Profiling of the peripheral blood mononuclear cell proteome in schizophrenia and mood disorders for the discovery of discriminatory biomarkers : a proof-of-concept study

PROFILING OF THE PERIPHERAL BLOOD MONONUCLEAR CELL PROTEOME IN SCHIZOPHRENIA AND MOOD DISORDERS FOR DISCOVERY OF DISCRIMINATORY BIOMARKERS - A PROOF OF CONCEPT STUDY

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Short Title: Peripheral blood mononuclear cell proteomics for diagnostic biomarker discovery in psychiatry

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Abstract

Introduction: Current diagnoses in psychiatry are solely based on the evaluation of clinical presentation by the treating psychiatrist. This results in a high percentage of misdiagnosis and consequential inefficient treatment; especially with regard to major depressive disorder (MDD), depression in context of bipolar depression (BD-D), bipolar disorder with manic symptoms (BD-M) and psychosis in the context of schizophrenia (SZ). Objective biomarkers allowing for efficient discriminatory diagnostics are therefore urgently needed.

Methods: Peripheral blood mononuclear cell (PBMC) proteomes of patients with MDD (n= 5) , BD-D (n= 3), BD-M (n= 4) and SZ (n =4) and of healthy controls (HC; n = 6) were analyzed by state-of-the-art mass spectrometry. Proteins with a differential expression of >2 standard deviation (SD) expression fold change from HC and between either MDD vs. BD-D or BD-M vs. SZ were subsequently identified as potential discriminatory biomarkers.

Results: In total, 4271 individual proteins were retrieved from HC. Of these, about 2800 were detected in all patient and HC samples. For objective discrimination between MDD and BD-D, 66 candidate biomarkers were found. In parallel, 72 proteins might harbour biomarker capacity for differential diagnostics of BD-M and SZ. A single biomarker was contraregulated vs. HC in each pair of comparisons.

Discussion / Conclusion: With this work, we provide a register of candidate biomarkers with potential capacity to objectively discriminate MDD from BD-D and BD-M from SZ. Although concerning a proof-of-concept study with limited sample size, these data provide a stepping-stone for follow-up research on the validation of true discriminatory potential and feasibility of clinical implementation of the discovered biomarker candidates.

Introduction

Psychotic and mood disorders are amongst the most prevalent and debilitating psychiatric illnesses. Partial overlap in clinical presentation of these disorders often renders diagnostic differentiation between these illnesses problematic to virtually unfeasible. Illustratively, depressive episodes occur both in context of unipolar depression (major depressive disorder, MDD) and of bipolar depression (bipolar disorder, BD) with studies reporting the presence of an actual diagnosis of BD-D in 31% to up to 69% of patients misdiagnosed with unipolar depression [1,2]. Often, also phenotypical distinction between manic and psychotic symptoms remains cumbersome[3]. Clinically utilized but arguably less valid intermediate disease types such as schizoaffective disorder [4], mood disorder with psychotic symptoms, schizophrenia with depressive episodes,... and high levels of comorbidity in psychiatric disorders [5] further confound accurate diagnostics. As the different syndromes each require a specific therapeutic strategy, erroneous diagnoses may lead to severely increased and/or prolonged patient suffering. Antidepressant monotherapy for example, is in general relatively ineffective for treating bipolar depression [6]. Consequently, treatment guidelines for this population advise the primary administration of antipsychotics and mood stabilizers all or not in combination with an antidepressant [7]. Unfortunately, Viktorin et al. [8] demonstrate that 35% of bipolar patients in Sweden were treated with antidepressant monotherapy with the risk of switching to mania only occurring in these patients, while being absent in patients treated with antidepressant add-on to a mood stabilizer.

In parallel, also when suffering from comorbid psychotic symptoms bipolar patients are often misdiagnosed. A recent study reports numbers as high as 61% of BD patients with psychotic symptoms to receive an initial other diagnosis, with 21% of misdiagnoses concerning a subtype of schizophrenia [9]. Although antipsychotics (AP) are in general effective in treatment of mania [10], the resulting absence of any type of mood stabilizer in BD-M patients treated with AP can result in a substantial increase in the duration of untreated illness of bipolar patients and frequent relapses or rapid cycling [11].

The above illustrates the cruciality of accurate diagnostics and underscores the urgent need for biomarkers enabling objective discriminatory diagnosis of unipolar vs. bipolar depression and of manic vs. psychotic episodes. Although several studies have investigated the potential of different types of biomarkers to categorize patients as either MDD or BD-D, so far, no studies have successfully identified a clinically usable, highly predictive biomarker using hypothesis-driven, targeted approaches (for review see Goossens et al., subm. and [12]). Non-targeted biomarker identification avenues (the so-called -omics approaches) however, might reveal

novel molecules with high discriminatory potential that have thus far not been related to any of the investigated pathologies.

A biomarker qualifies as clinically useful if it is easy to obtain, allows for stable and accurate detection and is present in high quantities in at least 1 of the comparative groups. As peripheral blood mononuclear cells (PBMC) are effortlessly collectable and intracellular protein levels are less fluctuating than those of free-circulating plasma/serum proteins [13–16], mapping of the PBMC proteome of MDD vs. BD-D and BD-M vs. SZ patients might prove a promising strategy for novel discriminatory biomarker discovery.

Therefore, this project aims to elucidate discriminatory biomarkers for unipolar vs. bipolar depressive, manic and psychotic symptoms in context of MDD, BD and SZ by performing large-scale non-targeted liquid chromatography - mass spectrometry (LCMS) proteomics on PBMC of patients with either of the aforementioned disorders.

Methods

Patient selection

MDD patients (n=5), BD patients in a depressed state (BD-D; n=3), BD patients in a manic state (BD-M; n=4), schizophrenic patients with active positive symptoms (n=4) were recruited from the Psychiatric Hospital Duffel. In addition, 6 age- and gender matched healthy controls (HC) were recruited via advertising. Diagnosis was made according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) and confirmed by the Mini international neuropsychiatric interview (MINI) version 5.0.0. Inclusion criteria were: men and women between the ages of 18 and 55; for patients: a score of ≥17 or more on the Hamilton Depression Rating Scale (HDRS) for depressed patients (both MDD and BD-D); a score of ≥13 on the Young Mania Rating Scale (YMRS) for manic patients; a total score of ≥14 on the positive scale of the Positive and Negative Syndrome Scale (PANSS) with either a score of ≥5 on at least 1 item or a score of ≥4 on at least 2 psychotic items (P2, P3, P5). Exclusion criteria were: recent occurence or a history of chronic inflammatory disorders, autoimmune diseases, acute physical diseases and substance dependence (in the last 6 months) and additionally for HC: a history of psychiatric disorders and having a first-degree relative with a history of psychopathology. All clinical scales were assessed by trained personnel and patients were matched to controls for age, gender, smoking status and body mass index.

The study was approved by the local ethics committee. All participants gave their written consent to take part in the study.

Collection of PBMC

Venous blood was collected sober between 7:30 and 10 AM in EDTA-coated collection tubes. Gradient centrifugation was performed using Histopaque-1077 (Sigma-aldrich, Missouri, USA) for 20 minutes at 700g and room temperature without brakes. Subsequently, the buffy coat was collected and washed 2 times with phosphate buffered saline (PBS;Thermo Fisher Scientific, Perth, United Kingdom). Finally, the supernatant was discarded en cells were stored dry at -80°C until LCMS analysis.

Quantitative Proteomics

Multiplexed iTRAQ (isobaric mass-tag labeling for relative and absolute quantitation) mass spectrometry liquid chromatography (LCMS) was performed as described hereafter. PBMC samples were solubilized in a protein extraction buffer (composition: 8 M urea, 2 M thiourea, 0.1% SDS and 50 mM triethylammonium bicarbonate). Next, protein concentrations were quantified using RC DC protein assays (Bio-Rad; California, USA). Equal amounts of proteins from each sample were then reduced by tris-2-carboxyethyl phosphine and alkylated by 5-methyl-methanoethiosulphate and finally subjected to trypsin digestion. The resulting peptides from each sample were labelled using iTRAQ reagents (Sciex, Massachusetts, USA) following the manufacturer's instructions. PBMC samples of HC and patient samples were then spread randomly across three different octaplex iTRAQ LC runs. To improve LC-MS/MS proteome coverage, samples were subjected to a 2D-LC fractionation system (Dionex ULTIMATE 3000, ThermoScientific, Massachusetts, USA). Peptide mixes were fractionated on a strong cationic exchange chromatography column (1 mm x 150 mm polysulfoethyl Aspartamide (California, USA, Dionex)) separated subsequently carried on a nano-LC C18 column (200 Å, 2 µm, 75 µm × 25 cm). The nano-LC is coupled online to a QExactive™-Plus Orbitrap(ThermoScientific) mass spectrometer (MS). The nano-LC eluents were infused to the Orbitrap mass-spectrometer with a capillary at 1.7 KV on a nano-ESI source at a flow rate of 300 nl/min. Data dependent acquisition in positive ion mode was performed for a selected mass range of 350-1800 m/z at the MS1 level with a resolution of 140,000 and at the MS2 level with a resolution of 17,500. The raw data were analyzed by

Proteome Discoverer 2.1 Software (ThermoScientific) using Sequest HT as the search engine against the human UniProt/SwissProt database. The threshold of confidence was set above 99% ensuring a false discovery rate of less than 1%. The list of identified proteins, containing iTRAQ ratios of expression levels over control samples, was generated. Proteome Discoverer 2.1 employs a global analytical methodology.

Statistical analyses

For demographics, group mean differences were calculated by ANOVA with Tukey honest significant differences (HSD) post-hoc comparisons for numerical data and with Fisher exact test for categorical variables (gender, smoking status). All analyses were performed using JMP® version 13 (SAS, Cary, North Carolina 27513, USA).

Only data of those proteins that were detected in all HC and patient samples were used. Using GraphPad Prism analysis of the distribution scope of the acquired MS data we only allowed datasets to transition to further levels of analysis if we found the mass level data to be normally distributed. All raw iTRAQ ratios (condition:Con) were $\log_2$ transformed before calculating the mean abundance ratio per experimental group. Subsequently, the fold change expression ratios of $\log_2$ transformed mean abundances of patient samples over HC on the one hand, and MDD over BD-D and BD-M over SZ were calculated. Finally, only proteins were withheld as biomarker candidates that showed fold change expression ratios of >2 standard deviations to the patient/HC or patient/patient abundance mean.

Our proteomic analysis pipelines and methodologies have previously been demonstrated to be both rigorous and able to generate highly effective and significant data outputs that give actionable high-dimensionality data appreciation [17–24].

Results

Demographics

After screening, 5 MDD patients, 3 BD-D patients, 4 BD-M patients and 4 SZ patients fulfilled all inclusion criteria. In parallel, 6 age and gender matched healthy controls were included. No significant between-group differences were found for age, gender, BMI and smoking status (Fisher exact, Tukey Honest significant difference). All patients except for 1 medication free MDD patient used some type of psychopharmacotherapeutics. Six patients (n = 3 SZ, n = 1 BD-M, n = 1BDD, n = 1 MDD) were on monotherapy, while all other patients (72%) took at least 2 different psychopharmacological drugs. For an overview of medication status per disorder, see supplementary table 1.

--INSERT SUPPL TABLE 1 HERE--

Compared to controls, all patients showed more depressive symptoms as measured by the Hamilton depression rating scale (HDRS). Among patient groups, BD-D and MDD had significantly higher HDRS scores than BD-M patients. As expected, psychotic symptoms as quantified by the Positive and Negative Syndrome Scale (PANSS) were only detected in SZ patients. SZ patients further scored highest on the Young mania rating scale (YMRS) for manic symptoms, followed by BD-M patients who in their turn differed significantly from bipolar depressed patients. No manic symptoms were demonstrated in MDD patients or HC. Demographic variables and symptom scores are presented in table 1.

--INSERT TABLE 1 HERE--

Protein retrieval capacity and differential expression from controls

To determine potential diagnostic discriminators for patients with either MDD, BD-D, BD-M or SZ, the proteomes of patients' peripheral blood mononuclear cells (PBMC) were mapped and compared with those of age and gender matched healthy controls.

Quantitative proteomic analysis led to the total detection of 4271 individual proteins in HC. Of these, about 90% were also found in the 4 psychiatric traits (PT). To ensure identification of biomarkers with highest certainty of retrieval, we subsequently quantified the number of HC-detected proteins that were only present

in all individuals for each experimental group and found a similar detection rate of ~70% for all experimental groups and HC. As pathological biomarkers are preferably distinguishing between the PT and HC, we last calculated the number of proteins detected in all patients per PT that were differentially expressed from controls. Differentially expressed proteins (DEP) were defined as proteins showing a >2 standard deviation (SD) fold change in expression from controls and on average ~5% DEP were found for all PT. Specifically, BD-D had the highest number of DEP (167), followed by MDD (132 DEP), BD-M (125 DEP) and SZ (102 DEP) (Table 2).

--INSERT TABLE 2 HERE--

Diagnostic biomarkers discriminating unipolar from bipolar depression

As clinical presentation of depressive symptoms hardly allows to discern the underlying pathology to be either MDD or BD, as such rendering adequate therapeutic intervention difficult, objective discriminative biomarkers are urgently needed. Therefore, we investigated which proteins showed both the greatest differential expression in patients with depressive symptoms vs. HC and in MDD vs. BD-D. Again, proteins with a fold change expression of >2SD were considered as substantially differentially expressed. Of the proteins showing differential expression from HC in either depressive condition, 66 reached the 2SD significance threshold for differential expression between MDD and BD-D and are as such potential discriminatory biomarkers for MDD vs. BD-D. Only 1 protein was found to be significantly contraregulated in MDD vs. BD-D. Interestingly, this concerns the protein HLA class II histocompatibility antigen, DRB1-16 beta chain (HLA-DRB1), a protein from the HLA-II class, which had been extensively implicated in several psychiatric disorders. HLA-DRB1 expression was significantly (i.e. >2 SD expression fold change from HC) downregulated in MDD (-0.21 fold change in expression from HC) while being significantly upregulated in BD-D (0.38 fold change expression from HC). Moreover, of the 65 additional discriminatory biomarkers, 7 more proteins were HLA molecules, confirming them to be of major interest with regard to the physiopathology of depressive disorders.

A list of all 66 discriminatory biomarkers for MDD and BD-D can be found in table 3.

--INSERT TABLE 3 HERE—

Diagnostic biomarkers discriminating manic from psychotic patients

Also manic and psychotic symptom presentations are not always readily discernible based on clinical phenotype and would benefit from objective biological categorization. Therefore, PBMC proteomes of patients with either manic symptoms within the spectrum of bipolar disorder or psychotic symptoms within the spectrum of schizophrenia were compared to identify symptom-specific discriminatory biomarkers. Of the 2651 proteins retrieved in all BD-M and SZ patients, 72 proved to be both significantly differentially expressed between BD-M and SZ on the one hand and between either of the pathologic conditions and HC on the other. Again, one protein (apolipoprotein C-III, APOC3) was found to be significantly contraregulated in BD-M vs. SZ. APOC3 is involved in triglyceride homeostasis and was downregulated in BD-M (-0.24 fold change in expression from HC) and upregulated in SZ (0.23 fold change in expression from HC). Three other proteins were however more strongly distinctiving BD-M from SZ, irrespective of their expression in control individuals. While APOC3 showed a downregulation of -0.47 in BD-M over SZ, these fold changes were stronger in hemoglobin subunit gamma-1 (-0.72 fold change expression BD-M / SZ), galectin-10 (-0.70 fold change expression BD-M / SZ) and HLA class II histocompatibility antigen, DRB1-11 beta chain (-0.54 fold change expression BD-M / SZ).

A list of all 72 discriminatory biomarkers for BD-M and SZ can be found in table 4.

--INSERT TABLE 4 HERE--

Discussion

With this work, we aimed at establishing a list of potential biomarkers that show great promise in objectively categorizing patients with MDD vs. BD-D on the one hand, and BD-M vs. SZ on the other. Non-targeted LCMS-based proteomics of patient PBMC revealed 66 proteins that may enable biological tracking of depressive symptoms to either a unipolar or bipolar depression context. Likewise, 72 proteins might biologically differentiate between manic and psychotic symptoms. For both comparisons, a single protein was found to be significantly contraregulated vs. HC between the 2 pathologies. In case of depression, this concerns the HLA class II histocompatibility antigen, DRB1-16 beta chain. When comparing differential expression vs. HC between BD-M and SZ, only apolipoprotein C-III was found to be significantly contraregulated.

By identifying proteins that both differ between the 2 comparative diseases and between either of the pathologies and healthy individuals, our data allow for 2 methods of clinical biomarking. The first method would be to utilize proteins that show contraregulation or differential regulation in either of the pathologies vs. HC. With this approach, a reference range of these proteins should be determined in a large cohort of HC that can subsequently be used as threshold value for patient samples. Preferably, we suggest the potential HC-based biomarker proteins to either show contraregulation vs. HC (i.e. HLA-DRB1-16b for MDD vs. BD-D and APOC3 for BD-M vs. SZ) or show less than 1 SD fold change differential expression in 1 of the 2 comparative pathologies while being significantly dysregulated in the other. Specifically, for MDD vs. BD-D, 35 proteins show < 1 SD fold change expression in either of the 2 pathologies and > 2SD fold change expression in the other and could thus be further validated as HC-based biomarkers for discriminatory diagnosis of MDD and BD-D (see Supplementary table 2).

--INSERT SUPPL TABLE 2 HERE--

With regard to objectively discerning BD-M from SZ, 31 proteins show < 1 SD fold change expression in either of the 2 pathologies and > 2SD fold change expression in the other and could thus be further validated as HC-based biomarkers for discriminatory diagnosis of BD-M and SZ (see Supplementary table 3).

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An alternative strategy to clinically implement proteins retrieved in this study as discriminatory biomarkers is to determine reference ranges for a large cohort of patients from both comparative pathologies in order to be able to quantifiably categorize patients to either of the 2 diagnostic groups. As this approach would per definition require biomarkers that show the largest discrepancy between the 2 comparative pathologies, the proteins ranked highest in the respective MDD / BD-D and BD-M / SZ biomarker lists (see tables 3 and 4) would theoretically show the largest predictive potential. Although considerably more time- and effort consuming, this approach would undoubtedly prove more sensitive and specific than less robust but considerably faster HC-based biomarker optimization.

Both approaches however, require thorough follow-up research in order to affirm true biomarker capacity of the candidate proteins identified within this project. In a first step, reproducibility of the results should be substantiated in a larger training cohort of patients as this project concerned a proof of concept with a limited sample size. Subsequently, retrieval capacity of the confirmed biomarker candidates by more efficient and accessible antigen-based methods like single or multiplex enzyme-linked immuno-assays (ELISA) should be evaluated as this would be the technology of choice for low-threshold implementation in clinic. For those candidate biomarkers retrievable as such, reference ranges should then be established for either a large HC or both patient populations and lastly, the predictive capacity of the final selection of candidate biomarkers is to be determined by comparing the accuracy rate of blind objective diagnostication of a large test sample of patients based on biomarker quantification with the phenotypical diagnosis made by the treating psychiatrist. If none of the above individual candidate biomarkers prove valid in having sufficient predictive capacity, machine learning algorithms might be applied to these data to distill a molecular fingerprint composed of differential regulation of multiple markers.

Although this cross-sectional study has merit in aiming to objectively discriminate between the aforementioned diagnoses to determine adequate treatment regimens, it does not provide information on biomarker stability throughout time. Longitudinal follow-up studies could provide insight in biomarker fluctuation according to disease course and moreover, potentially reveal biomarkers correlating to either disease state or treatment effectiveness. Of note, correlation analyses on our data revealed no correlation between the 5 most discriminating MDD vs. BD-D proteins and HDRS on the one hand, and between the 5 most discriminating BD-M vs. SZ proteins and either the YMRS or the PANSS-P clinical scales on the other (data not shown).

This is to be expected as both BD-D and MDD patients would score high on the HDRS and likewise, BD-M and SZ patients would have some overlap in YMRS and PANSS-P scoring.

As the aim of this study is to find biomarkers that are readily accessible, peripheral blood cells were chosen as investigation medium. This implies that at the current study stage we do not have any information on the biomarker's distribution in the brain. As some biomarkers might be subject to active transport across the blood-brain barrier following excretion from PBMC, they might additionally function as disease-related state or trait biomarkers in the brain. We are however hesitant to draw any definite pathophysiological conclusions from our data as this project concerns intracellular protein detection in peripherally circulating blood cells and may therefore be of little representative value for central mechanistic neurobiological processes. Notwithstanding, several of our biomarker candidates have already been implicated in psychiatric afflictions. With 16 out of the total of 138 (11%) potential biomarkers for MDD vs. BD-D and BD-M vs. SZ, the human leukocyte antigen (HLA) family is highly represented in our candidate list. HLA proteins are cell-surface proteins originating from the major histocompatibility (MHC) gene locus that regulate the body's immune responses and are present on all bodily cells except red blood cells. Notably, a recent study has indicated that the strongest genetic association for the risk of schizophrenia development is linked to greater expression of the *complement component 4 (C4) A* allele, contained on the MHC locus [25]. Although associations between certain HLA haplotypes and mood disorders and schizophrenia have been extensively described [26–30], the potential of HLA peptides or proteins as predictive diagnostic biomarkers has to our knowledge never been explored. In parallel, a considerable number of diagnostic protein candidates are involved in immune processes. While this is not unexpected considering the immune mediating function of PBMC, it is in line with the recently re-emerging hypotheses on the role of increased inflammatory processes in mediating psychiatric afflictions (for review see [31–33,34,35]).

Some contemplations might be taken into account when considering these data. First, as mentioned above, this project concerns a proof of concept study regarding the feasibility of proteome-based diagnostics in objectively discriminating MDD from BD-D and BD-M from SZ. While our small data cohort was able to generate distinctive and actionable data streams it is clear that further more advanced studies with considerably bigger patient cohorts will be more desirable for predictive diagnostic activities. These studies are currently underway in our laboratory.

Additionally, patients in this study had been recently started on medication, which might in itself influence

protein expression. Replication in drug-free patients and investigation of the potential influence of different types of psychopharmacological treatment regimens on expression of PBMC biomarkers in larger cohorts are envisaged. Lastly, we only considered proteins as candidate biomarkers if they were not only strongly differentially expressed between the 2 comparative pathologies, but were in addition also significantly dysregulated when compared to healthy individuals. In theory, it could be expected that some proteins with high pathological discriminative capacity would fall within the 2 SD fold change expression requirement with HC and are thus not registered in our final biomarker candidate bank.

Nonetheless, we believe that this work provides a major stepping stone towards imminent implementation of objective biological discriminatory diagnostics in the field of clinical psychiatry.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Author Contributions

VC performed biomarker candidate analyses and wrote the paper. ODW, JG, SM and JH wrote the paper. ODW, JG, JH and SM performed LCMS analyses. JVG and AA performed experimental LCMS procedures. TL and AVS performed clinical testing and demographic analyses. MM supervised the project. All authors revised the manuscript.

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