, validation of the dysregulated proteins by immunoblotting and ELISA

Bioinformation analysis with MetaCore database

**Fig. 1.** An overview of the work flow employed in this study. MDD, Major depressive disorder; HC, healthy control; iTRAQ, isobaric tags for relative and absolute quantitation; LC-chip-MS/MS, liquid chromatography-chip–tandem mass spectrometry; ELISA, enzyme-linked immunosorbent assay.

**Table 1.** The basic demographic and clinical characteristics of subjects

<table>
<thead>
<tr>
<th></th>
<th>Depressed patients</th>
<th>Healthy controls</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>21</td>
<td>21</td>
<td>–</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>12/9</td>
<td>12/9</td>
<td>–</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>29.52 ± 9.32</td>
<td>30.76 ± 7.18</td>
<td>0.632a</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.97 ± 3.31</td>
<td>21.38 ± 2.15</td>
<td>0.374a</td>
</tr>
<tr>
<td>HAMD</td>
<td>22.67 ± 3.67</td>
<td>3.67 ± 1.71</td>
<td>0.000a</td>
</tr>
<tr>
<td>BDI</td>
<td>20.14 ± 7.30</td>
<td>3.71 ± 1.71</td>
<td>0.000a</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>4.33 ± 2.44</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

BMI, Body mass index; HAMD, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory.

* Independent sample t tests.

Data are expressed as means ± S.D.
plasma proteins between depressed patients and healthy controls. Quadruplicate LC-MS/MS runs were carried out and 94 proteins were identified according to the aforementioned criteria. As the samples from each group were pooled for analysis, the main variable was technical variation. It has been reported that at least a 30% variation is required to be considered a technical variation in large-scale protein identification and quantification using an iTRAQ approach (Gan et al. 2007). Thus, a criterion of a 1.3-fold change was set as the cut-off, which has been employed in other studies (Chong et al. 2010; Choong et al. 2010; Hergenroeder et al. 2008). According to this criterion, nine proteins were found differentially expressed between the two groups. Among them, five proteins displayed increased expression and the other four proteins showed decreased expression in MDD subjects. The names and postulated functions of these nine proteins are listed in Table 2.

### Table 2. Up- or down-regulated plasma proteins in major depression using iTRAQ 2D LC-MS/MS

<table>
<thead>
<tr>
<th>Protein no.</th>
<th>IPI no.</th>
<th>Protein name</th>
<th>Fold change</th>
<th>State change</th>
<th>Major function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IPI00006662</td>
<td>Apolipoprotein D</td>
<td>1.69</td>
<td>↑ MDD</td>
<td>Lipid metabolism; cholesterol, bilirubin, steroids and arachidonic acid binding.</td>
</tr>
<tr>
<td>2</td>
<td>IPI00019943</td>
<td>Afamin</td>
<td>1.55</td>
<td>↑ MDD</td>
<td>Vitamin E binding, oxidative stress</td>
</tr>
<tr>
<td>3</td>
<td>IPI00022229</td>
<td>Apolipoprotein B-100</td>
<td>1.72</td>
<td>↑ MDD</td>
<td>Lipid metabolism, atherosclerosis</td>
</tr>
<tr>
<td>4</td>
<td>IPI00022895</td>
<td>α-1B-glycoprotein</td>
<td>1.46</td>
<td>↑ MDD</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>IPI00555812</td>
<td>Isoform 1 of vitamin D-binding protein</td>
<td>1.32</td>
<td>↑ MDD</td>
<td>Vitamin D metabolite and fatty acid transport, actin scavenging, macrophage activation</td>
</tr>
<tr>
<td>6</td>
<td>IPI00017601</td>
<td>Ceruloplasmin</td>
<td>0.35</td>
<td>↓ MDD</td>
<td>Copper transport, iron homeostasis, lipid and catecholamine oxidation, inflammation</td>
</tr>
<tr>
<td>7</td>
<td>IPI00022371</td>
<td>Histidine-rich glycoprotein</td>
<td>0.66</td>
<td>↓ MDD</td>
<td>Interactions with Zn²⁺, IgG, complement factors, heparin and FcγR</td>
</tr>
<tr>
<td>8</td>
<td>IPI00298003</td>
<td>Semaphorin-3F</td>
<td>0.62</td>
<td>↓ MDD</td>
<td>Axon guidance, limbic system development, synaptic transmission modulation</td>
</tr>
<tr>
<td>9</td>
<td>IPI00478003</td>
<td>α-2-macroglobulin</td>
<td>0.68</td>
<td>↓ MDD</td>
<td>Proteinase inhibition, cytokines and growth factors binding</td>
</tr>
</tbody>
</table>

iTRAQ, Isobaric tags for relative and absolute quantitation; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDD, major depressive disorder.

Validation of protein expression by immunoblotting and ELISA

Immunoblotting or ELISA was performed to verify the results of the previously described proteomic experiment. Crude plasma samples without depletion were employed in these assays. Six proteins (A1BG, AFM, Apo-D, Cp, HRG and VDP) were validated by immunoblotting. This was initially performed in pooled plasma samples used for iTRAQ analysis. All six proteins could be detected by immunoblotting, which strengthened the protein identification of MS/MS (Fig. 3a). Of the six proteins tested, four (Apo-D, Cp, HRG and VDP) displayed statistical difference (p<0.05; Fig. 3a). Therefore, these four proteins were
selected for further analysis in all 42 individual samples. The results are presented in Fig. 3b. The trends in the expression of these four proteins were consistent with iTRAQ results, but they did not reach statistical significance (p > 0.05; Fig. 3b).

An attempt was also made to validate the MS results of A2M, Apo-B100 and semaphorin-3F (S3F) using the immunoblotting method. However, this attempt was not successful due to either the poor performance of the antibodies or poor transfer to membrane. Thus, these three proteins were validated with ELISA assays. The ELISA results of A2M and Apo-B100 were consistent with the findings of the previous proteomic experiment with significant differences between MDD patients and healthy controls (p < 0.05; Fig. 4). Unfortunately, there is no commercial ELISA kit available for S3F.

Network and functional analysis by MetaCore
To further explore the probable roles of the differentially expressed proteins in MDD samples, the MetaCore database was used to analyse and build biological networks associated with these proteins. The top 10 ranking canonical pathway maps and GeneGo process networks associated with the differentially

Fig. 3. Western blot results of α-1B-glycoprotein (A1BG), afamin (AFM), apolipoprotein D (Apo-D), ceruloplasmin (Cp), histidine-rich glycoprotein (HRG) and vitamin D-binding protein (VDP). The immunoblotting was first performed in pooled plasma samples that had been used for isobaric tags for relative and absolute quantitation analysis (a). Then, four candidate proteins (AFM, Apo-D, Cp and VDP) were further analysed in individual samples from depressed patients (n = 21) and healthy controls (Con, n = 21; b). All samples were analysed in triplicate. The relative intensity of each protein was normalized by the total protein input of each lane. Quantitative analysis of protein bands was conducted using Quantity One analysis software (Bio-Rad, version 4.6.7). The results are illustrated by histogram, with each bar representing S.E.M. A representative subset of blots is also presented for each protein. Data were analysed with Mann–Whitney’s test. In order to verify the normalization of protein amounts, a loading control is also presented. MDD, major depressive disorder, HC, healthy control.
expressed proteins are listed in Table 3. Three pathway maps, namely, niacin-HDL metabolism, bile acids regulation of glucose and lipid metabolism via Farnesoid X receptor (FXR) and regulation of lipid metabolism by niacin and isoprenaline, showed the highest statistical significance ($p < 0.001$). In addition, the inflammation-associated IL-6 signalling and immune response-associated phagocytosis were found to be the most statistically significant GeneGo process networks ($p < 0.001$). Based on the shortest path algorithm, the potential interactions between the identified proteins and other molecules were analysed through the MetaCore database. Three of the six proteins could be mapped onto a network through links to certain

<table>
<thead>
<tr>
<th>GeneGo pathway maps</th>
<th>p value</th>
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<tbody>
<tr>
<td>1. Niacin-HDL metabolism</td>
<td>$1.332 \times 10^{-8}$</td>
</tr>
<tr>
<td>2. Regulation of metabolism: bile acids regulation of</td>
<td>$2.857 \times 10^{-4}$</td>
</tr>
<tr>
<td>glucose and lipid metabolism via FXR</td>
<td></td>
</tr>
<tr>
<td>3. Regulation of lipid metabolism by niacin and</td>
<td>$4.235 \times 10^{-4}$</td>
</tr>
<tr>
<td>isoprenaline</td>
<td></td>
</tr>
<tr>
<td>4. Atherosclerosis: role of ZNF202 in regulation of</td>
<td>$1.506 \times 10^{-2}$</td>
</tr>
<tr>
<td>expression of genes involved in atherosclerosis</td>
<td></td>
</tr>
<tr>
<td>5. Cholesterol and sphingolipids transport/recycling to</td>
<td>$1.648 \times 10^{-2}$</td>
</tr>
<tr>
<td>plasma membrane in lung (normal and CF)</td>
<td></td>
</tr>
<tr>
<td>6. Blood coagulation</td>
<td>$2.782 \times 10^{-2}$</td>
</tr>
<tr>
<td>7. Immune response: IL-1 signalling pathway</td>
<td>$3.133 \times 10^{-2}$</td>
</tr>
<tr>
<td>8. Mechanisms of CFTR activation by S-nitrosoglutathione</td>
<td>$3.274 \times 10^{-2}$</td>
</tr>
<tr>
<td>(normal and CF)</td>
<td></td>
</tr>
<tr>
<td>9. Regulation of lipid metabolism: G-$\alpha(q)$</td>
<td>$4.183 \times 10^{-2}$</td>
</tr>
<tr>
<td>regulation of lipid metabolism</td>
<td></td>
</tr>
<tr>
<td>10. Transport: intracellular cholesterol transport in</td>
<td>$6.321 \times 10^{-2}$</td>
</tr>
<tr>
<td>norm</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>GeneGo Process Networks</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Inflammation: IL-6 signalling</td>
<td>$1.515 \times 10^{-4}$</td>
</tr>
<tr>
<td>2. Immune response: phagocytosis</td>
<td>$9.649 \times 10^{-4}$</td>
</tr>
<tr>
<td>3. Regulation of metabolism: bile acid regulation of</td>
<td>$1.991 \times 10^{-3}$</td>
</tr>
<tr>
<td>lipid metabolism and negative FXR-dependent regulation</td>
<td></td>
</tr>
<tr>
<td>of bile acids concentration</td>
<td></td>
</tr>
<tr>
<td>4. Signal transduction: leptin signalling</td>
<td>$4.387 \times 10^{-3}$</td>
</tr>
<tr>
<td>interactions</td>
<td></td>
</tr>
<tr>
<td>6. Inflammation: kallikrein–kinin system</td>
<td>$1.293 \times 10^{-2}$</td>
</tr>
<tr>
<td>7. Blood coagulation</td>
<td>$8.882 \times 10^{-2}$</td>
</tr>
<tr>
<td>8. Transport: iron transport</td>
<td>$9.696 \times 10^{-2}$</td>
</tr>
<tr>
<td>9. Cell adhesion: amyloid proteins</td>
<td>$1.766 \times 10^{-1}$</td>
</tr>
<tr>
<td>10. Development: blood vessel morphogenesis</td>
<td>$2.037 \times 10^{-1}$</td>
</tr>
</tbody>
</table>

FXR, Farnesoid X receptor; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulatory.

Fig. 4. Enzyme-linked immunoadsorbent assay results of $a$-2-macroglobulin (A2M) and apolipoprotein B-100 (Apo-B100). All 42 samples were analysed in triplicate. Each bar represents s.e.m. Data were analysed with Mann–Whitney’s test. MDD, Major depressive disorder, HC, healthy control.
transcription factors, indicating the probable synergistic effects of pathways implicated in MDD (Supplementary Fig. S1).

**Discussion**

In this study, plasma samples from first-episode, treatment-naive depressed patients and healthy controls were differentially labelled with iTRAQ reagents and subsequently analysed by MS/MS. To our knowledge, this is the first proteomic-based study investigating putative peripheral biomarkers for MDD.

Sample homogeneity is critical to effective proteomic research. In order to improve sample homogeneity in this study, only first-episode, treatment-naive depressed patients with a short disease duration were enrolled. In the case of patients who were undergoing or had undergone treatment, or had a long disease duration, observed alterations cannot be solely attributable to the disease state, but may be related to treatment or chronic impairment. Using samples obtained from first-episode, treatment-naive subjects controls for the confounding effects of treatment exposure and chronic impairment.

Here, we have used a quantitative proteomic approach based on iTRAQ labelling coupled with 2D LC-MS/MS. Due to this method’s poor suitability in large sample analysis and resource constraints, samples from the same group were pooled. Sample pooling prior to proteomic analysis is an acceptable experimental technique and its advantages and disadvantages have been thoroughly discussed elsewhere (Diz et al. 2009; Karp et al. 2005). Depletion of high-abundance proteins was conducted using immunoaffinity chromatography in order to improve the detection of low-abundance plasma proteins. This method is a widely used initial step in plasma proteomic studies and considered to be the most promising depletion approach based on its strong reproducibility and specificity (Bjrhall et al. 2005; Brand et al. 2006; Liu et al. 2006; Whiteaker et al. 2007). The efficiency of the removal of the abundant proteins can be observed in Fig. 2. However, one limitation is that immunodepletion may cause a concomitant removal of non-targeted proteins, which interact with the targeted depleted proteins. Despite this limitation, this immunodepletion method remains a valuable tool for preliminary screening as it allows the detection of a higher number of measurable proteins.

With the iTRAQ-based differential profiling method, novel evidence of the pathophysiology of MDD at the protein level is revealed. The putative biological roles of these dysregulated proteins were mined with the MetaCore database. As observed in Table 3, the biological processes with the highest significance were centred around lipid metabolism and immunoregulation. These pathways have been previously implicated in the pathophysiology of MDD (Herbert & Cohen, 1993; Huang, 2005; Huang et al. 2003; Maes, 1995).

In the present study, some of the findings from the proteomic investigation were not replicated in individual samples when analysed with the aforementioned immunoblotting methods. A similar discrepancy has also been observed in other proteomic studies (Abdi et al. 2006; Airoldi et al. 2009; Cheng et al. 2011; Un-Beom et al. 2010). The lack of statistical significance in individual samples may be explained by either the differences of dynamic range between iTRAQ and immunoblotting (Abdi et al. 2006), or the intrinsic variability associated with the steps of proteomic and immunoblotting analysis. Additionally, as MDD is a complex mental disorder with poorly understood multifactorial aetiology, the individual variability cannot be excluded, although only first-onset, treatment-naive depressed patients were recruited in order to improve sample homogeneity. Dynamic measurement of potential biomarker levels for any given patient over time intervals may provide much more information than the evaluation of average levels (Wiederin et al. 2009). However, this needs to be investigated in a longitudinal study.

Our following discussion will be focused on the four proteins, namely Apo-B100, A2M, VDP and Cp, which displayed statistical significance or relatively low significance values in individual plasma samples. Apo-B100, a member of the apolipoprotein superfamily, was found to be elevated in the plasma of depressed patients. Apo-B100 is the major isoform of Apo-B, which is present in very low density lipoprotein, intermediate density lipoproteins and low density lipoproteins. Apo-B performs important roles in the regulation of lipid profiles, the assembly of atherogenic lipoproteins and the development of atherosclerosis (Olofsson & Boren, 2005). An elevated plasma Apo-B level has been proposed to be a predictor of cardio-cerebrovascular events (Benn, 2009; Benn et al. 2006; Sabino et al. 2008; Walldius & Jungner, 2005; Walldius et al. 2006). The finding of elevated plasma Apo-B levels in depressed patients is in accordance with previous studies (Sadeghi et al. 2011; Sarandol et al. 2006). However, it has also been reported that there is no significant difference in Apo-B levels between healthy controls and adults with elevated depressive symptoms (Lehto et al. 2010). In this investigation, only male subjects were enrolled.
and a full diagnostic psychiatric interview was not performed. This methodological disparity may contribute to the discrepancy in findings between the two studies. Convergent evidence suggests that MDD contributes to the development of cardio-cerebrovascular disease (Joynt et al. 2003; Lett et al. 2004; Pan et al. 2011a, b). However, the mechanism underlying this phenomenon is not clear. With regard to the aforementioned roles of Apo-B, it can be surmised that the abnormality in peripheral apolipoprotein levels may underlie the connection between MDD and cardio-cerebrovascular disease.

In this study, proteomic analysis also revealed some altered proteins that were associated with the modulation of the immune system. These proteins were A2M and isoform-1 of VDP. A2M is a broad spectrum proteinase inhibitor and can bind to several cytokines and growth factors (Feige et al. 1996; LaMarre et al. 1991). These properties may highlight the importance of A2M in regulating the immune response (Armstrong, 2001; James, 1980). Previous studies have investigated the possible relationship between MDD and plasma/serum A2M levels. However, the results are inconsistent. In a recent study consisting of 245 depressed patients, 229 schizophrenic patients and 254 healthy controls, a decreased plasma level of A2M was found in depressed patients as compared to healthy controls, a decreased plasma level of A2M was found in depressed patients (Joyce et al. 1992). Moreover, Maes et al. (1992b) observed a significant negative correlation between serum A2M levels and the severity of depression. In contrast, it has also been reported that elevated levels of serum A2M have been found in subjects with MDD (Seidel et al. 1995) and post-operative depression (Fujita et al. 2003). In another study, no significant difference in A2M levels between depressed patients and healthy controls has been reported (Rothermundt et al. 2001). The inconsistency of these findings may be partially attributed to the different diagnostic criteria applied in these studies and the possible confounding effects of antidepressant drug therapy. Despite these discrepancies, the decreased level of A2M found in the current study suggests a perturbation of the immune response in the pathophysiology of MDD.

VDP, also known as Gc-globulin, is a member of the albumin, α-fetoprotein and α-albumin/afamin gene family. In the present study, the plasma level of VDP was increased in subjects with MDD. Elevated levels of VDP in plasma/serum/CSF have previously been reported in several neuropsychiatric disorders, such as schizophrenia, multiple sclerosis and Guillain–Barre syndrome (d’Aguanno et al. 2010; Rithidech et al. 2009; Wan et al. 2007). Apart from its role in transporting vitamin D metabolites, VDP also exhibits several other functions including actin scavenging, fatty acid transport and modulation of immune and inflammatory responses (Gomme & Bertolini, 2004; Speeckaert et al. 2006, 2010; White & Cooke, 2000). An elevated VDP level may be associated with abnormalities in immunoregulation and the lipid profile found in MDD.

In addition to the aforementioned altered proteins in MDD, dysregulated expression of Cp was also discovered. Cp is the principal transporter of circulating copper, which was first purified from blood by Holmberg & Laurell (1948). Aside from its function in the transport of copper, Cp has also been demonstrated to be involved in other important functions, such as oxidation of the catecholamine neurotransmitter and lipids, iron (II) oxidation, iron homeostasis, and anti- and pro-oxidant actions (Healy & Tipton, 2007; Vassiliev et al. 2005; Virit et al. 2008). Previous investigations have found elevated plasma Cp concentrations in depressed patients (Joyce et al. 1992; Maes et al. 1992a). However, since the enrolled patients were undergoing antidepressant treatment or had a mere 2-wk wash-out period in these studies, the possible contribution of medication exposure to the Cp expression level cannot be excluded. Furthermore, only male subjects were recruited into the Joyce study, which may also contribute to the noted discrepancy. Plasma levels of Cp can be changed under different conditions (Chauhan et al. 2004; Jin et al. 2011; Vassiliev et al. 2005), implying that it is a non-specific marker of the inflammatory process or oxidative stress. This may explain the inconsistent Cp levels in the plasma of depressed patients. The likely role of Cp in MDD may be related to its role in the inflammatory process, oxidative stress and catecholamine metabolism.

Conclusions

In this study, several altered proteins were found in the plasma sampled from major depressive patients using an iTRAQ-based proteomic approach, demonstrating the utility of this method for blood-based biomarker discovery in MDD. Most of the differentially expressed proteins are associated with lipid metabolism and immunoregulation, suggesting that the early perturbation of lipid metabolism and immunoregulation may be involved in the pathophysiology of MDD. Further investigation on larger cohorts will be necessary to verify the potential role of these changed proteins as biomarkers applicable in the diagnosis, prognosis and disease monitoring of MDD.
Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145712000302.

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Statement of Interest

None.

References


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