



Proteomic changes in serum of first onset, antidepressant drug-naïve major depression patients

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Abstract

Major depressive disorder (MDD) is a complex and multi-factorial disorder. Although genetic factors and other molecular aspects of MDD have been widely studied, the underlying pathological mechanisms are still mostly unknown. We sought to investigate the pathophysiology of MDD by identifying and characterising serum molecular differences and their correlation to symptom severity in first onset, antidepressant drug-naïve MDD patients. We performed an exploratory molecular profiling study on serum samples of MDD patients and controls using multiplex immunoassay and label-free liquid chromatography mass spectrometry in data independent mode (LC-MS^E). We included two independent cohorts of first onset, antidepressant drug-naïve MDD patients ($n=23$ and 15) and matched controls ($n=42$ and 21) in our study in order to validate the results. The main outcome included the following list of circulatory molecules changing and/or correlating to symptom severity: angiotensin-converting enzyme, acute phase proteins (e.g. ferritin and serotransferrin), brain-derived neurotrophic factor, complement component C4-B, cortisol, cytokines (e.g. macrophage migration inhibitory factor and interleukin-16), extracellular newly identified receptor for advanced glycosylation end products-binding protein, growth hormone and superoxide dismutase-1. This study provides evidence of an increased pro-inflammatory and oxidative stress response, followed by a hyperactivation of the HPA-axis in the acute stages of first onset MDD, as well as a dysregulation in growth factor pathways. These findings help to elucidate MDD related pathways in more detail and further studies may lead to identification of novel drug targets, including components of the inflammatory and oxidative stress response.

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Introduction

Studies of major depressive disorder (MDD) are complicated due to the complexity and multi-factorial nature of this disorder. The potential role of genetic factors and other molecular aspects of MDD have been investigated, such as hypothalamic- pituitary- adrenal (HPA) axis dysfunction [reviewed in (Fava and Kendler, 2000)], association with immune response [reviewed in (Raedler, 2011)] and metabolic disorders [reviewed in

(Gans, 2006)]. However, the underlying pathological mechanisms are still mostly unknown.

Traditionally, psychiatric diseases have been regarded as brain disorders. However, it is not possible to obtain brain samples from living patients. Furthermore, post-mortem brain tissue analyses would not allow for the study of dynamic changes such as those which can occur before and after treatment with ADs. However, some insight into brain function in living patients has been obtained using neuroimaging techniques, neuro-psychological assessment tools and pharmacological challenge tests. In addition, recent studies have begun to study brain function through the analysis of peripheral body fluids, such as blood plasma, serum or cells. The study of serum or plasma molecules in cases of psychiatric disease is rational since many such molecules are

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Table 1. Demographic details of first onset MDD patients and matched control subjects of (a) cohort 1 and (b) cohort 2

| | Multiplex immunoassay | | | LC-MS ^E | | |
|--------------------------|-----------------------|-------------|---------------------|--------------------|-------------|---------------------|
| | Control | Patient | <i>p</i> -value | Control | Patient | <i>p</i> -value |
| <i>(a) Cohort 1</i> | | | | | | |
| Number | 42 | 23 | | 21 | 15 | |
| Gender (m/f) | 18/24 | 11/12 | 0.7962 ^F | 12/9 | 10/5 | 0.7317 ^F |
| Age (yr) | 40.57±11.17 | 40.83±11.64 | 0.9015 ^W | 41.14±12.67 | 39.53±13.09 | 0.4308 ^W |
| BMI (kg/m ²) | 25.17±3.54 | 25.20±6.07 | 0.7316 ^W | 24.87±3.03 | 25.80±5.42 | 0.9744 ^W |
| Smoking (yes/no) | 8/34 | 10/13 | 0.0458 ^F | 5/16 | 9/6 | 0.0410 ^F |
| HAMD | NA | 21.96±11.64 | NA | NA | 21.75±6.23 | NA |
| <i>(b) Cohort 2</i> | | | | | | |
| Number | 21 | 15 | | 21 | 15 | |
| Gender (m/f) | 7/14 | 3/12 | 0.4682 ^F | 7/14 | 3/12 | 0.4682 ^F |
| Age (yr) | 36.00±10.18 | 36.93±9.22 | 0.4791 ^W | 36.00±10.18 | 36.93±9.22 | 0.4791 ^W |
| BMI (kg/m ²) | 23.15±3.47 | 23.12±4.00 | 0.5106 ^W | 23.15±3.47 | 23.12±4.00 | 0.5106 ^W |
| Smoking (yes/no) | 6/15 | 8/7 | 0.1753 ^F | 6/15 | 8/7 | 0.1753 ^F |
| HAMD | NA | 17.33±6.84 | NA | NA | 17.33±6.84 | NA |
| Cohorts combined | | | | | | |
| Number | 63 | 38 | | 42 | 30 | |
| Gender (m/f) | 25/38 | 14/24 | 0.8349 ^F | 19/23 | 13/17 | 1.0000 ^F |
| Age (yr) | 39.05±10.98 | 39.29±10.79 | 0.8828 ^W | 38.57±11.65 | 38.23±11.20 | 0.8908 ^W |
| BMI (kg/m ²) | 24.50±3.62 | 24.38±5.08 | 0.4833 ^W | 24.01±3.33 | 24.46±4.88 | 0.9272 ^W |
| Smoking (yes/no) | 14/49 | 18/20 | 0.0144 ^F | 11/31 | 17/13 | 0.0139 ^F |
| HAMD | NA | 20.05±6.78 | NA | NA | 19.50±6.89 | NA |

The table shows number of samples, gender, age, body mass index (BMI), smoking and Hamilton Depression Scale (HAMD) scores of patients and controls used in the multiplex immunoassay and LC-MS^E study. Age, BMI and HAMD values are shown in average ±s.d. Note that the LC-MS^E profiling was limited to a subset of samples from cohort 1 due to reduced availability, whereas all samples from cohort 2 were analysed.

F=Fisher's exact test. W=Wilcoxon rank sum test.

known to affect or reflect brain function. These include circulating neuroendocrine hormones such as insulin, leptin, adrenocorticotrophic hormone (ACTH) and cortisol, along with numerous inflammatory factors including interleukin (IL)-1, IL-6, IL-18, interferon-gamma and tumour necrosis factor (TNF) alpha. Furthermore, the association between inflammation and MDD has been supported by the fact that cytokine treatment of hepatitis C and certain cancer types frequently induce depressive symptoms as side effects (Capuron et al., 2000; Lotrich, 2009). Therefore, there is a precedent for looking at inflammatory and other circulating factors in the early phases of MDD as performed in this study.

This study has two main aims: (1) to identify and characterise serum molecular differences that are associated with the disease onset in first onset, antidepressant drug-naïve MDD patients compared to controls and (2) to identify serum molecules associated with symptom severity of MDD. If successful, the latter will help to strengthen the case for the role of these proteins as putative markers for the disorder. For this purpose, serum samples of MDD patients and control subjects were subjected to molecular profiling analyses using multiplex immunoassay and liquid chromatography mass

spectrometry in data independent mode (LC-MS^E). This multiplex profiling approach was used to eliminate variability across individual measurements, thereby allowing reliable identification of molecules, which are co-regulated within and across molecular pathways. The platform targets the immune molecules and HPA axis hormones mentioned above, along with other molecular classes including growth factors, transport molecules and components of the clotting cascade, which may also be involved. An additional strength of this study is that the analysis of first onset, antidepressant drug-naïve MDD patients, thus eliminating the potential confounding influence of chronic AD medication, has not been performed previously.

Method

Study participants and sample collection

The study included two consecutive cohorts collected approximately one year apart at the Department of Psychiatry, University of Magdeburg, Germany (Table 1A and B). Cohort 1 consisted of 42 healthy controls (40.57±11.17 years) and 23 patients (40.83±11.64 years),

and cohort 2 was comprised of 21 healthy controls (36.00±10.18 years) and 15 patients (36.93±9.22 years). Controls were identified using M.I.N.I. (Mini International Neuropsychiatric Interview - German Version 5.0.0). All MDD patients from cohort 1 and 2 were diagnosed with first onset MDD and were antidepressant drug-naïve at the time of serum sample collection and none were taking other medications such as glucocorticoids. All patients met the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR) criteria for MDD and clinical tests including administration of the Hamilton Depression Scale (HAMD) assessment were performed by psychiatrists under good clinical practice-compliance to minimise variability. MDD patients with other psychiatric comorbidities were excluded and controls were screened for personal or family history of neuropsychiatric disorders using M.I.N.I. (German Version 5.0.0) and excluded if these were present. Patients and controls were free of acute and chronic infections, allergies, autoimmune diseases, cancer or systemic diseases as determined by self-report, doctors' report or by physical examination. In addition, patients and controls were excluded with a history of immune diseases, immunomodulatory treatment, cancer, chronic terminal disease, cardiovascular disorders, diabetes mellitus, substance abuse, and severe trauma or with clinical or paraclinical findings indicative of these disorders. All subjects gave informed written consent. Clinical investigations were conducted according to the Declaration of Helsinki, and the University of Magdeburg ethical committee approved the study.

Potential differences in demographic characteristics between patients and matched control subjects were assessed using either the Mann Whitney *U* test [age and body mass index (BMI)] or Fisher's exact test (gender and smoking).

Blood samples were collected from all participants after an overnight fast at around 08:00 a.m. on each occasion in the Department of Psychiatry, University of Magdeburg, Germany under the supervision of Dr Johann Steiner by venous puncture into S-Monovette 7.5 ml serum tubes (Sarstedt, Germany). Serum was prepared according to standard protocols by leaving the samples at room temperature for 2 h to allow coagulation followed by centrifugation at 1000 g for 5 min at 21 °C to remove the clotted material. The resulting supernatants were stored at -80 °C in Low Binding Eppendorf tubes (Germany) prior to analysis. A quality control (QC) sample was prepared from a pool of spare serum samples and split into aliquots for use in the LC-MS^E study to evaluate technical reproducibility.

Multiplexed immunoassay profiling

For cohort 1 and 2, a total of 190 molecules were measured in sera (200–250 µl) using the HumanMAP[®] v 1.0 platform in a CLIA-certified laboratory (Myriad-RBM,

USA; Supplementary Table 1) (Bertenshaw et al., 2008). This panel of assays targets inflammatory, metabolic, hormonal and neurotrophic pathways and has been applied successfully in previous studies of psychiatric disorders such as schizophrenia and MDD (Domenici et al., 2010; Schwarz et al., 2010).

LC-MS^E analysis

Sera were processed in random order to avoid potential sequential biases and were depleted of the 14 most abundant proteins using MARS14 (Agilent, USA) on a ÄKTA[™] purifier UPC 10 chromatography system (GE Healthcare, UK) using affinity chromatography as reported previously (Jaros et al., 2013). This was carried out to increase detection of a higher number of serum proteins, which are normally masked by the higher abundance proteins. QC samples were comprised of one randomly selected sample, which was divided into multiple aliquots. These were added to each sample preparation batch and prepared and analysed in parallel with the normal samples. Buffer exchange into 50 mM ammonium bicarbonate was carried out using spin columns (Millipore, USA) with a 5 kDa molecular cut-off. Disulfide bonds on proteins were reduced using 100 mM dithiothreitol (Sigma, UK) for 30 min at 60 °C and free sulfhydryl moieties were alkylated with 200 mM iodoacetamide (Sigma) in the dark at room temperature for 30 min. The proteins were enzymatically digested using porcine trypsin (Promega, USA), at a ratio of 1:50 (w/w trypsin/protein) for 17 h at 37 °C. Reactions were stopped by addition of 8.8 M HCl (ratio=1:60) to each sample and samples were stored at -80 °C.

LC-MS^E profiling was carried out in expression mode using a Waters quadrupole time-of-flight (QToF) Premier mass spectrometer (Waters, UK), as described previously (Levin et al., 2010). Resulting data were processed using the ProteinLynx Global Server (PLGS) v.2.4 (Waters) and Rosetta Inpharmatics Biosoftware Elucidator v.3.3 (USA) (Krishnamurthy et al., 2013). The human Swiss-Prot database (v.57, 20332 entries) search analysis was performed using PLGS with the ion accounting algorithm described previously (Li et al., 2009). The criteria for protein identification were set to ≥3 fragment ions/peptide, and ≥7 fragment ions and 2 peptides/protein. The maximum false identification rate was 4% using a randomised version of the database. Only peptides detected in both replicates and in 60% of samples were included in further analysis. Search results were imported into Elucidator for annotation of aligned features, resulting in a matrix that included intensities for each sample and peptide.

Statistics

Comparisons between groups were based on the non-parametric Mann Whitney *U* test for both cohorts and analysis platforms. To identify molecular signature

Table 2. Multiplex immunoassay profiling results of serum from first onset MDD patients vs. healthy controls (patient/control) (cohort 1: 23 MDD vs. 42 HC; cohort 2: 15 MDD vs. 21 HC; and both cohorts combined)

| Molecule name | Acc.No. | FC | Cohort 1 <i>p</i> -value | Cohort 2 <i>p</i> -value | Cohort 1 & 2 <i>p</i> -value |
|-----------------------------------|---------|-------------------|-----------------------------|-----------------------------|---------------------------------|
| Ferritin | P02794 | 3.70 4.59 3.36 | 0.0017 | 0.0047 | <0.0001 |
| MIF | P14174 | 1.65 2.32 2.74 | <0.0001 | 0.0064 | <0.0001 |
| EN-RAGE | P80511 | 2.45 2.53 2.24 | <0.0001 | 0.0016 | <0.0001 |
| Superoxide dismutase 1 | P00441 | 1.11 1.50 1.58 | *0.0611 | 0.0081 | 0.0011 |
| Interleukin-1 receptor antagonist | P18510 | 1.44 1.46 1.56 | 0.0013 | 0.0101 | <0.0001 |
| Interleukin-16 | Q14005 | 1.40 1.20 1.27 | 0.0001 | 0.0303 | <0.0001 |
| Tenascin-C | P24821 | 1.35 1.34 1.27 | 0.0086 | 0.0034 | 0.0002 |
| Brain-derived neurotrophic factor | Q9BYY7 | 1.15 1.16 1.16 | *0.0902 | *0.0983 | 0.0219 |
| Angiotensin-converting enzyme | P12821 | -1.13 -1.20 -1.16 | 0.0297 | 0.0237 | 0.0016 |
| Serotransferrin | P13726 | -1.14 -1.17 -1.26 | *0.0522 | 0.0315 | 0.0037 |
| Growth hormone | P01241 | -3.12 -4.90 -3.78 | *0.0754 | *0.0673 | 0.0100 |

The table includes Uniprot accession numbers (Acc.No.), fold changes (FC; cohort 1|cohort 2|cohort 1 & 2), *p*-values (Mann Whitney *U* test), and ANCOVA [bold text=analytes significant, *=analyte trending to significance (>0.05 and <0.1)] after correction for smoking covariate. Only molecules, which showed biological validation by being changed in both cohorts were included in this table. EN-RAGE, Extracellular newly identified RAGE-binding protein; MIF, Macrophage migration inhibitory factor.

differences between MDD and controls, analysis of co-variance (ANCOVA) was performed using smoking as co-variate. For ANCOVA, all variables were log₁₀-transformed to approximate normal distributions. Patients and control subjects from both cohorts, used for multiplex immunoassay and LC-MSE studies, did not differ significantly in age, gender or BMI. However, patients and controls differed in the ratio of smokers to non-smokers (data not shown) in both cohorts. Therefore, smoking was used as a co-variate in the analyses of molecular differences using both measuring platforms. No significant associations between HAMD scores and patient demographics were found (data not shown) in any of the cohorts. To identify markers of symptom severity as well as significant associations between demographics and HAMD scores, we applied non-parametric Spearman correlation (for age and BMI) and non-parametric Mann Whitney *U* (for gender and smoking) tests. *P*-values <0.05 were considered significant. Molecules showing a trend to significance (*p*<0.10) were included as these constituted potential consistency between the cohorts and platforms for validation purposes. Cohorts were not only analysed singly, but values of both cohorts (cohort 1 & 2) were also combined, normalised for batch effect and analysed, to increase the statistical power of the analyses by increasing sample numbers. All analyses were performed using the statistical software package R 2.12 (RDC Team, 2009) and Prism version 5 (GraphPad Software, USA).

Fold change (FC) values were calculated for each molecule as the average values of patients divided by those of controls (MDD/C). Resulting values <1.0 were multiplied by -1/*X* (where *Z* is the value) to give a negative FC.

For multiplex immunoassay analyses, values under the detection limit were replaced as described previously

(Stelzhammer et al., 2011). Furthermore, molecules, which had more than 40% of values outside the detection limit within a group were not included in the final list of changed molecules. For all LC-MS^E studies, coefficients of variation (CVs) were calculated using the QC measured intensities. The overall study CV was calculated as the mean of total protein CVs to assess technical variation. Significant analytes identified in the analysis of cohort 1 were used to carry out a predictive test on cohort 2 and the correct classification rate determined.

Results

Multiplex immunoassay

Molecules altered in major depressive disorder compared to controls

Multiplexed immunoassay analysis of cohort 1 and 2 sera resulted in the identification of 11 proteins, which showed reproducible differences between MDD patients and controls in both cohorts (Table 2; see Supplementary Table 2 for complete listing of significantly altered analytes in cohorts 1 and 2). The use of two cohorts provided a validation of the findings and the combination of both cohorts increased the statistical power of the findings. Six of these proteins [extracellular newly identified RAGE-binding protein (EN-RAGE), ferritin, interleukin-1 receptor antagonist (IL-1ra), IL-16, macrophage migration inhibitory factor (MIF) and tenascin-C) were significantly increased and one [angiotensin-converting enzyme (ACE)] was significantly decreased in both cohorts. Two proteins [serotransferrin (decreased) and superoxide dismutase 1 (SOD-1; increased)] were altered significantly in cohort 2 and trended to significance (*P*<0.1) in

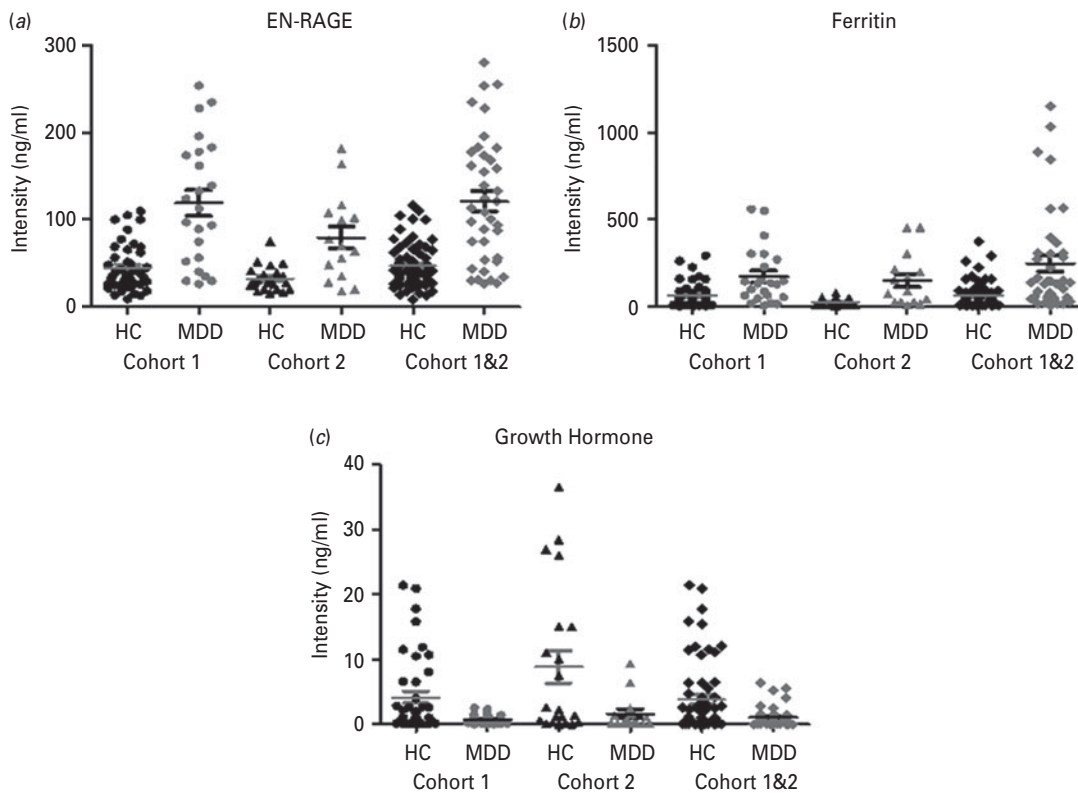


Fig. 1. Multiplex immunoassay result examples. The figure includes (a) EN-RAGE, (b) ferritin, and (c) growth hormone changes in first onset MDD patients (MDD) vs. healthy controls (HC) (cohort 1: 23 MDD vs. 42 HC; cohort 2: 15 MDD vs. 21 HC, and both cohorts combined). When cohorts 1 and 2 were combined, values were normalised between both cohorts for the comparison between MDDs and HCs.

cohort 1. EN-RAGE and ferritin showed the most marked differences as both were increased by more than 2-fold in MDD patients compared to controls in both cohorts (Fig. 1). Two other proteins [brain-derived neurotrophic factor (BDNF; increased) and growth hormone (GH; decreased)] were also included as they trended to significance in both cohorts and both were significant in the combined cohort. GH was decreased by more than 3-fold in MDD subjects compared to controls when both cohorts were combined (Fig. 1). All molecules which were either trending to significance in one or both cohorts showed significant changes after combining both cohorts due to the increase in statistical power. Cortisol was not changing or trending to significance in cohort 2, and therefore not included in Table 2, it was significantly increased in cohort 1 (FC: 1.67, p -value: 0.0072) and when both cohorts were combined (FC: 2.93, p -value: 0.0047).

Multivariate classification revealed that the combined panel of 11 differentially expressed analytes (ACE, EN-RAGE, ferritin, IL-1ra, IL-16, MIF, tenascin C, serotransferrin, SOD-1, BDNF and GH) found in cohort 1 (MDD=23, control=42) produced a separation between MDD ($N=15$) and control subjects ($N=21$) in cohort 2 with a correct classification rate of 89% using linear discriminant analysis. The sensitivity was 0.89 and the specificity was 0.95.

Table 3. Multiplex immunoassay symptom severity markers

| Molecule name | Cohort 1 | |
|--|---------------|-------|
| | p -value | r_s |
| Angiotensin-converting enzyme | 0.0732 | 0.39 |
| Macrophage migration inhibitory factor | 0.0495 | 0.42 |
| Superoxide dismutase 1 | 0.0323 | 0.46 |

The table shows significant associations (bold text) between molecular levels and HAMD scores at the time of sampling in cohort 1 (23 MDD). The results for cohort 2 (15 MDD) and both cohorts combined are not shown as these showed no significant correlations. The table includes p -value (Spearman's correlation test), correlation coefficient (r_s).

Symptom severity (multiplex immunoassay)

The second part of the study attempted to identify associations between molecule levels and HAMD scores at the time of sample collection. Of the 11 molecules that were present at different levels between first onset MDD patients and controls in cohort 1 and 2, two of these, MIF and SOD-1, were significantly positively correlated with HAMD scores in cohort 1 and ACE trended ($p < 0.10$) to a negative correlation (Table 3). None of the molecules showed a correlation to HAMD scores that

Table 4. LC-MS^E profiling results of serum from first onset MDD patients vs. healthy controls (HC) (cohort 1: 15 MDD vs. 21 HC; cohort 2: 15 MDD vs. 21 HC; and both cohorts combined)

| Name | Acc No | FC | Cohort 1 <i>p</i> -value | Cohort 2 <i>p</i> -value | Cohort 1 & 2 <i>p</i> -value | Peptides | CV | SC |
|------|--------|---|-----------------------------|-----------------------------|---------------------------------|----------|------------|-------------|
| HPR | P00739 | 1.60 2.21 1.90 | *0.0698 | 0.0047 | 0.0014 | 12 14 | 1.23 14.62 | 33.05 40.23 |
| CP | P00450 | - 1.09 - 1.13 - 1.11 | 0.0028 | 0.0059 | 0.0001 | 225 281 | 5.98 27.11 | 88.92 92.58 |

The table includes abbreviated protein names (haptoglobin-related protein=HPR; ceruloplasmin=CP), Uniprot accession numbers (Acc.No.), fold changes (FC; cohort 1|cohort 2|cohort 1 & 2), *p*-values (Mann Whitney *U* test), and ANCOVA *p*-value (A; cohort 1|cohort 2|cohort 1 & 2; bold values are significant) analysis results after correction of co-variate (smoking), peptide number (peptides; cohort 1|cohort 2), internal coefficient of variance (CV; cohort 1|cohort 2) and sequence coverage (SC; cohort 1|cohort 2). Only molecules were included in this table, which showed biological validation by being changed in both cohorts.

Table 5. LC-MS^E symptom severity markers

| Molecule name | Acc.No. | Cohort 1 | | Cohort 2 | |
|-----------------------------|---------|-----------------|-----------------------|-----------------|-----------------------|
| | | <i>p</i> -value | <i>r</i> _s | <i>p</i> -value | <i>r</i> _s |
| Complement C4-B | P0C0L5 | 0.0287 | 0.55 | 0.0058 | 0.45 |
| Plasminogen | P00747 | 0.0386 | -0.52 | 0.0550 | -0.32 |
| Ceruloplasmin | P00450 | - | - | 0.0199 | -0.39 |
| Haptoglobin-related protein | P00739 | - | - | 0.0085 | 0.43 |

The table shows significant associations (in bold text) between molecular levels and HAMD scores at the time of sampling in cohort 1 (15 MDD) and cohort 2 (15 MDD). The results for both cohorts combined are not shown as none of these associations were significant. The table includes Uniprot accession numbers (Acc.No.), *p*-value (Spearman's correlation test), and correlation coefficient (*r*_s).

was reproducible in both cohorts. Issues with reproducibility may be traced back to too subtle interactions between molecule intensities and HAMD scores, which could not be statistically shown due to limitations in sample size.

LC-MS^E

The CV of QC samples after LC-MS^E analysis was 25.47±18.67 in cohort 1 and 18.59±16.50 in cohort 2. Overall, 8517 and 9546 peptides were detected which corresponded to 430 and 408 proteins in cohort 1 and 2, respectively. In total, 169 proteins overlapped and were detected in both cohorts.

Molecules altered in major depressive disorder compared to controls

LC-MS^E analysis resulted in identification of two molecules (ceruloplasmin and haptoglobin-related protein), which showed reproducible changes in sera from patients and controls in cohorts 1 and 2 (Table 4). Although haptoglobin-related protein was only trending to significance in cohort 1, it was significantly changing in cohort 2 and when values of this molecule were combined from cohorts 1 & 2.

Symptom severity (LC-MS^E)

The greatest correlation was seen in cohort 2 as two molecules (complement factor C4-B and haptoglobin-related protein) were positively correlated and two (plasminogen and ceruloplasmin) were negatively correlated with HAMD scores (Table 5). However, only complement factor C4-B showed a reproducible correlation with symptom severity in both cohorts (Table 5). The correlation for plasminogen did not reach significance although it showed a trend (*p*=0.0550). When combining both cohorts none of these molecules interacted with HAMD scores. Again, issues with reproducibility may be traced back to too subtle interactions between molecule intensities and HAMD scores, which could not be statistically shown due to limitations in sample sizes.

Discussion

This is the first study to investigate molecular profiling differences, as well as symptom severity markers, in serum of two cohorts of first onset, antidepressant drug-naïve MDD patients. The findings from analysis of samples from cohorts 1 and 2 are most likely to be associated with the underlying pathophysiology of MDD as these were replicated and not biased by AD medication since all patients were antidepressant-naïve at the time

of sample collection. This is not the case with most previous studies, which used similar approaches (Domenici et al., 2010). Drug-free and drug-naïve samples are obviously difficult to obtain in psychiatric research.

This study showed evidence of pro-inflammatory changes (i.e. ceruloplasmin, EN-RAGE, ferritin, haptoglobin-related protein, IL-1 ra, IL-16, MIF, serotransferrin and tenascin-C) in first onset MDD patients. Although there has been already accumulating evidence that an increased immune response has an influence on the pathophysiology of MDD and may even induce depressive symptoms (Maes, 2011), reproducible changes found in inflammatory markers, such as ferritin and EN-RAGE, have not been shown before in first onset MDD patients.

Ferritin is a multifunctional protein involved in iron metabolism and has been shown to be elevated during inflammation (Alkhateeb and Connor, 2013). It is a positive acute phase protein (APP), which tends to increase in response to inflammation and is part of the innate immune system (Calandra and Roger, 2003). We showed that this protein was increased by more than 3-fold in both of the first onset MDD patient cohorts compared to healthy controls. Although the relationship between ferritin and MDD has rarely been studied before, Huang and Lee (2007) have shown that haemodialysis patients with MDD had significantly higher levels of ferritin than patients without MDD and that increased ferritin levels predicted depressive symptoms (Huang and Lee, 2007).

Ferritin has also been shown to have antioxidant properties (Alkhateeb and Connor, 2013), due to its ferroxidase activity and iron storage capacity. There is some evidence that the activation of immune responses may cause oxidative stress and overproduction of reactive oxygen species (ROS) and antioxidative enzymes (Bilici et al., 2001). Furthermore, there is mounting evidence that ROS are involved in the development of different forms of human pathologies such as MDD (Herken et al., 2007). This is consistent with our finding that SOD-1 was increased in both MDD cohorts and this was correlated with symptom severity in cohort 1. Similar to this study, Bilici et al., showed that SOD-1 levels were increased in MDD patients compared to healthy controls and these were normalised after three months of AD treatment (Bilici et al., 2001). Taken together, these findings suggest that an increased immune response and oxidative stress may be involved in the early stages of MDD (Fig. 2).

Previous studies have found that antidepressant treatment is associated with a decrease in inflammatory markers [reviewed by (Miller et al., 2009)]. Furthermore, clinical trials investigating the effects of anti-inflammatory treatment with Cox-2 inhibitors as add-on therapy to AD treatments have demonstrated positive effects (Muller, 2010; Raison et al., 2013). However, further evaluation of the effects of Cox-2 inhibition and anti-inflammatory therapy for MDD is required. For

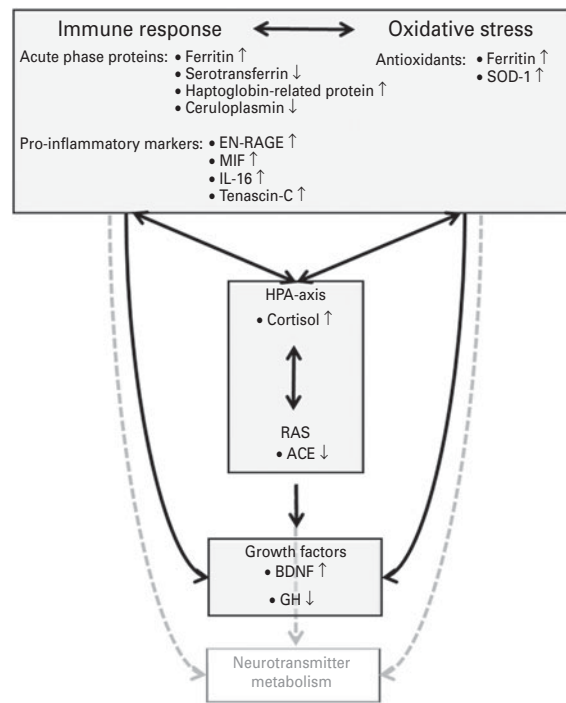


Fig. 2. Summary of molecular changes in this study. This study showed evidence of an activated immune response in first onset, antidepressant drug-naïve MDD patients, due to increases in positive acute phase proteins (APP) ferritin and haptoglobin-related protein and negative APP serotransferrin, as well as pro-inflammatory changes of extracellular newly identified RAGE-binding protein (EN-RAGE), macrophage migration inhibitory factor (MIF), IL-16 and tenascin-C. The multifunctional ferritin has immunological and anti-oxidant properties. These findings suggest increased oxidative stress in first onset MDD compared to controls which is further supported by increased levels of superoxide dismutase 1 (SOD-1). Changes in immune response were associated with effects on the hypothalamic-pituitary-adrenal (HPA) axis and renin-angiotensin-system (RAS), as shown by increased cortisol and decreased angiotensin converting enzyme (ACE) levels. These pathways may also be linked to effects on growth factors [brain derived neurotrophic factor (BDNF) and growth hormone (GH)] and neurotransmitter metabolism, which are hypothesised to be contributing factors to MDD. All molecular changes found in this study are indicated in black in this figure. Hypothesised links, which are solely based on literature findings are indicated in grey.

example, further studies should be carried out on the role of ferritin in MDD, considering the marked effects seen on this protein in the current study.

We also identified changes in the levels of other APPs in first onset MDD patients. Interestingly, increased levels of the positive APP, complement component C4-B, correlated with symptom severity in both cohorts. Furthermore, the haptoglobin-related protein (Nielsen et al., 2006) was increased in both cohorts. We also found a reproducible decrease in the negative APP serotransferrin in first onset MDD patients. These results suggest that an increased innate immune response, particularly including

the APP ferritin, is present early on in the disease course of MDD. This is consistent with the findings of a previous study, which showed increased levels of the positive APPs haptoglobin and alpha-1-antichymotrypsin in MDD patients, which correlated with symptom severity (Joyce et al., 1992).

We found that ceruloplasmin was decreased in cohort 1 and 2, which correlated with symptom severity in cohort 2. However, as it is a positive APP, this result does not agree with the other APP findings already discussed above. Interestingly, ceruloplasmin was previously reported to be increased in schizophrenia (Wolf et al., 2006; Morera et al., 2007). As we have seen the opposite regulation in first onset MDD patients, the potential of ceruloplasmin as a differential candidate marker between MDD and schizophrenia should be assessed in future studies.

As mentioned above, general alterations of inflammatory cytokines and other inflammation-related proteins have been previously described in blood cerebrospinal fluid and post-mortem studies of MDD patients [reviewed in (Maes et al., 1995; Raedler, 2011)]. In particular, our finding of increased levels of the pro-inflammatory proteins EN-RAGE, IL-16, MIF and tenascin-C support the case that there is an increase in inflammatory response in first onset MDD patients (Simon et al., 2008). Furthermore, MIF showed a significant positive correlation to symptom severity, indicating that patients with higher MIF levels had a higher symptom severity at the time of sample collection. Increased levels of MIF have also been previously linked to MDD (Musil et al., 2011). Therefore, MIF is a promising candidate in the neuro-immune interplay that may link depressive symptoms, altered immune state and HPA-axis dysregulation (Fig. 2).

Several studies have implicated altered HPA axis dysfunction in MDD pathophysiology, as increased stress vulnerability has been associated with MDD and acts as a trigger on the HPA axis, inflammatory and endocrine components of the disorder (Bartolomucci and Leopardi, 2009). Although normal activation of the HPA-axis is important for appropriate responses to acute stressors, chronic dysfunction of the HPA axis may result in long-lasting problems (Raedler, 2011), including effects on the immune system, mood, emotion and energy storage and expenditure (Engelmann et al., 2004). Most studies show a hyperfunction of the HPA axis in MDD patients, as shown by the presence of elevated circulating cortisol, corticotropin releasing hormone and ACTH (Nemeroff et al., 1984; Charlton et al., 1987; Roy et al., 1987; Breier et al., 1988). In this study, cortisol was significantly increased in cohort 1 and in the combined cohorts. In cohort 2, increased levels of cortisol showed a trend towards significance. However, this required control for smoking as a confounding factor. The effect of smoking on cortisol levels, as well as other inflammatory markers (e.g. c-reactive protein, IL-6 and TNF alpha), should also be considered in future MDD studies as it

has been shown to have an influence on the levels of these molecules (Steptoe and Ussher, 2006).

Interestingly, ACE is linked to HPA activity as part of the renin-angiotensin-system (RAS), and was decreased in first onset MDD. ACE was found to be changing in cohorts 1 and 2, and decreased levels of ACE correlated with symptom severity in cohort 1. This result supports previous studies suggesting that the RAS is connected to the aetiology of MDD (Saab et al., 2007) and that the change is present early on in the disorder.

Peripheral inflammatory markers, including inflammatory cytokines, have been shown to have the ability to cross the blood-brain barrier, thereby affecting brain function [reviewed by (Wichers and Maes, 2002; Yarlagadda et al., 2009)]. These factors are also involved in multiple aspects of MDD pathophysiology, including neurotransmitter metabolism, neuroendocrine function and neural plasticity (Miller et al., 2009).

Previous studies have shown that BDNF levels are decreased in MDD (Sen et al., 2008), leading to the 'neurotrophin hypothesis of depression'. In line with this hypothesis, other studies have shown that BDNF levels are increased after electroconvulsive stimuli or AD medication in both animal models (Angelucci et al., 2002) and MDD patient (Brunoni et al., 2008; Sen et al., 2008). We found that BDNF levels were increased in both cohorts, which is in contrast to previous literature results. However, as this is one of the first studies to investigate this protein in first onset, antidepressant drug-naïve MDD patients, the present findings suggest the potential presence of counter-regulatory protective mechanism in the early stages of the disorder. This is consistent with the current finding of increased levels of the anti-inflammatory cytokine IL-1ra, which may indicate a counter-regulatory mechanism in response to the elevation of the pro-inflammatory cytokines.

Limitations

Molecular studies in psychiatric research are generally limited by sample size, which increases the risk of false-positive results. This was certainly a limitation in the present study given the large number of analytes measured. However, we were able to test two independent cohorts, which strengthened the results. Nevertheless, the current findings should be considered as an exploratory study and further validation is required.

Although over 400 proteins were detected in cohorts 1 and 2, the serum proteome comprises several thousand proteins, which also include a variety of differential post-translational modifications of common core proteins. This limited the number of proteins that could be identified using this method. To increase this capacity, future studies could use complementary LC-MS platforms and protein modification-specific investigations (Brewis and Brennan, 2010).

It should also be noted that the finding of no change a given molecule does not necessarily mean that it is not a contributor to the aetiology of MDD. For example, it could be the case that an early increase in one or more molecules initiates a cascade of actions and then these molecules return to normal levels.

The focus of this study was to investigate molecular signatures related to MDD. Further studies are needed to compare our findings to known changes in other psychiatric disorders, such as schizophrenia and bipolar disorder, to establish the specificity of these markers to MDD.

Conclusions

This comprehensive study has shown multiple changes in first onset, antidepressant drug-naïve MDD patients, some of which have been reported previously in MDD research. We have also identified novel molecular and proteomic changes, which could help to elucidate MDD affected pathways in more detail and may be useful for the early detection of MDD. We found strong evidence of a link between an increased inflammatory response (i.e. ferritin, EN-RAGE, ceruloplasmin, IL-16, MIF, serotransferrin and tenascin-C) and oxidative stress (i.e. ferritin and SOD-1), as well as a hyperactive HPA-axis (i.e. cortisol), RAS (i.e. ACE) and changes in growth factors (i.e. BDNF and growth hormone), consistent with literature hypotheses. However, further studies are warranted to determine whether these molecular changes are causative factors in MDD or if they are an adaptive response to disease-related changes in the brain or periphery. Such studies of these pathways in MDD could lead to identification of novel drug targets.

Statement of Interest

Author Bahn, Cooper, Guest and Rahmoune have consulted for Myriad-RBM. All other authors do not report any conflicts of interest. This work was supported by the Stanley Medical Research Institute (SMRI), the Dutch Fund for Economic Structure Reinforcement (FES), under grant agreement number 0908 (NeuroBasic PharmaPhenomics project) and the European Union FP7 SchizDX research programme (grant reference 223427).

Supplementary material

For supplementary material accompanying this paper, visit <http://dx.doi.org/10.1017/S1461145714000819>.

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