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The Discovery of Clinically Applicable Biomarkers for Bipolar Disorder: A Review of Candidate and Proteomic Approaches

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Bipolar disorder (BD) is a severe psychiatric condition which affects innumerable people across the globe. The etiopathogenesis of BD is multi-faceted with genetic, environmental and psychosocial factors playing a role. Hitherto, the diagnosis and management of BD are purely on empirical grounds as we lack confirmed biomarkers for this condition. In this regard, hypothesis-driven investigations have been unable to identify clinically applicable biomarkers, steering the field towards newer technologies. Innovative, state-of-the-art techniques like multiplex immunoassays and mass spectrometry can potentially investigate the entire proteome. By detecting up or down regulated proteins, novel biomarkers are identified and new postulates about the etiopathogenesis of BD are specified. Hence, biological pathways are uncovered which are involved in the initiation and advancement of the disease and new therapeutic targets are identified. In this manuscript, the extant literature is thoroughly reviewed and the latest findings on candidate BD biomarkers are provided, followed by an overview of the proteomic approaches. It was found that due to the heterogeneous nature of BD no single biomarker is feasible, instead a panel of tests is more likely to be useful. With the application of latest technologies, it is expected that validated biomarkers will be discovered which will be useful as diagnostic tools and help in the delivery of individually tailored therapies to the patients.

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INTRODUCTION

Bipolar disorder (BD), an enduring, recurring, and impairing mood disorder, typically has its onset in early life, for example in teenage years. The characteristic course is marked by repeated affective exacerbations which could be manic/hypomanic and depressive in nature.[1] It is marked by persistence throughout the lifespan, among patients the presentations vary markedly and the disorder has myriad clinical forms.[2] Due to its masquerading nature, at initial presentation, the actual diagnosis is often missed and several years may elapse before the disorder is correctly identified.

BD is a spectrum disorder and classical presentation with recurrent manic and depressive episodes is seen in only 25% of the cases.[3] Commonly the patients have vague manifestations like mixed symptoms, anxiety spectrum disorders, psychotic features, subthreshold symptoms and rapid cycling.[4] In the youth, the clinical picture is further obscured by such externalizing disorders as attention deficit hyperactivity disorder, conduct disorder, and drug misuse.[5] BD is highly disabling, initially affecting young people, and causing severe impairments in the biopsychosocial realms of functioning.[6] In spite of these caveats, there are, as yet, no reliable biomarkers, so that the field immediately requires validated tests that are helpful in the identification and management of bipolar patients.[7]

In the literature a biomarker is defined as an exemplar which can be accurately quantified, and appraised as a gauge of the usual homeostatic responses, disease states, and medication response following a treatment intervention.[8] With regards to BD, biomarkers can have multiple potential applications.[9] Firstly, they can improve the diagnostic accuracy of the clinical process and 'state-specific'

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markers can differentiate unipolar from bipolar depression. Secondly, they can be used for the purpose of staging the disease trajectory and informing about the progression and outcome of the disorder. Thirdly, biomarkers may have a role for primary prevention in vulnerable individuals, with the advantage of potential 'trait' markers in identifying at-risk individuals. Fourthly, the biomarkers may also be valuable for timely identification of illness relapse, conceivably even before clinical symptoms have started, aiding in secondary prevention. Finally, the discovery of biomarkers carries a promise in terms of guiding further novel therapies to act on potential targets.[10]

BD is a highly complex disorder and research to date has identified probable factors which have etiologic and pathogenic implications.[11] Current knowledge assumes that vulnerability in certain genes, for example those involved in calcium signaling, predisposes a person for the development of the diathesis. Environmental conditions of a stressful nature cause the phenotypic expression of the disorder.[12] Once the disease sets in, ongoing damage occurs in critical mood regulating brain areas comprising the frontal-cortical-limbic circuits. Affective episodes act as allostatic states and working through such mediators as pro-inflammatory cytokines (PIC) and oxidative free radicals, damage neurons and glia. Coupled to this, decline in neurotrophic support and decreasing levels of brain derived neurotrophic factor (BDNF) causes apoptosis of neurons.[13] Additionally, abnormalities of the hypothalamo-pituitary-adrenal axis (HPA axis) are also present and contribute to the progression of the diathesis and play a role in its systemic manifestations. There is evidence for autonomic imbalance with an increase in sympathetic tone and withdrawal of parasympathetic neurotransmission.[14] At the sub-cellular level, abnormal functioning of the mitochondria and endoplasmic reticulum stress appear to play an increasingly important role in the pathogenic process.[15] Neuroinflammation is thought to have a mechanistic role and diversion of the tryptophan metabolism from serotonin/melatonin synthesis to neurotoxic kynurenine metabolites leads to neuronal damage.[16]

In BD there are multifarious interactions among biological, psychological, and social modules. Additionally, the animal models of the disease lack accuracy, such that investigations into the molecular and cellular basis of the diathesis are greatly hampered. While research has been directed at hypothesis driven and genomic studies, clinically applicable biomarkers have not been found, and resultantly this remains an unmet need.[17] Concurrently, recent advances in technology with proteomic analysis have enabled researchers to utilize hypothesis free paradigms in examining patients' biological specimens like peripheral blood, plasma and serum. Examination of the proteome at a given point in time provides valuable information about pathologically increased or decreased levels of circulating proteins, while furnishing clues about the etiopathogenesis of the disorder. The generation of a wealth of proteomic data has the promise of discovering dependable and confirmed biomarkers for the most serious psychiatric conditions which also embraces BD.[18]

Taking advantage of the extant literature, in the discussion that follows, an appraisal of approaches regarding the discovery of candidate biomarkers is provided. Subsequently, a summary of the current progress in the technology of protein analysis is given to develop an understanding of current methodologies used in the detection of biosignatures of human ailments, including BD. The objective behind this endeavor is to deliver a wide-ranging appraisal of the state of knowledge with respect to biomarkers for bipolar disorder.

CANDIDATE BIOMARKERS

1. Neuroimaging

In the context of bipolar disorder, there is an increasing amount of literature on anatomical and related functioning aspects of brain regions through the use neuroimaging techniques. Deviations have been consistently described in the cortico-limbic areas of the brain, which for instance, decrease in the gray matter in the left rostral anterior cingulate cortex and right fronto-insular cortex in patients with multiple episodes. These limbic areas, located anteriorly, are involved in decision-making and the processing of emotions, capabilities which are immensely affected in bipolar disorder.[19] Furthermore, with repeated episodes, morphometric studies show an increase in the size of lateral and third ventricles, and relentless reduction in hippocampal, fusiform, and cerebellar gray matter volume. There is also a sub-region specified decrease in gray matter density in the prefrontal cortex and more frequently occurring deep white matter hyperintensities. By and large, these findings support the premise of neuroprogression in BD, with incremental damage to brain structures occurring as the disorder advances.[20]

In this vein, studies utilizing functional MRI show that, in BD, brain areas involved in emotional regulation are activated which is generally supportive of the cortico-limbic hypothesis. However, the findings indicate that associations among the brain limbic areas are conceivably very intricate, such that sub-region specific abnormalities also need to be investigated.[21]

2. Genetics

Studies investigating genetic aberrations in BD indicate that several chromosomal regions and individual genes influence liability to disease development and these anomalous genes each have a small effect size, making BD a polygenic disorder. Taking twin studies into consideration, transmissibility of BD is likely to be >60%, one of the highest among psychiatric disorders.[22]

It is postulated that deregulation of gene function instigated by alterations, polymorphisms, and epigenetic modifications influence particular cellular signaling cascades, contributing towards disease onset and progression. The genetic vulnerability in BD is expressed as a pheno-

type, influenced by epigenetic changes and stressful life circumstances. Genome-wide association studies (GWAS) now have progressively larger sample sizes, such that more single nucleotide polymorphisms are detected, and this is helpful in elucidating the genetic architecture of bipolar disorder.[23]

A well cited meta-analysis showed that amongst the 226 genes with noteworthy influence in BD, nine varied in manifestation in the patients' dorsolateral prefrontal cortex: CACNA1C (encoding calcium channel, voltage-dependent, L type, α1C subunit), DTNA (encoding dystrobrevin α), FOXP1 (encoding forkhead box protein P1), GNG2 (encoding guanine nucleotide-binding protein Gi/Gs/Go subunit γ-2), ITPR2 (encoding inositol 1,4,5-trisphosphate receptor, type 2), LSAMP (encoding limbic system-associated membrane protein), NPAS3 (encoding transcription factor neuronal PAS domain protein 3), NCOA2 (encoding nuclear receptor coactivator 2), and NTRK3 (encoding tropomyosin receptor kinase C).[24] Initial scrutiny of the entire genome/transcriptome/proteome sequence points to the involvement of calcium, CREB, and potassium channels signaling.[25] A review of meta-analytic studies established a major effect for 46 genes (65 variants), while the most robust gene effects were described for 21 altered genes. The associated pathways comprised cell stimulation and multiplying, signal generation and propagation, cell death, chromatin remodeling, chemokine signaling, glutamate neurotransmission, immune reaction, cell adhesion and relocation, neurotransmitter metabolism, and cancer suppression.[26]

3. Brain derived neurotrophic factor (BDNF)

Neurotrophic factors, including BDNF, are specific entities which support the development of fresh neuronal connections and growth of new neurons. These lead to enhanced survival, diversity, and upkeep of healthy and regenerating brain cells. Abnormalities in growth factors and associated anomalies in neuroplasticity and the cellular ability to withstand various types of insults has been documented in BD.[27] BDNF is greatly concentrated in brain regions concerned with the control of perception, reasoning, and emotional reactions. BDNF localized in the brain crosses intact the blood–brain barrier (BBB) by a high-capacity saturable transport system, and peripheral circulating BDNF values are considered to be reflective of brain-tissue BDNF levels. It has a crucial part in the development of stress-linked psychiatric conditions; for example, acute stress caused by partial sleep denial prompted an upsurge in BDNF levels, while persistent stress resulting in sleep disruption and depression brought about a reduction in BDNF concentrations.[28] The part that BDNF plays in the pathophysiology of BD is further reinforced by the elevation of serum and brain BDNF concentrations by psychotropic medications bringing about symptomatic improvement in the patients.[29]

A meta-analysis comprising of 52 studies with 6481 BD patients matched to controls showed that circulating BDNF levels decreased mutually in manic as well as depressive episodes and were akin to healthy volunteers during periods of remission. Further, the severity of affective symptoms correlated negatively with BDNF levels.[30] Serum BDNF distinguished bipolar from unipolar depression, served as a marker of illness advancement and severity, discriminated early and advanced BD episodes, and reflected outcomes to treatment.[31]

A freshly published study examined serum BDNF levels in patients with BD as compared to age-, sex- and body mass index (BMI)-matched HC during a delineated twelve-year period. The serum BDNF level was lower in 83 patients with BD as compared to 222 HC samples (5.7±4.2 ng/mL vs. 12.2±7.5 ng/mL, F=46.784). The receiver operating characteristic curve (ROC) analysis indicated that BDNF concentrations showed a reasonable correctness in discriminating cases from controls (AUC=0.801); in this regard, an acceptable cut-off point was 6.74 ng/mL (sensitivity=82.0%, specificity=63.9%). The results were supportive of the fact that serum BDNF levels had practical value in distinguishing BD patients from healthy controls, but further studies with larger samples were needed to substantiate these findings.[32]

4. Inflammatory biomarkers

The higher frequency of medical comorbidities is well documented in BD, and with repeated episodes there is relentless decline in psychosocial faculties. While the neurobiological substrate of these findings is still debated, current line of evidence indicates that abnormalities in the inflammatory/immune system are implicated in the pathophysiologic process. In this regard, the important data can be summarized as follows. i) greater occurrences of autoimmune diseases; (ii) distinctive profiling of the immune cells; (iii) abnormal patterns of cytokine secretion by activated mononuclear cells; (iv) raised concentrations of peripheral circulating indicators of immune response, and (v) inflammation related alterations in the central nervous system.[9]

In order to investigate the issue of autoimmunity in BD, researchers studied the prevalence of autoimmune thyroiditis (AIT, Hashimoto's thyroiditis) with the highly specific anti-thyroid peroxidase antibodies. While the findings of individual studies were sometimes contradictory, a recently published systematic review concluded that an independent association existed between BD and AIT regardless of the confounding use of lithium, that autoimmune thyroiditis served as an independent risk factor and might be an endophenotype.[33] The relationship between BD and autoimmune diseases might be explained, in part, by the fact that the numbers of regulatory T lymphocytes (CD4+ CD25+ Foxp3+) were diminished in BD patients. These cells were connected to suppressing the propagation of effector T cells and checking of flawed immune responses, together with preventing the development of autoimmune diseases.[34]

Numerous studies, systematic reviews and meta-analyses have reported immune imbalance in BD. A recently published systematic review included original reports on

circulating immune markers, and had an analysis of genetic, post-mortem and cell studies with inflammatory biomarker examinations. It concluded that, in comparison to healthy controls, PIC were higher and anti-inflammatory cytokines decreased, mainly during manic and depressive phases. These aberrations tended to subside in euthymia, signifying that an inflammatory state existed during the acute phases of BD.[35] Another study utilized monocytes derived from initial versus advanced stages of BD. Macrophage polarization was induced and in vitro secretion of a number of cytokines including IL-1β, IL-6, IL-10, and TNF-α was measured. Patients in the advanced stage secreted lesser amounts of cytokines in comparison to early stage patients, while cytokine production was similar in early stage patients and HC. Although this investigation was restricted by a small number of participants (n=18), results suggested an increasing anomaly in the reaction of peripheral innate immune cells in late stage BD patients. The identified flaw in the control of immune system functioning might be connected to the involvement of so many bodily systems with the advancement of the diathesis in BD.[36] A meta-analytic review investigated the likelihood of determining a biomarker signature of illness phase in BD. In the analysis, 53 studies comprising 2467 cases and 2360 controls were included and it showed that in BD hs-CRP, IL-6, BDNF, TNF-α, and soluble TNF-α receptor 1 could discriminate a distinctive affective exacerbation. Individually, no biomarker distinguished mood phase in BD, but relating biomarker findings could discriminate patients with bipolar disorder from healthy controls, and taken together specified a precise marker of mood-phase.[37]

Over the past few decades, evidence has been accumulating regarding a neuroinflammatory state in BD, supported by data from peripheral, CSF and postmortem studies indicating an increased circulating level of PIC and microglia activation in the brain. Currently researchers have been studying brain gray matter changes as determined by sMRI morphometric studies and correlating this with circulating levels of PIC. An interesting study directly compared BD and partially remitted unipolar depressed patients and investigated gray matter changes along with circulating levels of PIC. Many areas of the brain including the orbitofrontal cortex, lingual gyrus, inferior frontal cortex, middle frontal cortex, and planum polare were statistically reduced in size in BD patients as compared to those with unipolar depression. Interestingly, the gray matter thickness alterations between BD and MDD were inversely related with soluble IL-6 receptor levels.[38] Another study by the same group of investigators included a larger sample size of BD and MDD subjects and included inflammatory and metabolic indices while comparing gray matter volumes. BD subjects as compared to MDD cases had greater loss of gray matter volume in several limbic areas and this negatively associated with circulating levels of IL-6 and sTNF-R1. The presented data showed that bipolar patients had greater BMI and higher pro-inflammatory cytokine levels in comparison to UD patients and this might have contributed to more severe gray matter loss in BD. Importantly, the results supported the neuro-inflammatory hypothesis of mood disorders.[39]

5. Oxidative stress markers

There is a burgeoning amount of evidence which indicates that there is increased oxidative stress in BD. There is decreased bodily concentration of antioxidants such as vitamin E, tryptophan, zinc, and coenzyme Q10, accompanied with diminished activity of antioxidant enzymes. Additionally, increases in oxidative and nitrosative stress causes lipid peroxidation, oxidation and nitration of proteins/DNA, injury to mitochondria and secondary eliciting of autoimmune responses, all of which are evident in BD.[40] Products of lipid oxidation (serum lipid hydroperoxides) may be valuable as clinically applicable peripheral biomarkers of BD. Meta-analysis of studies conducted on oxidative stress markers in BD established that lipid peroxidation, DNA/RNA damage, and nitric oxide were statistically significantly raised in patients compared to controls. Nonetheless, no noteworthy effect sizes were detected for antioxidant enzymes and products of oxidative protein damage.[41]

The relationship between augmented oxidative stress and decreased BDNF levels was recognized in BD. Serum thiobarbituric acid reactive substances (TBARS) which were produced as derivatives of lipid peroxidation could be utilized as biomarkers of disease advancement, and in manic patients compared to heathy volunteers, these were negatively correlated with serum BDNF levels.[42] In summary, the data was indicative that oxidative stress likely contributed to such phenomena as accelerated aging, metabolic abnormalities, and neuroprogression in the bipolar diathesis and had utility as biomarkers for different phases of the illness.[43]

PROTEOMIC BIOMARKERS

1. The description of proteomics

The proteome is the complete array of proteins manufactured by a living biological system changing with phase, organism's needs, level of stress, and varied external and internal influences. Proteomics denote an all-embracing, extensive examination of proteins in an organism at a specific interval and in a particular state. It complements genomic and transcriptomics, the other "omics" technologies, to explicate the distinctive protein profile of an organism, and it also elucidates the composition and biological properties of an identified protein.[44] It better delineates active pathophysiological processes as it aims to capture an overall view, providing a unified understanding of cellular functioning by examining the complete array of proteins, instead of studying each one individually. In medicine, laboratory examination of blood is the commonest investigative technique, and peripheral markers are helpful in diagnosis, while also supporting therapeutic measures. Recently, high-throughput proteomic technologies have hosted a

fresh period of biomarker discovery in medical sciences. Previous approaches utilized small groups of participants to determine lone biomarker candidates, with subsequent validation procedures through conventional immunoassays in larger cohorts. In the process, which employs plasma proteome analysis, the protein signatures of large cohorts are linked to their phenotypes during wellbeing and illness.[45] With regards to BD a complex, multifactorial disorder, proteomic examination, by emphasizing the here and now, has the potential to increase diagnostic accuracy while also facilitating discrimination between closely related disease phenotypes. Hypothesis-free paradigms used in the investigative process permit the generation of predictive representations regardless of a supposed etiology. Proteomics hold the potential for envisaging the onset of the disorder, while also predicting the disease trajectory and outcome.[46]

2. Proteomic techniques: a brief overview

In biological samples, protein detection is comprised of two main techniques, namely antibody centered approaches (immunoassays) or mass spectrometry (MS). The former is exemplified by enzyme-linked immunosorbent assay (ELISA) and Western blot, techniques applied for years as authentication means for identifying and measuring proteins of interest. More recently, with the application of multiplex immunoassay panels greater output having been achieved, concurrently hundreds of proteins can be detected and quantified.[47] In spite of advances in immunoassay procedures, there are in-built restrictions with multiplexing techniques, poor detectability of proteins with structural similarity, and incongruity with hypothesis-free lines of inquiry. In contrast, more recently, with technological advancements MS-based methods have taken precedence, and now these can characterize human plasma proteomes with unparalleled precision.[48]

Mass spectrometry based proteomics has many methods of protein separation, visualization and analysis. Initially, for relative measurement of proteins, gel-based two-dimensional electrophoretic methods were employed. However, these techniques had several limitations, such that nowadays, the most common method for hypothesis-free and large-scale protein analysis is called "shotgun proteomics" which can potentially characterize the entire proteome. Comparatively, in the technique termed "targeted proteomics" smaller number of proteins, numbering far fewer than 100, are studied and information is obtained with a high degree of accuracy. Mass spectrometry with liquid chromatography – mass spectrometry (LC-MS-MS) and matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF/TOF) are extensively applied tools and are principal amongst contemporary protein analysis techniques.[49]

1) A systematic review of the literature: In order to gauge the potential of proteomics for biomarker discovery, in February 2020, the PubMed electronic data base was queried by a variety of search terms including, "proteomics and bipolar disorder", "proteomics and mood disorders", "major depressive disorder and proteomic biomarkers", and "schizophrenia and proteomic biomarkers". A large number of articles, totaling more than 500, were recovered. The search was restricted to the last 10 years, and in addition, review articles, animal studies, human post-mortem investigations, and studies using other biological samples like saliva and cerebrospinal fluid were excluded. This narrowed the search substantially, with the exclusion of more than 300 citations. The examination was further refined by eliminating duplicates, and including only the least invasive studies utilizing blood, plasma or serum. Furthermore, inclusion criteria directed that those investigations be given consideration which employed typical MS-based proteomic procedures or multi-target immunoassays. This endeavor identified 40 original studies which were found eligible for this section of the manuscript. In May 2020, during the revision of the manuscript, one further study was identified in BD which met inclusion criteria and was also included. In this manner, a total of 14 studies were identified in BD and Table 1 gives an overview of these studies (Table 1).[50-63] Fig. 1 is the flow diagram of the search strategy adopted during the writing of this section of the manuscript (Fig. 1).

Included studies gave data on up- and down-regulated proteins; for proteins which were differentially expressed, and the considered factors of importance included: i) whether or not they were statistically significant, ii) fold change and the trend towards being low or high, and iii) linked mechanisms of biological processes. The proteomics data was cross-referenced with genetic loci identified in GWAS and the highest amplified established processes were identified through Ingenuity Pathway Analysis (IPA).

3. Examination of the data

The proteomic studies in major psychiatric disorders, as identified by the above-mentioned, systematic search, were marked by great heterogeneity. Differences were noted in the numbers of participants, biological samples utilized, processing techniques, study methodologies, mass spectrometry equipment, scrutiny of data, and analytical approaches. In the studies, a crucial aspect that was missing was the proper documentation of samples. Variations in case definitions made comparison among studies a challenging task, while diverse selection criteria further contributed to difficulties in comparing results of different studies. With respect to statistical procedures, few studies employed matching samples with a case-control format, and for the comparison of groups just used t-tests, while others applied regression modeling allowing for the analysis of confounding variables. Whereas few studies employed strict significance values ($p<0.01$), others applied higher thresholds ($p<0.05$) and did not correct for confounding variables. Moreover, it was found that some studies just described proteins which surpassed an absolute fold-change threshold, thereby ignoring data on proteins with lesser effect sizes. In this regard, it is worth mentioning that pro-

TABLE 1. Blood plasma and serum based investigations of proteomic biomarkers in bipolar disorder

Study	Subjects; diagnosis	Sample	Technique	Identified proteins	Biological pathways	Conclusion
Coppens et al. (2020)[50]	MDD (n=5), BD – depressed (n=3), BD – manic (n=4), SZ (n=4), HC (n=6)	PBMCs	State-of-the-art MS.	PBMC proteome was analyzed, yielding 4271 proteins. For discrimination between MDD and BD-D, 66 candidate biomarkers were found. 72 proteins might harbor a biomarker capacity for differential diagnostics of BD-M and SZ.	Examination of the entire proteome. No specific pathway investigated.	A register of candidate biomarkers with the potential to objectively discriminate MDD from BD-D, and BD-M from SZ. A proof of concept study, limited by small sample size.
Smirnova et al. (2019)[51]	33, SZ; 23, BD; 24, HC	Blood serum	Quantitative MS based on one-dimensional Laemmli polyacrylamide gel (PAG) electrophoresis.	27 proteins specific for schizophrenia, and 18 for BD.	Distinct protein sets in SZ and BD involved in diverse processes including immune response, cell communication/transport, cell/neuronal growth and maintenance.	Specific protein sets potentially useful for discovering novel biological pathways.
Ren et al. (2017)[52]	30, MDD; 30, BP; 30, HC	Blood plasma	Isobaric tags for relative and absolute quantification (iTRAQ) technology combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) with bioinformatics analysis.	9 proteins significantly altered between MDD and BP. B2RAN2, B4E1B2, APOA1, ENG, SBSN and QSOX2 up-regulated; ORM1, MRC2 and SLPI down-regulated.	Most identified proteins related to the immune system. Bioinformatic analysis showed that B2RAN2 and ENG may serve as candidate biomarkers for distinguishing between MDD and BP.	The study identified a protein panel that may distinguish BP from MDD. Particularly B2RAN2 and ENG need further investigation.
de Jesus et al. (2017)[53]	14, BD using Li; 4 other psychiatric (OD) using Li; 23, SZ; 12, HC	Blood serum	2-D DIGE and nano LC-MS/MS analysis.	37 protein spots found differentially abundant in the serum of patients compared to controls.	From detected spots, 13 different proteins identified including ApoA1, ApoE, ApoC3, ApoA4, Samp, SerpinA1, TTR, IgK, Alb, VTN, TR, C4A and C4B.	Proteomic analysis allowed distinction of BD from SZ. The findings contribute towards pathophysiology/biomarker discovery for major psychiatric disorders.
Haenisch et al. (2015)[54]	29, BD (manic); 17, BD (mixed); 53, HC	Plasma	Multiplex immunoassay analysis.	C-peptide, progesterone, insulin, and antigen 125 altered in both manic/mixed states. Peptide YY and sortilin changed only in mania. Haptoglobin, CC4 and matrix metalloproteinase 7 (MMP-7) altered specifically in mixed states.	Mania and mixed mood patients had similar changes in proteins related to insulin signaling (trait markers). Additionally, mania patients showed changes in hormonal and growth factor functions and mixed mood patients had changes in inflammation-related molecules.	Further studies can increase the understanding of the biological pathways involved in different BD mood states, leading to the identification of novel biomarkers and new drug targets.

teins with smaller fold change values might have contributed to falsely identifying differentially expressed proteins. Lastly, several studies failed to reveal whether or not key confounding variables like diet, smoking, and exercise were accounted for. The discrepancies in workflows posed serious problems in formally comparing proteomics data

TABLE 1. Continued

Study	Subjects; diagnosis	Sample	Technique	Identified proteins	Biological pathways	Conclusion
Chen et al. (2015)[55]	20, BD-II; 30, MDD; 30, HC	Plasma	2-DE coupled with MALDI-TOF MS/MS; ELISA for validation.	Two-fold differences for at least 25 distinct protein spots in BD II versus MDD; 3 proteins differentially expressed in cases versus controls. Results: C3, MDD>bipolar II>HC CFI and C4BPα, HC>MDD>bipolar II.	Enriched biological processes in BD-II relative to MDD were immune regulatory, acute inflammatory and wound response. Differently expressed proteins when mapped onto the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, showed that the complement and coagulation cascade pathways were significantly enriched ($p<0.01$).	Autoimmune dysregulation involved in the pathophysiology of bipolar II/ MDD.
Frye et al. (2015)[56]	52, Unipolar; 49, BD-II; 46, BD-I; 141, HC	Blood serum	Multiplex profiling of 320 proteins using Myriad RBM multi-analyate profiling (MAP) platform.	73 proteins showed nominally significant differences; 6 proteins different after Bonferroni correction.	Statistical analysis showed that MMP-7 significantly different in mood disorder vs HC. MMP-7, GDF-15, HPN significantly different in bipolar (BD-I+BD-II) vs controls. GDF-15, HPX, HPN, RBP-4 and TTR significantly different in BD-I vs controls.	Good diagnostic accuracy (ROC-AUC>0.8) obtained for GDF-15, RBP-4 and TTR when comparing BP-I vs controls. This discovery sample suggested applicability of proteomic panels to identifying and distinguishing mood disorders, in particular bipolar I disorder.
Song et al. (2015)[57]	10, euthymic BD-I; 20, depressed BD-I; 15, manic BD-I; 20, HC	Plasma	2-D electrophoresis/ tandem MS. Proteomic results validated by immunoblotting.	32 proteins identified with 1.5-fold changes in expression compared with HC.	16 proteins perturbed in BD independent of mood state, while 16 proteins specifically associated with particular mood states. Two mood-independent proteins identified-apolipoprotein (Apo) A1 and Apo L1.	BD pathophysiology may be associated with early perturbations in lipid metabolism. Down-regulated carbonic anhydrase 1, a mood-dependent protein, may be involved in the pathophysiology of bipolar depression.
Haenisch et al. (2014)[58]	17, BD outpatients; 46, HC	Plasma	Human Discovery MAP multiplexed immunoassay	190 proteins measured, identifying 26 dysregulated proteins in BD compared to controls.	Identified proteins comprised mostly of growth factors, hormones, lipid transport and inflammatory proteins. Decreased apolipoprotein A1 previously associated with BD was confirmed in the study.	Future studies needed to increase understanding of BD pathophysiology, paving the way for patient stratification/ better treatment outcomes.
Schwarz et al. (2012)[59]	250, first/ recent onset SZ; 35, MDD; 32, euthymic BD; 329, controls	Blood serum	Human MAP multiplex immunoassay .	Identification of a signature that comprised of 34 analytes in a cohort of closely matched SZ (n=71) and control (n=59) subjects.	Partial least squares discriminant analysis using this signature gave a separation of 60-75% of SZ subjects from controls across cohorts. The same analysis also gave a separation of ~50% of MDD patients and 10-20% of BD subjects versus HC.	A biological signature for SZ identified in the serum. It laid the groundwork for a diagnostic test for distinguishing SZ from HC as well as related psychiatric illnesses.

TABLE 1. Continued

Study	Subjects; diagnosis	Sample	Technique	Identified proteins	Biological pathways	Conclusion
Schwarz et al. (2012)[60]	75, pre-proximal SZ; 110, BD; 75+110, controls	Blood serum	Discovery MAP immunoassay.	Samples drawn within 1 month before estimated onset of illness. Identification of 20 molecules altered in pre-SZ and 14 in pre-BD compared to controls.	Only two of these molecular changes were identical in both data sets and predictive testing confirmed that the biomarker signatures for pre-SZ and pre-BD were dissimilar. Identified molecules related to inflammation and immune response.	Distinct serum alterations occurred before clinical manifestations of SZ and BD. The findings could lead to diagnostic tests to identify vulnerable patients early, allowing for earlier and more effective therapeutic intervention.
Alsaif et al. (2012)[61]	24, BD; 21, HC	Blood serum and plasma	Multiplex immunoassay	Total of 190 proteins measured in serum and plasma.	In the disease cohort 6 proteins changed significantly in serum and ten in plasma with an overlap of two proteins.	There were expressed differences in proteome coverage/reliability of measurement when comparing serum and plasma. This could have significant impact on identifications made in biomarker studies.
Herberth et al. (2011)[62]	16, euthymic BD-I outpatients (remitted); 16, euthymic BD-II outpatients (remitted); 32, HC Validation cohort: 7, BD-I; 7, BD-II; 14, controls	Blood serum (and PBMCs)	LC-MS and Human MAP	About 60 differentially expressed molecules identified which were involved in cell death/survival pathways.	In PBMCs, this was manifested in cytoskeletal and stress response-associated proteins, whereas most serum analytes associated with the inflammatory response. The predicted effect of serum analytes on physiological systems was tested by treating PBMCs with serum obtained from the same patients, resulting in reduced cellular survival.	BD patients carried a peripheral fingerprint that had detrimental effects on cell function and could be used to distinguish BD from HC despite being in a remission phase. Additional studies of BD in the manic and depressed phases could lead to the identification of a molecular fingerprint used for predicting episodic switching/ guiding treatment.
Guest et al. (2010)[63]	6, first-onset/acutely psychotic SZ; 10 euthymic BD; 78 HC	Blood serum	Fluorescence assays and immunoassay.	5 molecules differentially expressed in the cohorts	Identified proteins involved in dysregulated glucose metabolic pathways.	Insulin-related molecules and other co-secreted proteins may be potential biomarkers.

BD: bipolar disorder, BP: bipolar depression, HC: healthy controls, MDD: major depressive disorder, PBMC: peripheral blood mononuclear cells, SZ: schizophrenia.

and performing meaningful analyses.

INTERPRETATION OF THE PROTEOMIC DATA

1. Differentially abundant proteins

Analysis of the proteomic data revealed that roughly two hundred and two peptides were specifically abundant in schizophrenia (SZ), one forty one in major depressive disorder (MDD) and ninety nine in BD. In the proteome of patients with SZ, raised levels of insulin-related peptides were repeatedly found[64]; additionally, interleukins including IL10, IL12β, IL17α, IL5 and growth factors such as BDNF were variably expressed.[65] A number of original studies reported a reduction of apolipoproteins, and at least two found dysregulation of APO A_1, APO A_2, APO A_4, and APO C_1.[66]

One BD study described interesting results of a grouping of 20 proteins/metabolites which were meaningfully altered preceding any clinical manifestations. These included cortisol, CTGF (connective tissue growth factor), APCS (Serum amyloid P component) and TFF3 (Trefoil factor 3). Concluding from these results, the authors estab-

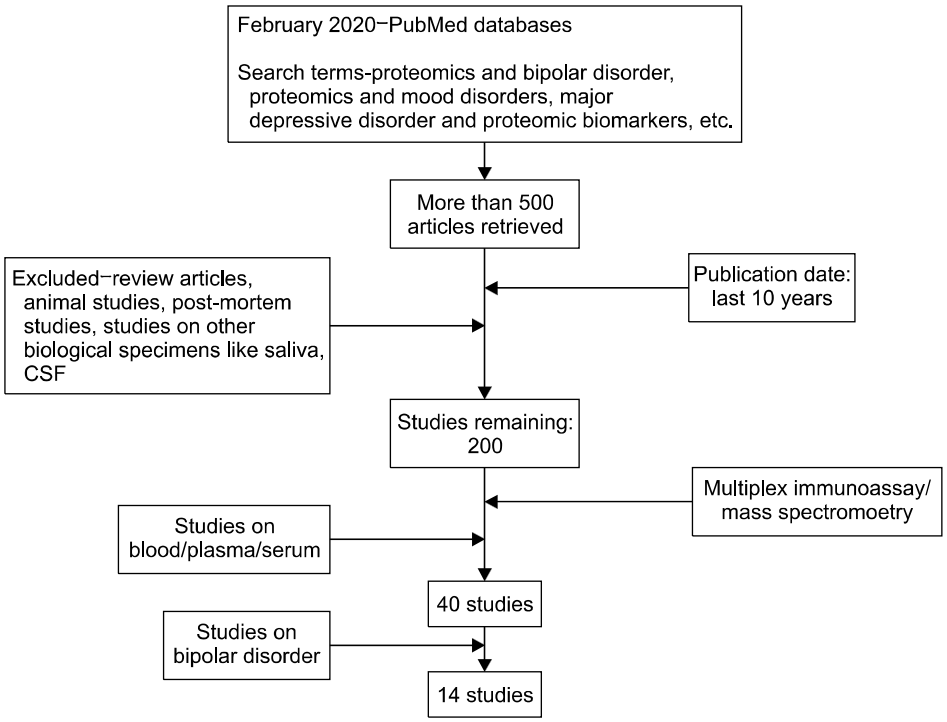

FIG. 1. Flow diagram illustrating the literature search for proteomic studies on blood/plasma/serum in bipolar disorder.

lished that the findings could be possible biomarkers applied as diagnostic tests in helping to detect susceptible patients either prior to onset or just at the beginning of the illness.[60] An interesting study underlined the alterations in proteomic analyses and consistency of results in a group of bipolar subjects compared to healthy volunteers. The investigators discovered that separate proteins were identified with discernable variances in measurement in two biological fluids, i.e. serum and plasma. The authors recognized that these discrepancies might well make true changes in proteomic analysis doubtful.[61] Another study evaluated the likelihood of mitochondria associated proteins in differentiating bipolar cases from HC and discriminating subcategories of mood disorders. The results indicated that GDF15 (Growth/differentiation factor 15), RBP4 (Retinol binding protein 4), and TTR (Transthyretin) were useful in predicting bipolar disorder type I with a ROC AUC of 0.81. Additionally, peripheral concentrations of GDF15, HPX (Hemopexin), NPN (Nephrocan), MMP7 (Matrix metalloproteinase-7), RBP-4, and TTR were greater in BD-I versus unipolar and BD-II patients, as well as controls.[56] Of note, one study found that there was disparate abundance of molecules concerned with cell death/survival in BD[62]; yet other research study determined that the pathophysiology of BD may be related to alterations in lipid metabolism. Remarkably, in the later mentioned study, APO A1 and APO L1 had differential modes of expression individually for the mood state.[57] See also Table 1 for a synopsis of all studies in BD (Table 1).

A number of MDD studies discovered a significant increase in pro-inflammatory cytokines and molecules involved in the oxidative stress response. A study had findings related to three complement proteins found to be differentially expressed, and the results were authenticated with ELISA. These were C3 - MDD>BD-II>HC; CFI and C4BPA - HC>MDD>BD-II subjects.[55] Another study found proteomic markers linked to cellular communication and signaling, immune response (CXCL1) and coagulation (VWF), and these variations were related to the severity of depressive symptoms.[67] An investigation determined that insulin was the molecule with the greatest statistical significance in MDD versus control.[68] Another study found that a panel consisting of six serum proteins could serve as combined biomarkers (APO D, APO B, GC, CP, HRNR, and PFN1), differentiating MDD patients from HC with a precision of 68%.[69] In an attention-grabbing study, the investigators tried to ascertain any protein abundances which could discriminate patients from controls, and considering three indicators with the most prospect of potential biomarkers, they detected cases with only a few false positive results (AUC=0.92).[70]

Scrutinizing the studies in the three populations, namely SZ, MDD and BD, there existed a small overlay of variably abundant proteins and twenty-one altered molecules corresponded through these diagnoses. Among the proteins, Compliment C3 (up), Macrophage Migration Inhibitory Factor (up), and Immunoglobulin M (down) were variably expressed, but trended similarly across the three disorders. Rather than depending on one biomarker for major psychiatric disorders, the findings were in agreement with the broadly believed notion that a suite of tests was needed for diagnostic purposes.[71]

2. Overlap with gwas findings

Interestingly, analysis in SZ patients revealed that there was a greater presence of proteins associated with C3 and C4 components of the complement cascade. This finding was shared by GWAS studies which implicated the genes encoding for complement factors *C3* and *C4* as related to augmented possibility for the development of SZ.[72,73] Variable gene expression at this location influenced the mRNA levels of C4A in the brain, emphasizing that C4 and C3, which had similar functions could possible serve as SZ biomarkers in the periphery. Nonetheless, there was decreased specificity, as C3 and C4 were chief mediators of innate immunity, and their concentration was subject to change in other conditions marked by inflammation. With respect to mood disorders there was no correspondence with genome-wide associated loci in the examined studies. However, future GWAS studies with larger sample sizes might uncover hitherto undiscovered overlaps between genetic risk loci and the peripheral proteomic profile in these disorders.

3. Biological correlates

In this section of the manuscript, analysis performed by Comes et al was referred to, as it was very important to determine the biological processes which were revealed by the proteomic data.[74] These authors, utilizing the Ingenuity Pathway Analysis (IPA), determined the five most augmented formal pathways for the conditions of BD, MDD and SZ. They determined that for BD, SZ, and MDD, respectively, the topmost involved pathways were FXR/RXR Activation (p=2.89E-30), Acute Phase Response Signaling (p=2.90E-31) and LXR/RXR Activation (p=1.62E-23). They also carried out bioinformatic enrichment analyses with Gene Ontology annotations to ratify IPA findings utilizing PANTHER GO-Slim Biological Process, PANTHER Protein Class, and PANTHER Pathways analysis. It was found that there were commonalities among diagnoses with respect to boosted biological processes, confirming the IPA results of enhancement of pathways concerned with immune/inflammatory signaling. Significantly, implicated biological pathways in these disorders involved response to interferon-gamma, the cytokine-mediated signaling pathway, blood clotting and complement activation. For all three diagnoses, the classes of proteins which were found to be enriched belonged to the complement component, chemokine, and growth factors.

Evidently, these augmented processes were relatively comparable across diagnoses and also incriminated in numerous medical conditions such that the specificity and validity of the proteomics data became questionable. This was likely because of the inherently intricate task of determining alterations in peripheral protein abundances that precisely replicated molecular changes in the brain. Additionally, the extraordinary technical requirements of thorough and exact investigation of the plasma proteome posed a huge challenge. Nonetheless, there was future potential in continuing with efforts for biomarker exploration via the latest proteomics technology.[75,76]

4. The promise of proteomics

The framework of cross-sectional criteria for diagnosis as provided by DSM-5 and ICD-11 has grave limitations with regards to precision and this flaw can only be rectified by a classification system that incorporates benchmarks ranging from changes at the cellular/molecular level to phenotypic manifestations. A paradigm change is required to shift from the "atheoretical" criteria-guided clinical entities to biological marker-based diagnoses that have actual scientific value.[77] Such a model based on precise pathophysiological mechanisms can direct the timing of the needed clinical interventions as specified by illness requirements, ensuring that appropriate steps are undertaken in the overall management of psychiatric patients. Changes in biological entities that can be consistently identified in the peripheral circulation of the patients allow for minimally invasive and cost-effective monitoring during different phases of the disease. Hence, modern 'omics' methodologies ideally can provide potent means for research in major psy-

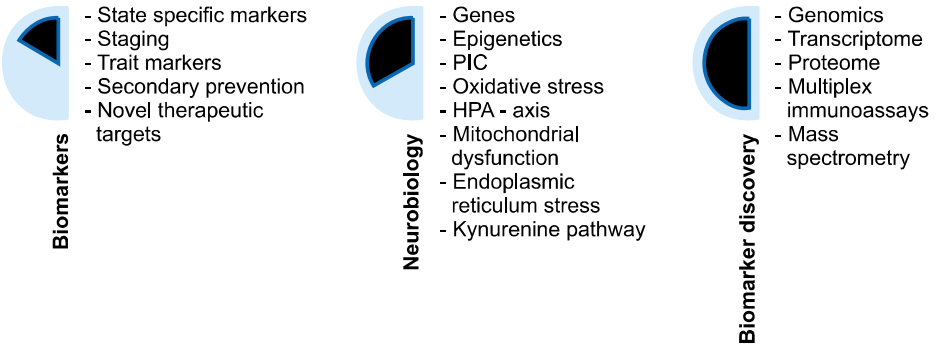

FIG. 2. The process of biomarker discovery for bipolar disorder - validated/replicated. Bipolar disorder is a heterogeneous condition whose neurobiology is hitherto not fully elucidated. The therapeutic process is purely empirically based and validated biomarkers are still not available. Research into candidate biomarkers has not been successful thus far, and with the help of 'OMICS' (genomics, transcriptomics and proteomics) the field is moving towards hypothesis free investigations of bipolar patients. It is hoped that state of the art technologies (mass spectrometry, bioinformatics) will ultimately lead to the discovery of clinically applicable biomarkers.

chiatric illnesses.[78,79]

5. Future directions

Proteomics has grown from mundane detection and documentation of proteins to incorporated ventures integrating genomic, epigenomic, transcriptomic and metabolomic data.[80] In order for proteomic investigations to precisely determine biomarkers, it is essential to select an accurately defined clinical population, have adequate sampling technique, and employ standardized procedures for sample processing. A large enough sample size is needed to permit adequate statistical power after stratifying for possible confounding variables (i.e., medication use, life style factors). Future studies should ideally be longitudinal in design, and allow for repeated measures while accounting for population-wise variances in protein profiles. Furthermore, findings from small preliminary studies must be verified in bigger samples.[81] As has previously happened in GWAS, proteomic analyses with adequate detection and verification samples will significantly contribute towards discovering and ratifying biomarkers. More recent investigations have demonstrated that having speedy and thorough proteome reporting pipelines is technically feasible.[82]

Lately, the 'Omics' field has seen rapid growth and huge amounts of information is now available. Consequently, employing technologies that adequately interpret this enormous data is indispensable and the creation of internationally available and fully accessibly databases is critical. In the context of studies examining the proteome, this step would enable multicenter partnerships and pooling of vast amounts of data from several layers of exploration.[83] Presently, even protein reporting sometimes lacks agreed terminology for gene symbols rendering the process of comparing of different studies challenging. The endeavors to incorporate proteomic data with other test site and clinical findings could lead to the discovery of omics-based innovative markers which have multiple uses and provide new understanding of complex diseases, based ultimately on pathophysiological mechanisms. However, this goal can only be achieved through interdisciplinary joint efforts which require collaboration from biochemists, biologists, molecular genetic experts, statisticians, alongside clinicians.[84]

CONCLUSION

In this manuscript an effort has been made to bring to highlight the latest findings related to the discovery of validated biomarkers for bipolar disorder, which undoubtedly, is a severe psychiatric condition. Firstly, an outline of candidate biomarkers is given, with the caveat that such investigations have thus far being unable to find clinically applicable tools in this regard. BD is a heterogeneous disorder with varied etiologic and pathophysiologic connotations, such that a single biomarker may not be realized. More likely, in this challenging condition a panel or suite of tests is expected to be needed to identify, monitor progression, and help in therapeutic decision making. The exciting era of proteomics holds promise in uncovering BD pathophysiology and finding practically useful biomarkers, while the integration of this information with other 'omics' data can lead to real progress in the management of this recalcitrant disorder. Fig. 2 provides a pictorial summary of the prevailing views regarding discovery of biomarkers in BD which have practical value (Fig. 2).

CONFLICT OF INTEREST STATEMENT

None declared.

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