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Thesis

Search for peripheral biomarkers in patients affected by acutely psychotic bipolar disorder: a proteomic approach

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ABSTRACT

Data on neurobiological mechanisms underlying mood disorders are elusive; the aetiology of such states is multifactorial, including genetic predisposition and environmental factors. Diagnosis is currently being made only on an interview-based methodology. Biological markers, which could improve the current classification, and in perspective, stratify patients on a biological basis into more homogeneous clinically distinct subgroups, are highly needed. We describe here a comparative proteomic analysis of peripheral lymphocytes from patients affected by acute psychotic bipolar disorder (PBD) (n = 15), major depressive episode (MDE) with no personal or family history of psychosis (n = 11), and a group of demographically matched healthy controls (HC) (n = 15). All patients were evaluated by means of Structured Clinical Interview for DSM-IV-Patient version (SCID-I-P), Positive and Negative Symptoms Scale (PANSS), Young Mania Rating Scale (YMRS), Hamilton Anxiety Rating Scale (HAM-A) and Hamilton Depression Rating Scale (HAM-D-17) questionnaires. Blood lymphocytes were obtained by gradient separation, and 2-DE was carried out on protein extracts. Significant differences in protein patterns among the three groups were observed. Thirty-six protein spots were found to be differentially expressed in patients compared to controls, which collapsed into 25 different proteins after mass spectrometry identification. Twenty-one of these proteins failed to discriminate between PBD and MDE, suggesting common signatures for these disorders. Nevertheless, after the western blot validation only two of the remaining proteins, namely LIM and SH3 domain protein 1, and short-chain specific acyl-CoA dehydrogenase mitochondrial protein, resulted in being significantly up regulated in PBD samples suggesting additional mechanisms that could be associated with the psychotic features of bipolar disorder.
INTRODUCTION

Bipolar Disorder

Bipolar disorder (BD) and severe clinical depression are a group of mental diseases, which often manifest themselves with dramatic changes in mood, energy, cognition, and behavior that fluctuate over time. Psychotic states characterized by auditory or visual hallucinations, and paranoid or delusional beliefs can also be present in the acute phase. The aetiology of these disorders is multifactorial including a complex interaction between genetic predisposition, with many genes each accounting for a small effect, and environmental factors, such as developmental abnormalities, trauma, substance abuse and social factors. Despite the abundance of research, data on neurobiological mechanisms underlying mood disorders are elusive, and diagnoses rely on clinical assessment of these dynamic symptoms mainly through interview-based methodology. Currently most used classification systems are the Diagnostic and Statistical Manual of Mental Disorders 4th Edition and the International Statistical Classification of Diseases (DSM-IV-TR) and Related Health Problems 10th Revision (ICD-10)[3] that are not evidence-based and many diagnostic criteria overlap with many other diseases with different aetiologies, such as neurological or organic conditions. There is no clear consensus on how many types of bipolar disorder exist[1] in the DSM-IV-TR and ICD-10, bipolar disorder is conceptualized as a spectrum of disorders occurring on a continuum. The discrimination between the various clinical subtypes of bipolar spectrum is essentially based on the time course and the symptom of emotional connotation intercurrent. The DSM-IV-TR lists three specific subtypes[2]:

Bipolar disorder I be characterized by the presence of at least a Mixed Episode, or by the presence of at least one major depressive episode, interspersed by at least one manic episode spontaneous. In most cases, or mixed manic episodes alternated with one or
more depressive episodes, though these are not required for diagnosis. In fact, in 2-10% of cases occur exclusively manic relapses, the latter forms that show more often a late-onset.

**Bipolar disorder II** on the other hand, shows a clinical course is characterized by at least one major depressive Episode (see dedicated paragraph), supplemented by at least one Hypomanic Episode spontaneous.

**Ciclotimico** is a disorder characterized by the development of numerous hypomanic episodes, alternating with periods characterized by the presence of depressive symptoms, which however are not sufficient to diagnose major depression. Not surprisingly, the course of the disorder remains unpredictable, and treatment options are unspecific leaving the practice mostly to the clinician’s personal experience. In order to be clinically useful, a diagnosis should be able to predict the prognosis, the course of illness and eventually the guide treatment choice, and such aims are better achieved when the classification of illnesses moves closer to underlying biological mechanisms. The identification of biological markers could improve the diagnosis and classification of mood disorder subtypes, as well as stratify patients into more homogeneous, clinically useful subpopulations. Biomarker studies on psychiatric disorders present peculiar hurdles such as the inherent difficulties in accessing relevant biological materials, since the main alterations appear to be in the brain. Almost all large-scale proteome studies in psychiatry have aimed to profile protein expression differences compared to control samples in a hypothesis-free manner. Proteomic profiling for psychiatric disorders has been initially carried out in brain tissue. Samples from major depression (MD), schizophrenia, BD and control subjects have been simultaneously analyzed[4]-[6].

**Biomarkers in Psychiatry**
According to the official definition by the National Institutes of Health (NIH), “a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention”[7]. The utilization of biomarkers for brain disorders is not a recent concept. In the nineteenth century, Kraepelin established a writing scale to stratify patients suffering from psychiatric disorders by measuring their writing pressure curves [8]. Due to the phenotypic heterogeneity and the lack of quantitative measures for disease symptoms, biomarker discovery in the field of neuroscience has been confronted with considerable challenges. This holds true especially for neuropsychiatric disorders where, despite tremendous progress in understanding brain function, the exact molecular underpinnings of mental dysfunction remain elusive. Because biomarkers can differentiate between distinct biological states, their availability is critical in clinical settings for premorbid diagnosis, patient stratification, and monitoring of disease progression and treatment. The use of biological markers in other areas of medicine has come a long way with advances in the fields of pathology, biochemistry and most notably genetics. For example, prevalent and debilitating diseases such as heart failure can be diagnosed with high sensitivity and specificity by measurement of levels of B-natriuretic peptide (BNP). Certain types of cancer can be screened and monitored by specific tumor markers. The search for biological markers for psychiatric diseases has been going on for decades; however, previous experimental attempts have selected candidate biomarkers based on current models of disease pathogenesis. Due to the presumed high level of etiologic heterogeneity and the overlap of dimensions across mood disorders, there aren’t specific markers. Recent technological advances made it possible to leave the hypothesis-based approaches of biomarker discovery in favor of non-hypothesis-driven profiling experiments. Biomarkers may be in the form of genes, proteins and other molecules, or morphological characteristics, in any case they should
relate closely to the neurobiology representing the molecular correlate of the disorder, not merely the symptom or disease consequences. Diagnostic biomarkers, depending on the information they can provide, be used as prediction tools (e.g. subclinical markers, risk or vulnerability markers), this is of high importance in psychiatry as there is evidence that delays in diagnosis and intervention lead to poorer prognosis. Biomarkers could also be identified as diseases signatures (e.g. disease markers, stage or progression markers)[9], adding information on prognosis and course of illness. Moreover, in order to be translated into clinical practice biomarkers should also be measurable and reproducible over time, non invasive, easily available, cost-effective and provide with high sensitivity and specificity for the disease[10][11].

**Proteomics in psychiatric disorders**

The first proteomics publication in psychiatric disease dates back to 2000 [12]. Biomarker studies on psychiatric disorders present peculiar hurdles such as the inherent difficulties in accessing relevant biological materials, since the main manifestations appear to be in the brain. Almost all large-scale proteome studies in psychiatry have aimed to profile protein expression differences compared to control samples in a hypothesis-free manner. Proteomic profiling for psychiatric disorders has been initially carried out in brain tissue. Studies in brain tissues, however, carry some limitations and the reasons are both methodological and theoretical. Indeed, brain samples need to be analyzed carefully to avoid post-mortem artefacts, they are a very valuable source for the generation of hypotheses with regard to the aetiology of a disorder; however, they can only give molecular information from the time of death. One additional limitation of post-mortem research is that results cannot be exported into animal models. Sampling peripheral tissues is important for diagnostic purposes as well.
as for investigating treatment effect, therefore proteome of blood or cerebrospinal fluid is necessary to integrate findings between brain and periphery.

**Biological samples studies**

- **Post mortem tissue.**

In schizophrenia brain tissue, deregulation of pathways associated with reactive oxygen species has been observed [13] as well as alterations in mitochondrial oxidative phosphorylation and glucose metabolism. Proteome analyses have identified differential expression of proteins involved in those processes, such as key enzymes associated with glucose metabolism including aldolase C (ALDOC), gamma enolase (ENO2), aconitase (ACO2), hexokinase (HK1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a number of subunits of mitochondrial ATPase and proteins associated to oxidative stress [14]. Oligodendrocytes are responsible for myelination of axons in the central nervous system. Reduction or malformation of the myelin sheath can result in leakage and reduced propagation of nerve impulses. In addition, proteome analyses have confirmed this through identification of differentially expressed proteins such as Myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and 20,30-cyclic nucleotide,30-phosphodiesterase (CNP)[14]. Proteome analyses of human brain tissue of patients with bipolar disorder have also been performed. Dihydropyrimidinase-related protein-2 (DPYSL2) and glial fibrillary acid protein (GFAP) were found to be decreased in the frontal cortex (FC-BA10) suggestive of effects on brain development. The differential expression of several tubulin subunits suggested that cytoskeletal dysfunction may be an important component of bipolar disorder through an analysis of the anterior cingulate cortex (ACC-BA24)[16]. One common finding in proteomic studies of distinct brain regions from bipolar disorder patients is suggestive of a
dysfunction in energy metabolism[12][16], more prominently in the dorsolateral prefrontal cortex (DLPFC-BA9) where half of the identified differentially expressed proteins were involved in these pathways. At present, only two brain regions, the frontal cortex (FC) and accumbens (ACC), from depressed patients have been subjected to proteomic analyses, revealing differentially expressed proteins[12][16]. Altered expression of DPYSL2 was common to both studies, although this was down regulated in FC and up regulated in ACC. DPYSL2 plays a role in nervous system development and cell differentiation by regulating axonal guidance, neuronal growth cone collapse and cell migration, suggestive of alterations in brain development in depressed patients. The differential expression of carbonic anhydrase (CA2) and ALDOC was also common to both studies, implicating effects on energy metabolism. Both of these studies used brain tissue samples from the Stanley Neuropathology Consortium and analyzed samples from major depression, schizophrenia, bipolar disorder and control subjects simultaneously.

➤ *Serum*

Serum can be obtained in relatively large quantities, it can be sampled with minimal discomfort to the patient and therefore remains one of the most suitable methods to investigate diagnostic biomarkers and findings can be easily exported and used to develop large scale tests. While there is yet no clear biomarker, there is increasing evidence to suggest that disease related changes can be detected outside the brain. Multiple contributing factors including growth factors and/or pro-inflammatory cytokines are deregulated in psychotic disorders. And also multiple endocrine (Thyroid, sex steroids, HPA axis) and metabolic (insulin resistance) function appear altered in mood disorders. Serum levels of the inflammatory markers sTNFR1 and sTNFR2, were
found to be higher in chronic institutionalized patients with schizophrenia than in
controls, however no correlation with symptom severity was found[17]. Dietrich-
Muszalska and Olas[18]showed that collagen-stimulated platelet aggregation was
significantly lower in the schizophrenic patients group compared to healthy controls. In
another study the activity of the platelet antioxidative enzyme superoxide dismutase
(SOD), and the levels of thiobarbituric acid reactive species (TBARS) were measured as
oxidative stress indicators. Results suggested an enhanced generation of reactive oxygen
species and significantly lower SOD activity in schizophrenia patients compared to
healthy controls[19]. A recent review of 185 publications described a total of 273
schizophrenia biomarkers identified in serum and/or plasma. The findings suggested an
ongoing immunological and inflammatory process in schizophrenia, accompanied by
altered cortisol levels which suggested activated stress response and altered
hypothalamic-pituitary-adrenal axis function in these patients [20].

➤ **Lymphocytes**

Metabolism and regulatory processes of viable tissue of subjects from with central
nervous system (CNS) disorders are difficult to assess. Sampling peripheral tissues (i.e.
blood and cerebrospinal fluid)[21] is important for diagnostic purposes as well as for
investigating treatment effects.

Abnormalities of the anterior cingulate cortex have previously been described in
schizophrenia, major depressive disorder and bipolar disorder. In fact, there is now an
increasing body of evidence pointing to a close integration between the central nervous
system (CNS) and immunological functions with lymphocytes playing a central role.
Numerous studies showed similarities between receptor expression and mechanisms of
transduction processes of cells both in the nervous system (e.g. neurons and glia) and in
lymphocytes [21]. In several neuropsychiatric disorders, the alteration of metabolism and cellular functions in the CNS, as well as disturbances in the main neurotransmitter and hormonal systems, are concomitant with altered function and metabolism of blood lymphocytes. Taken together the authors suggest further detailed exploration of the expression of genes and gene products in lymphocytes that could be used as markers for a variety of psychiatric disorders and could predict responses to psychotropic drugs. Furthermore, these parameters may help to identify responsible genes that underlie process of the disease. The lymphocyte may therefore serve as an easily accessible model to investigate alterations in immune–neuroendocrine interactions observed in psychiatric disorders. The authors therefore suggest that they be used as a neuronal and genetic probe in experimental and clinical research in psychiatry[22]. Recently, proteomic analysis has been performed on lymphocytes of schizophrenic[23] and bipolar patients [24][25] suggesting the presence of potential biomarkers such as alpha defensins and phosphoglyceratemutase 1 (PGAM1) respectively.

**Proteomics**

In the last few years, it has become widely recognized that the genome only represents the first layer of complexity. Biological function is carried by the dynamic population of proteins, moreover, only the characterization of the proteins themselves can reveal posttranslational modifications (e.g., phosphorylation, sulfation, glycosylation, ubiquitination, and methylation) and give insight into protein-protein interactions and subcellular localization, thus providing clues about function. For these reasons, there is increasing interest in the field of proteomics: the large-scale identification of proteins contained in cells, tissues or body fluids[26]. The proteome was originally defined as the complete protein complement expressed by a genome[26]. However, this definition
does not take into account that the proteome is a highly dynamic entity that will change based on cellular state and the extracellular milieu. Therefore, the definition of a proteome should specify that it is the protein complement of a given cell at a specified time, including the set of all protein isoforms and protein modifications [27]. Proteomic analyses can be used to identify the protein content in complex biological samples such as biological fluids and tissue extracts, and to determine the quantitative or qualitative differences for each polypeptide contained in different samples. It is expected that the proteomic profiling patterns resulting from such analyses define comprehensive molecular signatures in health and disease. The exploitation of a proteomic approach for the study of different diseases has led to the hypothesis that multiple biomarkers or a panel of biomarkers shown by proteomic profiling may correlate more reliably with a specific disease than a single biomarker or protein. Expression pattern of a known biomarker or correlation of expression of several known biomarkers can be a valuable research and clinical tool for monitoring disease or treatment progression [28].

**Two-dimensional electrophoresis.**

The identification of proteins from complex biological sample has traditionally been performed using 2-D PAGE coupled with mass spectrometry (MS). Two-dimensional electrophoresis (2-DE) separates proteins by both their isoelectric point (pI) and molecular weight. With this technique proteins are resolved into discrete spots, each of which represents a single protein that can be selectively excised and identified by MS. The high resolution of 2-DE allows the researcher to pick only the proteins of interest while bypassing the more abundant or less interesting proteins [29].
Sample preparation.

Preparation of samples for 2-D PAGE involves solubilization, denaturation and reduction to completely break up the interactions between the proteins [30]. Although desirable, there is no single method of sample preparation that can be universally applied due to the diverse samples which are analyzed by 2-DE gel electrophoresis [31]. The ideal sample solubilization procedure for 2-D PAGE would result in the disruption of all non-covalently bound protein complexes and aggregates into a solution of individual polypeptides [32]. However, whatever method of sample preparation is chosen, it is most important to minimize protein modifications which might result in arte-factual spots on the 2-DE maps [31]. Samples containing urea must not be heated as this may introduce considerable charge heterogeneity due to carbamylation of the proteins by isocyanate formed from the decomposition of urea. Generally speaking, samples should be subjected to as minimum handling as possible and kept cold at all times [33]. Protein extracts should not be too diluted to avoid loss of protein due to adsorption to the wall of the vessel (glass or plastic). If samples are rather diluted and contain relatively high concentrations of salts which can interfere with IEF, samples may be desalted [31]. Alternatively, proteins can be precipitated with ice-cold TCA / acetone to remove salts. Diluted samples with a low salt concentration may also be applied directly without further treatment, if the dried IPG strips are reswollen in sample solution. In this case, solid urea, CHAPS and dithiothreitol (DTT) are added to the sample until the desired concentration is obtained [31][34].

First dimension.

Iso-electro focusing (IEF) represents the first dimension of 2-DE and it is performed in individual immobilized pH gradients (IPG) strips. Each sample protein applied to an
IPG strip will migrate to its isoelectric point (pI), the point at which its net charge is zero. There are strips with broad or narrow pH gradient (e.g., 3-10; 4-7; 4-9; 6-10; 5-6; 9-12; 10-12). Dried gel strips containing immobilized pH gradient were commercially introduced in 1991 (Pharmacia Biotech, Immobiline® DryStrip Gel), their adoption for the first dimension of 2-DE has produced significant improvement over the classical O’Farrell carrier ampholyte-based 2-DE separation [35]. In the original 2-DE the required pH gradient is established by the migration of individual species of carrier ampholytes to their respective pI. Variations of the complex carrier ampholyte mixtures result in variations in the shape of the pH gradient [35]. The use of commercially prepared IPG DryStrip, introduced by Bjellqvist et al. [36] and Gorg et al. [37] eliminates these variations. The pH gradient is immobilized by covalent incorporating Immobiline® acrylamide buffers into the acrylamide matrix during polymerization. Since Immobiline consists of discrete, relatively simple molecules, they can be manufactured very reproducibly pure, eliminating batch effect as demonstrated by interlaboratory comparison [35][38]. Further, pI resolution to 0.01 pH unit can be achieved [36]. The acrylamide matrix with the Immobiline, acrylamide buffers is cast onto a baking sheet, polymerized, washed and dried. The backing gives the strips size stability and simplify handling. The dried strips can be rehydrated in various buffers and additives that would inhibit polymerization if included at the time of casting [35].

Second dimension.

Prior to the second dimension (Sodium Dodecyl SulphatePolyAcrylamide Gel Electrophoresis-SDS-PAGE), the IPG strips are equilibrated twice with gentle shaking in a solution containing urea and glycerol in order to diminish electroendosmotic effects [37] which are held responsible for reduced protein transfer from the first to the second dimension. DTT, a reducing agent, is added to the first and iodoacetamide (IAA) to the
second equilibration step [39]. IAA is added to the equilibration buffer in order to remove excess DTT (responsible for the "point streaking" in silver stained patterns) [40]. After equilibration the strips are applied to vertical SDS gels in order to perform electrophoresis and to separate proteins according to their molecular weight. Polypeptides separated can be visualized by Coomassie Blue, silver staining, fluorescence or autoradiography, or by "specific" stains such as glycoprotein staining or immunochemical detection methods [31]. Whereas the "general" protein stains are carried out in the electrophoresis gel directly, immunochemical detection methods are usually performed after electrophoretic transfer ("blotting") of the separated polypeptides from the electrophoresis gel onto an immobilizing membrane [41][42]. Today, among the variety of methods used for protein detection after gel electrophoresis, fluorescent methods offer an interesting compromise, especially for detection linearity and for compatibility with mass spectrometry[43]. Stained spots excised from the electrophoresis gel can be identified by mass spectrometry.

**Mass Spectrometry, nanoLC-ESI-MS/MS**

In traditional protein chemistry, proteins were identified by de novo sequencing using automated Edman degradation. Today, this technique is replaced by mass spectrometry, which is becoming one of the most powerful techniques in protein chemistry. The reason for this is the increase in sensitivity (until 1000fold) and the speed of analysis. Today, the Nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC–MS/MS) has become an essential tool in the field of proteomics, due to your enormous analytical advantages when dealing with sample-limited situations. Tandem mass spectrometry has been used as a microscale de novo sequencing tool for peptides because collision-induced dissociation (CID) followed by product ion scanning provides systematic fragment information of amino acid sequences. Further improvement in
peptide sequencing sensitivity was accomplished by the development of nanoelectrospray combined with a peptide sequence tag approach for protein identification in databases. Because even one peptide is sufficient to identify a unique protein, this approach is more powerful for protein identification in proteome-scale experiments than the peptide fingerprinting approach where several peptide masses from one protein are used for identification[44]. For proteomic analysis, proteins are normally first cleaved into peptides by enzymatic digestion (i.e., usually by trypsin) and subsequently, separated by reversed phase (RP) Nano LC before data-dependent MS/MS analysis [52]. The typical microcolumns for nanoLC are prepared using RP materials with a 3-10μm diameter packed into fused silica capillaries with a 12-100μm diameter. In ESI-MS, a spray needle is used as a restrictor for packed particles to prepare a fritless column, to minimize the post-column dead volume [44]. Generally, smaller columns at a lower flowrate combined with real nanoelectrospray conditions give higher sensitivity. For peptides, in combination with C18 stationary phases, acidic conditions are usually used: Trifluoroacetic acid (TFA) is one of the most popular reagents because of higher peak capacity with smaller peak width while Acetonitrile or Methanol have been used as an organic solvent in off-line infusion. Finally, because of the low flowrate, and the small size ranges of proteomic samples (<100μl), trap columns are useful to reduce the injection time. Spots of interest are excised from the gel and treated with tripsin, an enzyme that cleaves C-terminal to arginine (R) and lysine (K). The mixture of protein fragments (peptides) obtained after digestion is purified and subjected to mass analysis.
The aim of the present study was to carry out a non-hypothesis based proteomic investigation, in order to identify potential specific disease-related biomarkers in acute psychotic bipolar disorder (PBD). We performed a comparative proteomic analysis by using two-dimensional electrophoresis (2DE) coupled to mass spectrometry (MS) of lymphocyte protein samples from patients affected by PBD, Major Depression (MDE) and Healthy Control (HC) to identify a potential proteins able to discriminate between the three classes of patients and to suggest associated dysfunctional molecular pathways in the acute psychotic features.
METHODS

Patients

Forty-one subjects (26 patients and 15 healthy) participated in the study. The patients were recruited from the Day Hospital and Inpatient Service of the U.O. Psichiatria II, AOUP. All patients were given an in-house case report form (CRF) for socio-demographic assessment; record of current pharmacological treatment at the time of taking blood and basic medical information. The 26 patients were divided into two classes: 15 patients (4 males and 11 females; mean age 40.5 years ±9.31), were enrolled in the PBD class. The mean age at the onset of illness was 28.1 years (±11.32) and mean illness duration was 12.50 years (±9.6). Eleven patients (2 male and 9 females; mean age 36.5 ± 9.36), were recruited in the MDE class. The mean age at the onset of illness was 27 years (±11.2) and the mean illness duration was 9.53 (±7.14). All patients in the PBD class were admitted to hospital in the acute phase, after a PBD. At the time of blood collection they had been on medication (a combination of mood stabilizers, benzodiazepines and anti-PBDs) for 2 days (±1), with the exception of one patient who was totally unmedicated. All patients belonging to this class showed acute full-blown psychotic episodes in the context of BD. Seven patients had a diagnosis of the most recent manic episode, 5 had a diagnosis of the most recent depressive episode, and 3 had a diagnosis of most recent mixed episode. None of these patients had a family history of psychosis; however, 9 patients out of 15 had a family history of mood disorders. All patients included in the MDE class had a diagnosis of major depressive episode, 6 patients had a second Axis I diagnosis of panic disorder, 5 patients had a family history of mood disorders, but none of these patients had a family history of psychosis. In terms of medication, 6 patients were in mono-therapy with SSRIs, 1 patient was in combined treatment of SSRIs and
benzodiazepines (BDZ), 1 patient in combination of bupropion and gabapentin and 3 patients in combination of SSRIs and mood stabilizers. The healthy subjects (10 female and 5 male; mean age around 38.9 ± 12.1), were recruited from among the hospital staff. The inclusion criteria were age range between 20 and 50 years and fasting at the time of blood collection. All the subjects were admitted to the study after screening for the absence of metabolic diseases (obesity, metabolic syndrome, diabetes, hormonal disorders), current fever or infectious disease, current substance abuse or dependence, pregnancy or refusal to sign informed consent to the study.

**Clinical features**

The patients were assessed from the socio-demographic point of view by using the Structured Clinical Interview for DSM-IV Patient version (SCID-I-P), according to DSM-IV in which are established the criteria to determine the presence or the absence of DSM IV Axis I disorders. Patients were also assessed by means of PANSS [45], YMRS [46], HAM-A and HAM-D-17 [47][48] questionnaires.

**Lymphocyte preparation and protein extraction**

From all subjects, a peripheral venous blood sample of 25 ml was obtained and placed in EDTA tubes. The blood samples were immediately processed to obtain lymphocyte fraction as previously described.25 Briefly, blood was centrifuged for 15 min at 300g to obtain a platelet-rich plasma (PRP) and a pellet containing red cells, platelets, granulocytes, lymphocytes (P1). Platelets were precipitated from PRP by centrifugation at 3000g for 10 min to obtain platelet-free plasma (PFP). P1 was diluted 1 : 1 with Emagel (Behring AG, Marburg, Germany) and centrifuged for 30 min at 550g at room temperature over a density gradient of Lymphoprep (Nycomed, Oslo, Norway). A
lymphocyte ring was thus obtained at the interface between the plasma and the
Lymphoprep. Lymphocytes were harvested and washed in phosphate buffered saline
(PBS) at 300g for 15 min. The resulting pellet was diluted with 1 ml PBS, stratified in
7 ml of PFP and centrifuged at 600g for 15 min. Lymphocytes were washed twice in
PFP to obtain a sample that was completely separated from platelets. The pellets of cells
were resuspended in rehydration solution (7 M Urea, 2 M thiourea, 4% CHAPS, 60 mM
DTT, 0.002% bromophenol blue) in which the Protease Inhibitor Cocktail (5 μl ml⁻¹)
(Sigma-Aldrich; MO, USA) and Halt Phosphatase Inhibitor Cocktail (10 μl ml⁻¹)
(Thermo Fisher Scientific; IL, USA) were added, and incubated for 30 min at room
temperature. After incubation, the samples were centrifuged for 5 min at 16 000g to
remove undissolved material. Protein concentrations were measured using a RC–DC
protein assay from Bio-Rad using bovine serum albumin as the standard. All samples
were stored at -80 °C until analysis.

2DE, staining and image analysis

The 2DE analysis was performed in triplicate. 200 μg of proteins, for each sample,
were filled up to 450 μl in rehydration solution (supplemented with 1.2% (v/v)IPG-
buffer, pH 3–10 NL). Iselectrofocusing (IEF) was carried out by using 18 cm
Immobiline Dry-Strips (GE Health Care, WI, USA) with a non-linear, pH 3–10 gradient
(IPG strips). IEF was performed on an EttanIPGphor II apparatus (GE Health Care) at
16 °C and the proteins were focused for up to 70000 V h. The second dimension (SDS-
PAGE) was carried out by transferring the proteins into 12.5% polyacrylamide gel,
running at 16 mA per gel and 10°C for about 16 h, using the PROTEAN-II Multi Cell
system (Bio-Rad, CA, USA) [49]. At the end of the 2DE, the gels were stained with
Ruthenium II tris (bathophenanthrolinedisulfonate) tetrasodium salt (SunaTech Inc.;
Suzhou, P. R. China)(RuBP) [50]. The images were acquired upon fluorescence
imaging using ‘‘Image Quant LAS4010’’(GE-Healthcare). The analysis of images was performed using Progenesis Same Spot (v4.1, Nonlinear Dynamics; Newcastle Upon Tyne, UK)[49][50].

**NanoLC-ESI-MS/MS Analysis and protein identification.**

Spots of interest were cut out from gel reference and the nanoLCESI-MS/MS analysis by LTQ-OrbitrapVelos was performed. Peak lists were generated from raworbitrap data using the embedded software from the instrument vendor (extract_MSN.exe). The monoisotopic masses of the selected precursor ions were corrected using an in-house written Perl script [51]. The peak list files were searched against the UniProtKB/Swiss-Prot database (Release-2011_08 of 21-Sep-2011)using Mascot (Matrix Sciences, London, UK). Human taxonomy(20 323 sequences) was specified for database searching. The parention tolerance was set to 10 ppm. Variable amino acid modifications were oxidized methionine and fixed amino acid modifications were carbamidomethylcysteins. Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. The mascot search was validated using Scaffold 4.0 (Proteome Software, Portland, OR). Only proteins matching with two different peptides with a minimum probability score of 95% were considered identified.

**Western Blot analysis**

All samples were processed using WB analysis to validate different protein expressions found by 2DE analysis. 25 µg of proteins were resolved by 12% SDS-PAGE gels and transferred onto nitrocellulose membranes (0.2 μm) using a voltage of 25 V for 7 min (Trans-Blots Turbot Transfer System; Bio-Rad). After electrophoresis, we progress the standardization as follows: the membranes were fixed in 7% acetic acid (v/v) and 10%
methanol(v/v) for 15 min, subsequently rinsed in water, and stained with 1 mM RuBP in 1% phosphoric acid and 30% ethanol for 15 min. After this the membranes were rinsed in water prior to the acquisition of the total proteins by “ImageQuant LAS4010” (GE HealthCare). After acquisition, the membranes were blocked and then incubated with appropriately diluted primary antibodies. The primary antibodies used were anti-LASP1 (rabbit polyclonal, 1 : 1000 dilution; Novus Biological, CO, USA), anti-SCAD (rabbit polyclonal, 1 : 300 dilution; Novus Biological, CO, USA), anti-STIP1anti-Hop (D6E3) (rabbit monoclonal, 1 : 1000 dilution, Cell Signaling Technology Inc., MA, USA) and anti-TPD52L2 (goat polyclonal, 0.2 mg ml_1 dilution; Thermo Fisher Scientific, IL, USA). HRP-conjugated goat anti-rabbit(1 : 20 000 dilution; Stressgen, NY, USA) and HRP-conjugated donkey anti-goat (1 : 5000 dilution; Santa Cruz Biotechnology Inc., CA, USA) were used as secondary antibodies. The chemiluminescent images were acquired by LAS4010 (GE Health Care). For the comparison of protein expression levels among the three classes (PBD, MDE and HC), the antigen specific bands and the total proteins were quantified using the Image Quant-TL (GE HealthCare). In order to normalize the volume of proteins of interest, the ratio of optical density of the antigen-specific bands with those of total proteins was calculated. The results were express as a ratio of optical density.

**Statistical Analysis**

In 2DE analysis, the comparison between PBD, MDE and HC was performed. The significance of the differences of normalized volume for each spot was calculated by the software Progenesis Same Spot including the analysis of variance (ANOVA) test. The protein spots with a Z1.5-fold spot quantity change and p<0.05 were cut out from the gel and identified using (nanoLC-ESI-MS/MS) analysis. Data obtained from the WB
analysis were subjected to statistical analysis with unpaired Student’s t-test making a comparison of protein expression levels between the three classes.

**Signalling pathway analysis**

Functional pathway and network analyses were generated using the IPA software v7.1. Proteins that met the expression ratio with a cut-off of $\geq 1.5$, and a p value cut-off of $<0.05$ for differential expression, and those that were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base, were considered for the analysis. Swiss-Prot accession numbers were inserted into the software along with the corresponding comparison ratios between the groups. The network proteins associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. These networks were scored for the degree of relevance, with values $>3$ having a 99.9% confidence level of not being generated by random chance alone. The genetic networks that were created describe the functional relationships between gene products based on known associations in the literature.
RESULTS

Clinical assessment

The demographic characteristics of all the subjects enrolled in the study are shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>PBD (n=15)</th>
<th>MDE (n=11)</th>
<th>HC (n=15)</th>
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<tr>
<td>Gender (male/female)</td>
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<td>2/9</td>
<td>10/5</td>
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<tr>
<td>Age (years) mean (±SD)</td>
<td>40.5 (±9.31)</td>
<td>36.5 (±9.36)</td>
<td>38.9 (±12.1)</td>
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<tr>
<td>Age at onset mean (±SD)</td>
<td>28.1 (±11.32)</td>
<td>27 (±11.2)</td>
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<tr>
<td>Illness duration mean (±SD)</td>
<td>12.5 (±9.6)</td>
<td>9.53 (±7.14)</td>
<td>-</td>
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<tr>
<td>Smokers</td>
<td>6/15</td>
<td>3/11</td>
<td>2/15</td>
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Table 1. Demographic details of enrolled subjects

In Table 2 are reported the clinical assessment data for each class. The two classes differ significantly in Positive and Negative Symptoms Scale (PANSS) total score (p < 0.001), positive symptoms subscale (p < 0.001), negative symptoms subscale (p = 0.005), and psychopathology subscale (p < 0.001). No statistical difference was observed for Hamilton Depression Rating Scale (HAM-D-17) and Hamilton Anxiety Rating Scale (HAM-A) mean scores, while Young Mania Rating Scale (YMRS) mean total score was statistically different (p = 0.001) between the two classes.

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<th>p-value</th>
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<td>PANSS_P</td>
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<td>PANSS_G</td>
<td>44.8 (±15.6)</td>
<td>25.5 (±4.5)</td>
<td>&lt;0.001</td>
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<tr>
<td>HAM-D</td>
<td>15.2 (±16.7)</td>
<td>10.9 (±5.5)</td>
<td>NS</td>
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<tr>
<td>HAM-A</td>
<td>12.2 (±6.3)</td>
<td>11.6 (±3.5)</td>
<td>NS</td>
</tr>
<tr>
<td>YMRS</td>
<td>23.3 (±13.1)</td>
<td>1.9 (±3.2)</td>
<td>=0.001</td>
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Table 2. Clinical assessment of patients
Proteomic analysis

A comparative proteomic analysis was performed on lymphocyte samples from patients with PBD, major depressive episode (MDE) and healthy control (HC) subjects by using 2DE coupled to nano-liquid chromatography electro spray ionization tandem MS (nanoLC-ESI-MS/MS). Fig. 1 shows a representative gel image of lymphocytes from PBD patients.

**Figure 1.** Representative 2DE gel map of PBD lymphocyte protein extracts. A total of 200 mg of proteins was separated by 2DE using an 18 cm pH 3–10 NL strip and 12.5% SDS-PAGE. Proteins were detected by RuBP staining. The maps were analyzed by Progenesis Same Spot (Nonlinear Dynamics) software. Spots numbers indicate all the proteins identified by MS/MS and refer to the number reported in Table 3.
By computational 2DE gel image comparison, 36 protein spots, which collapsed into 25
different proteins, were found to be differentially expressed. These spots exhibited
≥1.5-fold change of the mean value of normalized spot intensities in the lymphocyte
preparations of PBD and MDE patients with respect to HC subjects. The list of
identified proteins is given in Table 3: here, MS (matched peptides, coverage and best
ion score) and 2DE (ratio, p-values) are also reported. Fig. 2 shows the histogram of the
normalized optical density volumes (mean SEM) of the proteins found indifferent
quantities in the comparison among the three classes.
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<th>#</th>
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<th>Gene Name</th>
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<th>MW a</th>
<th>Match. pep.</th>
<th>Cov. (%)</th>
<th>Best Ion Score</th>
<th>BDP vs Ctr</th>
<th>MDE vs Ctr</th>
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Table 3. MS/MS data of protein spots differentially expressed among PBD, MDE and HC samples.
Figure 2. Comparison of differentially expressed proteins. Histograms of the normalized optical density volumes (mean ± SEM) of the 25 protein spots found in significantly different quantities from the comparison between PBD and HC (*p<0.05, **p<0.01, ***p<0.001) and MDE and HC (#p<0.05, ##p< 0.01, ###p<0.001). Significant differences from HC is based on unpaired Student t test.
Validation of proteins in PBD, MDE and HC lymphocyte preparations by WB analysis

Immunoblot analysis was performed to confirm the ability of short-chain specific acyl-CoA dehydrogenase mitochondrial (SCAD), LIM and SH3 domain protein1 (LASP1), tumor protein D54 (TPD52L2) and stress-induced protein 1 (STIP1) to discriminate PBD from MDE as suggested by 2DE observations (Fig. 3A). After statistical analysis, all these proteins resulted in being differentially expressed in a significant manner in PBD when compared to HC samples, with increased values of 2.2-, 14-, 16- and 2.7-fold for SCAD, LASP1, TPD52L2 and STIP1 respectively. Moreover, the efficacy of LASP1 and SCAD to discriminate PBD from MDE was confirmed. Representative western blots are shown in Fig. 3B.
Figure 3. WB validation of selected differentially expressed proteins. SCAD, LASP1, TPD52L2 and STIP1 differential expressions by immunoblot analysis.

Conventional SDS gels were run with lymphocytes protein extracts from all subjects using 12% resolving capacity. Twenty-five micrograms of total proteins were loaded into each lane, proteins were transferred onto nitrocellulose membranes and incubated with specific antibodies against the target proteins. Panel A: representative 2DE enlarged images of validated proteins. Panel B: the western blot is representative of one sample appertaining to PBD, HC and MDE class, respectively. The bar graph shows the mean ± SEM of the optical density values. The staining by RuBP was used as a protein loading control. Statistically significant differences were determined by the unpaired Student t test and the p values are indicated. (PBD vs. HC: *p<0.05, **p<0.01; MDE vs. HC: #p<0.05; PBD vs. MDE: $p<0.05).
Network construction for biological processes

The assignment of biological processes and the subsequent construction of networks were done using the IPA software v7.1 (Ingenuity® System Inc., Redwood City, CA, USA. www.ingenuity.com). Twenty-five proteins that showed significant change in the expression between psychiatric disorders (PBD and MDE) and HC lymphocytes samples were included in the analysis. Each identified protein was converted to its gene and mapped onto its corresponding gene object in the IPA knowledge base. A network with a score value of 38 was generated and the director indirect relationships exhibited by the proteins among each other within the network are shown in Fig. 4.

The network shows 35 proteins that work together in neurological diseases, psychological disorders and the amino acid metabolism network and 15 of 25 proteins were included in the network. We utilized IPA to retrieve the known functions of each protein. The major categories of network-associated functions included cellular movement, cell-to-cell signaling and interactions inflammatory response and molecular transport. All in all, 16 of our proteins were found to be involved in neurological disease (p-value $2.9 \times 10^{-10}$ to $3.1 \times 10^{-2}$), 14 in inflammatory disease (p-value $7.7 \times 10^{-8}$ to $2.6 \times 10^{-2}$) and 16 in psychological disorders (p-value $2.9 \times 10^{-10}$ to $1.1 \times 10^{-2}$). The collocation of our proteins in the context of the canonical pathways involved in acute phase response signaling, LXR-RXR activation, glycolysis, 14-3-3-mediated signaling, NRF2-mediated oxidative stress-response and IL-8 signaling (Fig. 4) is noteworthy.
Figure 4. IPA network analysis of differentially expressed proteins. Twenty-five proteins that showed significant change in expression between psychiatric disorders (PBD and MDE) and HC lymphocytes samples were included in the analysis. The network shows 35 proteins that work together for Neurological diseases, Psychological disorders and Amino acid metabolism network. Solid lines correspond to direct protein-to-protein interactions/regulations, dashed lines correspond to indirect interactions/regulations. Proteins involved in acute phase response signaling, LXR-RXR activation, glycolisis, 14-3-3 mediated signaling, NRF2-mediated oxidative stress-response and IL-8 signaling canonical pathways are indicated.
DISCUSSION

The proteomic approach leads to the suggestion of molecular distinguishing markers for many diseases, which are lacking for psychiatric disorders, despite the large incidence of psychiatric disorders such as BD, schizophrenia and major depression. The most difficult tasks remain the multifactorial characteristics of psychiatric disorders, the overlapping of symptoms among all, and drug abuse. Nevertheless, information derived from a protein fingerprint could provide new insights into the molecular interaction involved in the disease progression and then improve then characterization of the disease.

In this study, we performed a comparative proteomic analysis of lymphocyte protein samples from patients affected by PBD, MDE and HC. This approach should identify potential proteins able to discriminate between the two classes of patients and to suggest associated dysfunctional molecular pathways in the acute psychotic features. The patients enrolled for the study underwent accurate characterization, and the sample collections followed standard procedures to improve the reliability of the results. Lymphocytes were chosen since different studies demonstrated that alterations in peripheral blood cell neurotransmitter signaling pathways reflected the changes observed in CNS, together with much evidence of altered immune functions in these diseases[21][22]. By using high performance 2DE coupled with nanoLC-ESI-MS/MS analysis we identified 25 proteins in PBD lymphocytes that were differentially expressed with respect to HC. Twenty-one of these proteins were also differentially expressed in MDE lymphocytes when compared with controls converging in a common signature for these disorders. However, of the remaining up regulated proteins (LASP1, STIP1, SCAD and TPD52L2) only SCAD and LASP1 were confirmed as discriminatory proteins between PBD and MDE after WB validation. The acyl CoA dehydrogenases (ACAD) are a family of enzymes that catalyze the alpha and beta dehydrogenation of
the acyl-CoA esters, transferring electrons to electron transferring flavoprotein (ETF). In humans, very long, medium and short chain ACAD (VLAD, MCAD, and SCAD, respectively) catalyze the first step in the beta-oxidation cycle with substrate optima of 16, 8 and 4 carbon chains, respectively. SCAD acts within mitochondria, the energy-producing centers within cells, and it is essential for fatty acid oxidation, which is the multistep process that metabolizes fats and converts them into energy. Our results suggested a significant increase of SCAD in PBD lymphocyte preparations with respect to MDE and HC. An alteration of expression of this protein has been also recently reported by Kazuno and co-workers[25] in whole cell lysate derived from lymphoblastoid cells of monozygotic twins discordant for BD, even if the observed decrease of protein expression in the patient was found to be not significant. Therefore, we can hypothesize that the increase of SCAD in PBD lymphocytes may be seen as a compensatory effect to maintain a constant velocity of acetyl CoA production in the cell. As far as LASP1 is concerned, it is important to note that the up regulation observed in the patients was particularly high in the PBD. LASP1 is a multi domain protein that may recruit signaling molecules to the actin based cytoskeleton and an association with susceptibility to schizophrenia has been suggested[53]. In this context the observed differential expression of cytoskeletal proteins, i.e. tubulin beta chain, actin, vinculin, vimentin could also be explained. On the other hand, as regards cytoskeletal proteins appertain to serum but we found their significant differential expression in PBD and MDE lymphocyte preparations when compared with HC. At the same time, as suggested by Herbert et al.[23] in PBMC and by Politi and co-workers[54] in endothelial cells, different circulating hormonal or inflammatory factors or serum analytes may interact in different manners with these cells to determine detrimental effects or sensibilization phenomena, which might be responsible for internalization of the molecules. Nevertheless, these proteins were unable to
discriminate PBD and MDE inflammatory profiles showing very similar values of increase and decrease with respect to HC. It is noteworthy that the IL-8 specific pathway seems different in PBD in light of the direct interaction with LASP1 that in turn modulates cytoskeletal proteins. Our results agree with those reported by Herberth et al.[23] that showed the differential expression of cytoskeletal and stress response proteins in peripheral blood mononuclear cells (PBMC) together with serum-associated proteins linked to inflammatory response, as signatures of BD. To examine if the 25 identified proteins found were related to each other and constituted a global molecular network and pathway, we applied IPA analysis to our data. The results showed that the differentially expressed proteins work together for neurological diseases, psychological disorders and amino acid metabolism network, and the principal network-associated functions were cellular movement, cell to cell signaling and interactions, and inflammatory response. Worthy of attention was the collocation of our proteins in the context of the canonical pathways that focused on the involvement of acute phase response signaling, LXR-RXR activation, glycolysis, 14-3-3 mediated signaling, NRF2-mediated oxidative stress-response and IL-8 signaling. In our network the inflammatory response is associated with proteins such as ApoA1, HP, TTR, TF, and FGB, which correlate directly or indirectly with pro-inflammatory cytokine and different protein kinases. Intriguingly, all the above named proteins appertain to serum but we found their significant differential expression in PBD and MDE lymphocyte preparations when compared with HC. At the same time, as suggested by Herbert et al.[23] in PBMC and by Politi and co-workers[54] in endothelial cells, different circulating hormonal or inflammatory factors or serum analytes may interact in different manners with these cells to determine detrimental effects or sensibilization phenomena, which might be responsible for internalization of the molecules. Nevertheless, these proteins were unable to discriminate PBD and MDE inflammatory profiles showing very similar
values of increase and decrease with respect to HC. It is noteworthy that the IL-8 specific pathway seems different in PBD in light of the direct interaction with LASP1 that in turn modulates cytoskeletal proteins. If this way might be preferential in PBD with respect to other ones, the relation between STIP1 and the peptide hormones LH and FSH, whose increase has been observed and correlated with side effects such as depression, triggering mania and aggression in treated BD subjects, needs to be investigated.

CONCLUSIONS

The novelty of this work is the analysis of patients with psychosis in the context of BD and in particular all patients belonging to this class showed an acute full-blown psychotic episode (manic or depressive episode) in the context of BD. Most of the proteome studies reported in the literature have been carried out on samples from patients with schizophrenia, while mood disorders samples have only been used in a validation context for schizophrenia. Our choice has limited the number of patients included in the study and surely a larger number of patient samples, ideally collected in different hospital settings, would increase the confidence of our findings. Nevertheless our results confirm the reliability of lymphocytes as a peripheral model to study psychiatric disorders, and to prove the difficulty in defining specific biomarkers due to the significant overlap of disease clinical profiles. However, we believe that the increase of SCAD found in PBD lymphocytes might provide new insights into the molecular pathways involved in acute full-blown PBD symptoms.
REFERENCES


