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Blood biomarker discovery in drug-free schizophrenia: the contributionof proteomics and multiplex immunoassays.

Sophie Sabherwal
Royal College of Surgeons in Ireland, sophiesabherwal@rcsi.ie

Jane A. English
Royal College of Surgeons in Ireland, janeenglish@rcsi.ie

Melanie Föcking
Royal College of Surgeons in Ireland, mfocking@rcsi.ie

Gerard Cagney
University College Dublin

David R. Cotter
Royal College of Surgeons in Ireland, drcotter@rcsi.ie

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REVIEW

Blood biomarker discovery in drug-free schizophrenia: the contribution of proteomics and multiplex immunoassays

Sophie Sabherwal, Jane A. English, Melanie Föcking, Gerard Cagney and David R. Cotter

Department of Psychiatry, Royal College of Surgeons in Ireland, ERC Beaumont Hospital, Dublin, Ireland; Proteome Research Centre, UCD Conway Institute of Biomolecular and Biomedical Research, School of Medicine, and Medical Sciences, University College Dublin, Dublin, Ireland

ABSTRACT Introduction: Recent evidence supports an association between systemic abnormalities and the pathology of psychotic disorders which has led to the search for peripheral blood-based biomarkers. Areas covered: Here, we summarize blood biomarker findings in schizophrenia from the literature identified by two methods currently driving biomarker discovery in the human proteome; mass spectrometry and multiplex immunoassay. From a total of 14 studies in the serum or plasma of drug free schizophrenia patients; 47 proteins were found to be significantly altered twice or more, in the same direction. Pathway analysis was performed on these proteins, and the resulting pathways discussed in relation to schizophrenia pathology. Future directions are also discussed, with particular emphasis on the potential for high-throughput validation techniques such as data-independent analysis for confirmation of biomarker candidates. Expert commentary: We present promising findings that point to a convergence of pathophysiological mechanisms in schizophrenia that involve the acute-phase response, glucocorticoid receptor signalling, coagulation, and lipid and glucose metabolism.

KEYWORDS Schizophrenia; blood biomarker; proteomics; mass spectrometry; multiplex immunoassay; coagulation; acute-phase; metabolism; HPA axis; high-throughput

1. Blood-based protein biomarkers to predict schizophrenia

The value of identifying the molecular correlates of schizophrenia cannot be underestimated. The clinical outcome in schizophrenia, which affects up to 1% of the population worldwide, is significantly improved by early identification and treatment [1]. Symptoms generally occur in early adolescence; however, criteria for full disorder are not usually met until late adolescence or adulthood. This gap provides researchers with the opportunity to study this period preceding progression to disorder. Therefore, biomarker studies involving at-risk mental state (ARMS), clinical high-risk (CHR), first-episode, and nondrug treated schizophrenia have great potential to facilitate earlier intervention and improve patient outcomes [2]. The field of blood-biomarker discovery for brain disease is coming to fruition, with recent clinical trials for blood-based biomarkers of the neurodegenerative disorder Alzheimer's disease, and significant studies in Parkinson's disease [3–5]. This is of significance to the psychiatry and neurology fields as patient blood is an easily accessible biological sample [6]. In addition, blood can be taken at any stage during the course of illness, unlike cerebrospinal fluid (CSF) for instance [3,7]. There is now strong evidence supporting an association between systemic abnormalities and schizophrenia pathology which is driving the search for peripheral, blood-based biomarkers for the disorder [8,9]. There have been many interesting findings in this field, implicating processes such as

inflammation, stress response signaling, innate/adaptive immune signaling and energy metabolism in the pathology of schizophrenia [6,10–12]. Schizophrenia, and indeed any psychiatric or neurological disorder, is multifactorial in etiology and heterogeneous in expression [13]. Therefore, the use of a multidisciplinary approach, including imaging techniques, neuropsychology, electroencephalograms (EEGs) and integrative omics in the predictive biomarker discovery pipeline has received much attention in recent times [14]. Furthermore, investigations of schizophrenia pathology have been carried out in various biological samples including postmortem brain, CSF, blood serum and plasma, and sweat [8,15–20]. Here, we will discuss the current state of the art in the field of high-throughput protein biomarker discovery, and focus on studies of blood serum or plasma involving largely drug-free schizophrenia, first-episode psychosis and CHR subjects. We will also briefly relate these findings to those observed in the CSF.

2. The platforms driving high-throughput biomarker discovery workflows

2.1. Mass spectrometry

'Bottom-up' proteomics refers to an approach in which information about proteins in a samples are reconstructed from individually identified fragment peptides. The earliest proteomic experiments investigating schizophrenia pathology combined two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) with mass spectrometry (MS) [21,22]. Most mass spectrometry instruments used at this time were matrix-assisted laser desorption/ionization time of flight (MALDI-TOF), and identified proteins by matching the tryptic peptide products of protein digestion to patterns predicted using protein sequence databases (a 'bottom-up' approach). In an attempt to reduce uncertainty from gel-to-gel differences in 2-D PAGE, difference gel electrophoresis (DIGE) was introduced in 1997 [23,24]. The introduction of tandem mass spectrometry (MS/MS), multiple stages of mass spectrometry with fragmentation of precursor ions to fragment ions, has led to significant improvements in the rate and reproducibility of protein identification and quantification [25]. There have also been numerous improvements made to mass spectrometry apparatus over recent years including the introduction of hybrid, or high resolution/mass accuracy (HR/AM), instruments which typically permit the routine identification of a few thousand proteins in a complex sample within several hours [26]. Current proteomic biomarker discovery studies generally use 'bottom-up' approaches. An exception to the norm being a study by Ding and colleagues who employed a 'top-down' approach (identification of proteins prior to digestion into peptides) using surface-enhanced laser desorption/ionization, time-of flight mass spectrometry (SELDI-TOF MS [27]). The currently used 'bottom-up' approaches employ different acquisition methods such as data-dependent, data-independent and targeted, depending on the approach (discovery or hypothesis driven) and the type of instrument available. These acquisition methods differ in their respective methods of precursor selection for fragmentation, and the methods by which fragment ion signals are recorded [28]. Here we will briefly introduce these approaches in the context of blood biomarker discovery in schizophrenia.

2.2. Data-dependent acquisition

Shotgun, or discovery, proteomics is the most widely used 'bottom-up' method in this field. This method aims for global protein identification and systematic profiling of complex proteomes. Shotgun-based methods employ a data-dependent acquisition (DDA) mode [29]. In DDA mode, the most abundant peptide ions (precursor/parent ions) detected in a survey (MS1) scan are selected for fragmentation. A significant limitation of DDA is low reproducibility, which occurs as a result of the stochastic nature of the approach. For a detailed review on the DDA acquisition method and its strengths and limitations see review by Wasinger and colleagues in 2013 [26]. A single liquid-chromatography tandem mass spectrometry (LC-MS/ MS) run can identify thousands of proteins [30]. The quantification of proteins in a shotgun comparative experiment can be based on 'labeled' or 'label-free' peptides [31]. Each method has its strengths and weaknesses which have been addressed extensively in the literature (see review by Chahrour and colleagues in 2015 [32]). Most biomarker investigations have employed label free approaches because they are not limited by the number of labels/tags and can facilitate a larger number of samples being run, where sample size and statistical power are key factors considered in the design of clinical biomarker investigations [31].

2.3. Targeted proteomics

Targeted proteomics has not been utilized in blood-based schizophrenia studies; however, it was the method of choice in a study involving brain tissue from a phencyclidine (PCP) rat model of schizophrenia [33]. Single reaction monitoring (SRM), also known as multiple-reaction monitoring (MRM), is the fundamental acquisition mode in targeted proteomics. It is highly reproducible across samples, and even different laboratories, making it a suitable tool for biomarker validation [28]. Before an SRM experiment, a single transition (or multiple transitions in MRM) and the retention time for the protein of interest must be predefined. Current SRM experiments are typically limited to the quantitation of up to 100 peptides per run, inferior in this respect to DDA [34].

2.4. Data-independent acquisition

DIA methods have begun to emerge prominently in this field [20,35]. In DIA the precursor mass range is selected, the mass range is then divided into a series of isolation windows (for example 20 m/z per window), tandem MS data is acquired for all detected precursor ions per isolation window and, finally, tandem MS spectral libraries are used to identify peptides of interest from acquired data. DIA mode can be used for discovery or pseudo-targeted proteomic investigations [28], since high numbers of proteins can be measured in an unbiased fashion [34,36]. For a detailed review on the strengths and limitations of DIA for discovery and hypothesis-driven experiments see study by Sajic and colleagues in 2015 [28]. DIA as an approach is still quite immature [37]. In particular, statistical handling of data lags slightly behind other approaches [37,38]. Several DIA workflows have been reported in the schizophrenia biomarker literature [39]. They all use unbiased MS/MS data acquisition but differ in their mode of data collection and analysis, and in the type of mass-spectrometer used. DIA approaches can be broadly divided into two categories; full m/z range and selected m/z range [28]. MSE (a method for tandem mass spectrometry data acquisition) is DIA workflow which involves the fragmentation of all ions within the full m/z range by the mass-spectrometer. MSE has been used in two blood-based proteomic studies of schizophrenia [20,35].

2.5. Multiplex immunoassays

The introduction of biomarker discovery databases such as the multi-analyte profile (MAP) platform by Myriad Rulesbased medicine (RBM) has transformed the use of immunoassays in biomarker research. The MAP platform was developed using microsphere technology in order to provide researchers with reproducible, quantitative multiplex immunoassay data for hundreds of proteins (from relatively little quantities of blood plasma or serum). This technology has caused a shift from the use of immunoassays for purely targeted protein quantitation, to the identification of biomarker panels. MSE was used to identify a serum biomarker panel capable of distinguishing first-onset drug-naive schizophrenia subjects from healthy subjects. This panel was then validated by immunoassay and later became the first commercially available blood-based laboratory test for schizophrenia [40]. In this way, the multiplex immunoassay (MIA) approach to biomarker discovery is dependent on candidate identification from mass spectrometry (MS) or other discovery studies. Findings from both methods will be included in this review, as they are both valuable methods to biomarker discovery.

2. **Search strategy for blood-based protein biomarker studies in schizophrenia**

The objective of this review is to summarize blood biomarker findings in schizophrenia from the literature identified by mass spectrometry and multiplex immunoassay. We used the 'Preferred Reporting Items for Systematic Reviews and Meta Analyses' (PRISMA) guidelines for reporting in systematic reviews [41]. Relevant articles were identified by searching for titles in search engines (PubMed and Web of Science) using the following search terms: (Schizophrenia[Title/ Abstract] OR psychosis[Title/Abstract] Or psychotic[Title/ Abstract] OR ARMS[Title/Abstract]) AND (Blood[Title/Abstract] OR plasma[Title/Abstract] OR serum[Title/Abstract]) AND (mass spectrometry[Title/Abstract] OR multiplex immunoassay[Title/ Abstract] OR proteomic[Title/Abstract]) NOT (rat[Title/Abstract] OR mouse[Title/Abstract]) NOT review[Title/Abstract] The searches returned 153 records after duplicates were removed. Fourteen records reached inclusion criteria after screening of abstracts and full texts. Please refer to PRISMA Flow diagram in Supplementary Figure 1. Abstracts from the search records were screened using the following criteria for inclusion/exclusion: only experimental published papers in English were included; reviews, meta analyses and non-English language papers were excluded. Papers were included if they measured serum or plasma protein levels in males and/or females in late adolescence or adulthood with schizophrenia (e.g. paranoid subtype or first episode psychosis) or clinical psychosis risk (e.g. clinical high risk) and (a) Analysis was conducted using a high-throughput approach (mass spectrometry or multiplex immunoassay). (b) Compared subjects to a healthy control group or those at clinical high risk who did not covert to disorder. (c) Was published before January 2016. (d) Subjects were drug-free or effects of treatment were excluded by an untreated control group and (e) Did NOT solely focus on the effects of antipsychotic medications on protein levels. (f) Did NOT solely focus on unrelated variables e.g. comparing patients with TD (long-term side effect of antipsychotics) and those without or those who have attempted suicide vs. those who haven't. (g) Did NOT solely focus on proteins measured in brain tissue.

Here we have summarized all studies in the serum or plasma of schizophrenia patients (at various stages) who were drug-naïve (n = 10), non-medicated for a period of at least 8 weeks (n = 1), or for

whom the effect of treatment could be excluded (by including an untreated control; n = 3). This resulted in the inclusion of six mass spectrometry and eight multiplex immunoassay studies in this review (Table 1 [8,9,20,22,23,27,29,35,42– 47]). Please refer to Table 1 for a summary of studies included, along with demographic details for the patients and controls in each investigation. We then systematically collated the protein expression data from these independent investigations, and created a table summarizing the biomarker candidates identified in each study (Table 2).

4. Summary of biomarker candidates collated from our systematic review of the literature

A total of 149 significantly differentially expressed proteins were collaged from the 14 studies reviewed (Supplementary Table 1). Of these, 50 biomarker candidates (47 proteins and 3 steroid hormones) were identified in two or more studies, in the same direction (Table 2). We then used Ingenuity Pathway Analysis (IPA), a commercial bioinformatics application, to determine the key protein pathways implicated, as previously described [18]. Pathway analysis of the 47 proteins listed in Table 2 was conducted and the results are summarized in Figure 1 (cortisol, progesterone and testosterone were excluded from analysis as the focus of this review is on the proteome). In Table 3, the top 10 canonical pathways are listed. The full list of pathways implicated by IPA in this data set can be found in Supplementary Figure 2. Given the lack of specificity of peripheral biomarker findings in psychiatry it is unlikely that any one protein could have the potential to greatly impact diagnostics and treatment [13]. Therefore, biomarker panels with discriminatory power between cases and controls, and also between different psychiatric disorders, are the way forward.

5. Findings by ingenuity pathway analysis

The top pathway implicated by IPA was the acute-phase response (Figure 1). Eleven proteins from the acute-phase pathway were identified by this review as biomarker candidates of schizophrenia out of a total of 171 in the canonical pathway (Table 3). The remainder of the top 10 most significant pathways defined by IPA function in the immune system (communication between innate and adaptive immune cells, hepatic stellate cell activation), lipid and glucose metabolism (LXR/RXR and FXR/RXR), blood formation and clotting (hematopoiesis of multi/pluripotent cells and coagulation respectively), and the stress response (glucocorticoid receptor signaling). Atherosclerosis has links with lipid metabolism and inflammation. Here we discuss some of the top pathways implicated by IPA for the candidate proteins in Table 2. These are highly relevant to the current peripheral hypotheses of schizophrenia pathology and provide a bridge linking protein changes in the periphery and the neuropathology of schizophrenia. We will also compare the proteins and pathways implicated in this review to those of similar studies conducted in CSF, in order to discern whether there is a possible connection with the CNS pathology of schizophrenia.

5.1. Immune system; acute-phase response signaling

Canonical pathways within the immune system were heavily represented in both proteomic and MIA studies. This suggests immune system dysregulation plays an important role in schizophrenia pathology. The immune system is split into two different categories; innate and adaptive. The innate is considered the ‘first line’ of defense as it promotes defense against invading pathogens, but without conferring any

specificity toward recent additions to the immune system. The innate humoral system involves the acute-phase response and complement cascade, which function primarily in phagocytosis of invading pathogens. Acute-phase response signaling was the top pathway identified in our data set by IPA, with 11 (HP, C3, APOA1, APOH, APCS, APOA2, CFB, VWF, SERPINA1, FGA, A2 M) acute-phase proteins out a total of 171 being present in the analysis. There are two different types of acute-phase proteins involved in the acute-phase response; positive and negative. Positive acute-phase proteins increase in concentration in response to inflammation and function in protecting against invading microbes or as negative regulators of inflammation. Seven positive acute-phase proteins were found here to be up-regulated, indicating an overall increase in the inflammatory response. Conversely, the concentration of negative acute-phase proteins decreases in response to inflammation, therefore these protein scan be used as markers of inflammation. Negative acute phase proteins APOA1 and APOA2 concentrations were found to be decreased (Table2), therefore providing further evidence for an increase in inflammation in psychotic disorders (Figure 1). Interestingly, however, higher concentrations of the positive acute-phase proteins tissue plasminogen activator (tPA) and serum amyloid p (APCS) at birth were previously found to be protective in terms of risk of developing non affective psychosis later in life [47]. This suggests those with lower levels of acute phase proteins at the time of birth are more susceptible to psychosis later in life, as a result of increased risk of infection. The acute-phase response also plays a role in central nervous system development suggesting lower levels at birth could also lead to neurodevelopmental deficiencies. Furthermore, APOA1 and TTR (negative acute-phase protein) concentrations were found to be dysregulated in the CSF of first-episode schizophrenia, by Western blotting and mass-spec based methods [16,19,49]. This suggests that acute-phase response dysregulation occurs not only in the periphery, but also in the central nervous system (CNS).

5.2. Immune system; cytokines

Cytokines are the key signaling molecules that coordinate the innate and adaptive processes. This coordinating pathway was implicated in the top 10 significant pathways by IPA for Table 2 proteins. Seven proteins (CXCL8, CD40LG, IL10, IL15, IGHA1, CCL5 and CSF2) out of a total of 90 molecules in the IPA cytokine signaling canonical pathway were present in our analysis(Table3). There are numerous enzyme-linked immunosorbent assays (ELISAs) studies reporting evidence of altered levels of cytokines in the plasma, serum and cerebrospinal fluid of patients with psychoticdisorders.Interleukin12(IL-12),interleukin-6(IL-6),interleukin 1 beta (IL-1β), serum interleukin 2-R (sIL-2r), interferon gamma (IFN-γ), transforming growth factor (TGF-β) and tumor necrosis factor alpha (TNF-α) are amongst the differentially expressed proteins found in drug-naive first-episode psychosis subjects, shown by meta-analysis of ELISA, and other non-proteomic, studies [11,50]. IL-1β was also found to be decreased in the CSF of schizophrenia subjects compared to control, by meta-analysis of antibody-based methods [50]. TNFandIL-1β were amongst the top five upstream regulators implicated by IPA (Supplementary Figure 3). These signaling molecules play a pro-inflammatory role in the immune system and they also share the ability to activate the acute-phase response.

5.3. The hypothalamic–pituitary–adrenal axis; glucocorticoid receptor signaling

Stress-related biomarkers have also been investigated in the blood of those suffering from psychotic disorders. The classic neural-diathesis stress model of schizophrenia theorizes that psychosocial stress activates the hypothalamic–pituitary–adrenal (HPA) axis which causes an increase in peripheral cortisol.

Glucocorticoids, a major subclass of steroid hormones, regulate a large number of immune, metabolic, cardiovascular and behavioral functions. They are produced and released from the adrenal cortex under the control of the HPA axis [50]. Glucocorticoids produce their effect on responsive cells by activating the glucocorticoid receptor (GR) to directly or indirectly modulate the transcription of target genes (IPA). Glucocorticoid receptor signaling was implicated among the top 10 pathways by IPA for our data set. Seven proteins (CXCL8, PRL, IL10, CCL5, CSF2, CCL11, A2 M) out of a total of 293 in the IPA canonical pathway were present in the analysis (Table 3). Interestingly, we found cortisol to be up-regulated in five multiplex immunoassay studies (Table 2). It was subsequently excluded from the IPA which focused exclusively on protein interactions, and is therefore not represented in Figure 1. In healthy individuals, glucocorticoids, the products of HPA axis activation, negatively regulate the peripheral immune system. Conversely immune system activation results in stimulation of the hypothalamus [12]. This reciprocal process is protective during periods of stress and is also helpful in modulating immune responses in inflammatory disease. There is, however, evidence for disruption to the bidirectional communication between the HPA axis and immune system in schizophrenia. This includes the finding that in schizophrenia subjects there is a significantly positive correlation between salivary cortisol and IL-6 concentration in response to environmental stress [52]. The HPA axis is one of the main biological responses to stress, however other stress-related systems have also been shown to be associated with schizophrenia. Prolactin (PRL), which is an anterior pituitary hormone, has been shown in a number of studies to be increased in response to psychosocial stress, in the serum of healthy men and women [53]. Significantly, it has also been found to be increased in the blood of up to 39% of FEP cases (drug-naive). Furthermore, there is evidence for prolactin as a predictor of the transition from ARMS to psychosis in serum (Labad et al. 2015 [54]; Aston et al. 2010 [55] Riecher-Rössler et al. 2013 [56]).

5.4. Coagulation

Free-protein S and functional protein C are natural anticoagulants that form complexes that inhibit tissue plasminogen activator (tPA) inhibitors i.e. promote tPA activity. The observation that psychotic patients on chronic warfarin therapy for deep-vein thrombosis showed long-term remission of psychotic symptoms led Hoirisch-Clapauch and colleagues to hypothesize that dysregulation of the coagulation pathway, specifically low tPA activity, could be involved in the pathology of the disorder [57]. In addition to their classic function in blood clotting, coagulation factors are also considered positive acute-phase proteins. Furthermore, the products of coagulation factors are in some cases chemotactic for phagocytic cells, providing another link for these proteins to immune system functioning. The coagulation canonical pathway was implicated by IPA among the top 10 most significant pathways (five coagulation proteins; VWF, SERPINA1, F7, FGA, A2 M out of a total of 35 in the IPA canonical pathway were present in our analysis; Table 3). This finding is supported by a high prevalence of conditions affecting tPA activity in drug-naive schizophrenia, such as antiphospholipid antibodies, elevated cytokine levels, hyperinsulinemia and hyperhomocysteinemia. It was later found that free-protein S deficiency was highly prevalent (22%) among schizophrenia patients, but absent in controls [58]. There was no difference in protein C levels between patients and controls, suggesting that protein S may have a role in

schizophrenia pathology independent of protein C [58]. Johnson et al. [59] found the presence of two 40kDa spots in 2-D gels of CSF from schizophrenic patients which were absent in the controls. These spots were later identified as fibrinogen beta chain (FGB), also a coagulation factor, however not observed in this review. It is important to note that there is significant overlap between acute-phase responses, coagulation and complement system proteins. There are only 35 and 37 proteins in the coagulation and complement canonical pathways respectively, in comparison to 168 proteins in the acute-phase response canonical pathway, meaning the latter is more likely to be implicated by discovery protein biomarker studies.

5.5. Glucose and lipid metabolism dysfunction; LXR/RXR and FXR/RXR activation

Comorbidities exist between schizophrenia, metabolic syndrome and diabetes mellitus [60,61]. Metabolic syndromes are characterized by insulin resistance and glucose tolerance, abdominal obesity, hypertension, low high-density lipoproteins (HDL) and hypertriglyceridemia. The Retinoid X receptors (RXRs) are nuclear receptors that mediate the biological effects of retinoids. The Farnesoid X receptor (FXR) is also a nuclear receptor and activated by bile acids and their intermediates. The Liver X receptor (LXR) on the other hand is a type II nuclear receptor that is activated by oxysterol ligands. Coupled with the RXR, FXR plays a crucial role in linking bile acid regulation with lipoprotein, lipid and glucose metabolism thus acting as a modulator of bile, lipid and glucose homeostasis. Similarly, the LXR/RXR heterodimer is involved in the regulation of lipid metabolism, inflammation, and cholesterol to bile acid catabolism. LXR/RXR (6/128; C3, APOA1, APOH, APOA2, SERPINA1, FGA) and FXR/RXR (7/137; C3, APOA1, APOH, APOA2, INS, SERPINA1, FGA) were found by IPA to be significantly represented by the protein biomarker candidates in Table 2 (Figure 1 and Table 3). Furthermore, 40 of the proteins in Table 2 were also found to be specifically involved in lipid metabolism by IPA (Supplementary Figure 3). In support of this, a study conducted in 2013 by Wu et al., using chemiluminescence, found that first-episode schizophrenia patients differ from healthy controls in their fasting glycometabolism parameters and lipid profiles [62]. It was also found that APOA1 levels were down regulated in schizophrenia by Wu and colleagues, consistent with the findings presented in this review (Table 2). Furthermore, APOA1 has also been found to be down regulated in the CSF of first-episode schizophrenia patients [15]. Finally, the hypothesis surrounding metabolic abnormalities in psychotic disorders is supported here in that IPA identified metabolic disease among the most represented disorders in the data set (see Supplementary Figure 3).

5.6. Specificity of biomarkers to schizophrenia

In order to examine the specificity of the current findings to schizophrenia, biomarker candidates found in two or more studies involving comorbid psychiatric disorders i.e. major depressive disorder and bipolar disorder, were identified. This literature search followed the same criteria for inclusion/ exclusion as the schizophrenia studies, and resulted in three major depressive disorder [9,63,64] and two bipolar disorder [8,63] studies being assessed (see Section 3). From these studies we identified two drug-free schizophrenia biomarkers that are likely to be shared with major depressive disorder; growth hormone and α2 macroglobulin. These were found to be differentially expressed in two or drug-free major depressive disorder studies, in the opposite direction to schizophrenia findings (Table 2).

Domenici and colleagues [65] found 28 of the 34 significant major depressive disorder proteins to also be significant in schizophrenia. This suggests that there may be significant overlap between blood biomarkers of the two disorders, and that future studies are needed to address this. The same study, however, also identifies a number of proteins with high discriminatory power between cases and controls for each disorder and also specificity for each disorder, respectively. This was achieved by plotting the variable importance of contribution (VIP) from partial least squares discriminant analysis (PLS-DA) for each analyte on x-axis for major depressive disorder and on the y-axis for schizophrenia. No overlapping biomarkers were identified for bipolar disorder. The lack of reproducible results in bipolar studies of a similar nature may be due simply to the number of studies passing inclusion/exclusion criteria. The study by Schwarz and colleagues in 2011 found an overlap of two proteins found to be significantly differentially expressed in the same direction in bipolar disorder and schizophrenia; calbindin and cancer antigen 125. Three of a total of 16 candidates in Haenisch and colleagues' (2016) [66] bipolar disorder study were reported previously in schizophrenia by Schwarz and colleagues (2011) [8] and three of a total of 60 in schizophrenia study by Chan and colleagues in 2015. The latter validated a panel of 22 analytes in two separate cohorts of first-onset schizophrenia patients and controls, and found it to have a sensitivity of 87% and specificity of 97%. It was also found to have high accuracy for identifying pre-schizophrenic patients, but failed to accurately identify prebipolar patients, indicating high differential diagnostic power of the panel [43]. This suggests a higher degree of specificity of biomarker candidates for bipolar disorder than major depressive disorder, with regards to schizophrenia. Finally, Perkins and colleagues (2015) [46] demonstrated the high accuracy of a 15-analyte panel which could identify clinically high-risk patients who eventually developed schizophrenia, and were able to discriminate preschizophrenia patients from premajor depressive disorder and prebipolar disorder patients [46].

5.7. Potential limitations of this review

In this instance it is also important to note the disparity between the plasma and serum proteome. Plasma is produced from blood by addition of an anticoagulant such as heparin, EDTA or sodium citrate, followed by removal of cells and cell debris by centrifugation. Production of serum differs in that no anticoagulant is added and the resulting material composed of clotting factors, cells, and cell debris is removed. In this review, the majority of studies assessed were in serum (Table 1; 10/14) and the remainder were plasma (4/14) so disparities between the sample types are expected. Indeed, a study published by Alsaif and colleagues (2012) [67], found that despite similarities in coverage on a multiplexed platform, marked differences in variation between plasma and serum of healthy individuals were observed [67]. Our aim was to identify protein pathways consistently implicated in blood-based biomarker studies schizophrenia, irrespective of sample type but some inconsistent findings could be attributed to sample type differences. It has been suggested that combining profiling results from plasma and serum may increase the probability of discovering and validating novel biomarkers of psychiatric and other disorders [67]. Another potential limitation of biomarker studies is the uncertainty as to whether the findings reflect causality or epiphenomena. Many of the studies included in this review attempted to overcome this by identifying and matching cases and controls for potential confounding factors (outlined in the comparison section of Table 1). Confounding variables can also be adjusted for during statistical analysis. The presence of a number of factors in patients and/or controls which could significantly influence findings include the presence of systemic diseases (such as diabetes

mellitus, hyperlipidemia, hypertension, cardiovascular or immune diseases), other neuropsychiatric disorders (such as a family history of mood or anxiety disorders), or substance abuse issues [68]. This is discussed in further details in the Supplementary Methods section.

6. High-throughput biomarker discovery: antibody based approaches versus mass spectrometry

As previously mentioned, the approaches currently driving high-throughput biomarker discovery are the multiplex immunoassay and mass spectrometry. Each approach gives rise to different protein candidates, because of the unique properties of each technique (Supplementary Table 1). Here, we will briefly discuss the strengths and weaknesses of antibody based methods and mass spectrometry based methods for biomarker discovery. First, it is important to note that the two approaches are not necessarily mutually exclusive; mass spectrometry can be used as a semi-quantitative method and discovery tool in order to generate candidate biomarkers that are subsequently translated into immunoassay platforms (which tend to be more accessible in a clinical setting). The analytical specificity (ability to discern one molecule from another) of mass spectrometry is generally superior to any other method because in nearly all cases diagnostic ions capable of identifying the analyte can be isolated. For example, vitamin D is measured on automated immunoassay platforms using a binding assay that does not discriminate between vitamin D2 and vitamin D3. Mass spectrometry based methods can discriminate between the two forms, cholecalciferol and ergocalciferol, since they have different molecular mass [69,70]. Despite analytical superiority to automated immunoassays, mass spectrometry lags behind antibody-based platforms in terms of reproducibility. This is clearly evident in Table 2 in that there are only four replicated mass spectrometry findings in comparison to 45 multiplex immunoassay findings (full list of protein candidates available in Supplementary Table 1), even though the number of studies using each method is comparable. However, it is important to note that some of the results of the pathway analysis on Table 2 are representative of mass spectrometry studies also. This is evident in that the immune system signaling, the acute-phase response, LXR/ RXR, lipid metabolism and coagulation pathways have been implicated by pathway analysis (IPA and KOBAS) in three of the reviewed mass spectrometry papers [20,29,35]. Furthermore, those biomarkers identified by mass spectrometry which were replicated (APOA1, FGA, HPT, SERPINA1, APOA2) are involved in FXR/RXR signaling (APOA1, APOA2, SERPINA1, FGA), coagulation (FGA and SERPINA1) and the acute-phase response (APOA1, APOA2, HPT). This limitation in reproducibility perhaps reflects the natural variation in chromatography, and the presence of missing data which arise as a consequence of the DDA sampling procedure. However, this is also the result of the true discovery nature of the approach (as opposed to the use of specific antibodies), and indeed of the analytical specificity which results in the identification of various isoforms of the same protein. Furthermore, the studies which were assessed in this review used a range of mass spectrometry sampling methods and analysis techniques, which may also account for the lack of reproducibility across studies. DIA approaches however provide fragment level quantification, which can be considered the repeated measure of abundance of peptides across samples [38]. Immunoassays allow greater sensitivity of detection than mass spectrometry [36]. DIA may be less sensitive than DDA, due to the increased instrument time required to progress through the isolation windows [71]. However, this can be compensated for by increased signal-to-noise in MS2 spectra [71]. The targeted SRM approach, however, is of comparable sensitivity to immunoassays. Furthermore, antibody-based methods are not easy to scale up and are also limited to the availability and quality of antibodies. For example, the multiplex immunoassays in this review failed to fully analyze the

complement pathway's involvement in schizophrenia pathology as a result of the inclusion of only three complement protein (C3, CFB and CFHR1) antibodies in the most recent MAPs. The complement system is defined as a cascade of enzyme activations that bridge the innate and acquired immune systems. Biological effects of the complement system include opsonization, lysis of foreign cells, clearance of immune complexes and apoptotic cells, activation of inflammation and augmenting the antibody response. The complement canonical pathway has been previously implicated by pathway analysis in schizophrenia pathology, including in studies by Jaros and colleagues in 2012 and Li and colleagues in 2012 [29,35]. Complement C3, the first protein in the alternative complement pathway, is a component of the classical pathway and a convergence point between all three pathways in the complement system. C3 has been reported to be up-regulated in the blood of schizophrenia patients by hemolytic assay [72]. Moreover, an up-regulation of the alternative complement pathway, in particular, has been reported in the serum of drug-free schizophrenia subjects (also by hemolytic assay; Boyajyan et al. 2010 [73]). Complement C3 alterations were previously reported in the CSF of schizophrenia patients by proteomic methods, however this was not significant, most likely as a result of the small sample size used in the study [16]. Discovery proteomic workflows are biased toward highly abundant peptides which led to an issue which previously complicated proteomic work; the need to deplete high abundant proteins to allow assessment of mid to low abundant candidate biomarkers. Such depletions however can now be reliably conducted using high-performance liquid chromatography [74]. This reduces protein concentration variability but there is the risk of co-depletion of potentially significant biomarkers due to nonspecific binding or loss of biomarkers bound to higher-abundance carrier proteins [75]. Another factor in comparing these approaches is cost. Multiplex immunoassays are less costly than mass spectrometry approaches in terms of instrument and training costs; however, developing antibodies for a particular protein of interest makes this approach economically dependent on the predefined MAPs mentioned above. The most recent human MAPs are fixed with up to 300 antibodies per array for proteins present in serum or plasma but only a portion of these (33–50%) are quantifiable in an experimental setting (Table 4). For a protein to be suitable for quantitation different studies set different criteria. For example, the proteins must have given robust reading, within the range of quantitation, in70% of samples(Ramseyetal.2013[46]; Chanetal.2015) [42]. Conversely, a recent mass spectrometry method (MSE) has the ability to identify approximately 1500 proteins and quantify up to 320 of these. Criteria are often similar to those of MIA studies in that peptides found in>70%samples (in2/3injections) are quantified [20,35]. However, some studies have less stringent criteria, for example Li et al., 2012, quantify peptides found in as few as 50% of samples [29].

7. Expert commentary

Immune system disturbances in schizophrenia pathology were heavily supported by this review, with the implication of the acute-phase response, coagulation and cytokine signaling pathways in the top 10 by IPA. There is evidence for immune molecule imbalances locally in the prefrontal cortex [76]. Some pro-inflammatory molecules, such as TNF-α, have been shown to directly have negative effects on synaptic transmission, long-term potentiation (a form of synaptic plasticity involved in cognition) and also to lead to increased glutamatergic synaptic transmission and subsequently excitotoxicity [77– 79]. Glucocortiocoid signaling was also implicated by IPA. HPA axis over-activation has the ability to cause enhanced DA release in the brain, thus contributing the emergence of psychosis in predisposed individuals [51]. Interestingly, there is evidence for disruption of the bidirectional communication

between the HPA axis and immune system in schizophrenia, which provides another possible mechanism for their contribution to the neuropathology of schizophrenia [12]. Metabolic disturbances were highly evident in this review with the implication of lipid metabolism as one of top molecular processes and LXR/RXR and FXR/RXR implicated among the top canonical pathways. There is evidence that the ingestion of omega-3 or correction of an omega-6/omega-3 imbalance can contribute to balancing the cholesterol system and alleviating psychiatric symptoms in schizophrenia. Furthermore, it has been shown that there are decreased phospholipid polyunsaturated fatty acids (PUFAs) in brain and peripheral (red blood cell) membranes of schizophrenia subjects, which is consistent with myelin abnormalities and neurotransmitter system impairment observed in the disorder [80–82]. Significantly, there is evidence for a causative link between immune system dysregulation and lipid imbalance in schizophrenia [83], providing another possible mechanism of action for the effects of peripheral and central immune disturbances and brain pathology in the disorder. In summary, immune system disturbances may affect schizophrenia neuropathology directly via the effect of immune molecules on synaptic plasticity, or indirectly via cross talk with the HPA axis and/or its downstream effects on lipid metabolism. Similarly, HPA axis over-activation has the potential to directly or indirectly affect neuropathology. The relationship between the brain and blood based proteomes is of significance to understanding pathophysiology of brain disease. This is referred to in more detail in a review by English and colleagues (2011) [18].

8. Five-year view

For the discovery phase of biomarker development shotgun proteomics using DDA and targeted approaches using SRM are a more established workflow (especially in terms of data analysis) and widely accepted within the proteomic community. However, DIA has huge potential for both the discovery and validation of potential biomarkers [28]. In addition, there are currently limitations in independent validation methods for proteomic experiments. At present, the results of most proteomic experiments must be validated by low-throughput, and often costly. The gold standard for validation experiments is by ELISA. However, alternative techniques such as Western blot, fluorescent bead, chip immunoassay arrays, or surface plasmon resonance (SPR) are also commonly used. DIA has the ability to

validate data-dependent discovery proteomic findings in a high-throughput manner, similarly to targeted approaches like SRM [84]. Therefore, we foresee the development of the DIA workflow and a surge in its use in biomarker development. There is the possibility of the replacement/validation of immunoassay findings with targeted mass spectrometry techniques such as SRM/PRM–which can detect low abundance compounds with sensitivity comparable to that of immunoassays. Therefore, the exceptional analytical specificity of mass spectrometry, mentioned previously, could be better utilized, whilst allowing greater sensitivity (for example to detect low abundance cytokines) than discovery approaches. As previously mentioned, it may be that these approaches are integrated along with other biomarkers discovery approaches in the future in order to provide more accurate and personalized prediction/diagnosis of psychotic disorders [14]. In fact, several groups are working on incorporating clinical, socio-environmental, molecular, neuroimaging and neurophysiological findings for Alzheimer's disease, depressive disorder, schizophrenia in order to identify a particular multifactorial signature specific to each [85–88]. The issue of antibody availability, and thus lack of comprehensive proteome coverage, may eventually be overcome. The Human Protein Atlas is an antibody-based platform which

contains expression and localization profiles for 86% of the predictive human genome. Forty-four different human tissues and annotation data for 83 different cell types are included. The ultimate goal is to continue to extend this analysis to the majority of human proteins [89]. It is clear from this study that there are relatively few proteomic studies conducted in first-onset drug-naive schizophrenia subjects. This is as a result of low recruitment rates of these patients; between 10 and 30 per year, which leads to longer duration studies, and thus longer storage time for samples, which could potentially influence biomarker stability [43]. Another factor to consider in the future with regards to studies which focus on untreated psychosis is that this group is not representative of the whole population of subjects who go on to develop schizophrenia. Future studies will have to account for this possibility in sample collection and interpretation of data. Despite these limitations, studies of this nature are extremely important to ensure earliest possible clinical intervention; the search for disorder specific psychiatric biomarker is hoped to ultimately lead to less subjective and more accurate diagnosis, but also to provide an insight into pathology. The studies discussed here also pave the way for future biomarker studies involving prodromal patients. Furthermore, Perkins and colleagues [90] described the use of clinical high-risk patients who converted to psychosis within 2 years in comparison to those that did not convert (results not included [46]). Chan and colleagues (2015) [43] also described testing the predictive performance of their biomarker panel on help-seeking individuals who developed schizophrenia up to 2 years after baseline, and found an area under the curve (AUC) of 0.82 [43]. These studies demonstrate the potential for investigations involving ARMS or clinical-high risk patients in order to facilitate early intervention and even potentially prophylactic treatment; currently impossible due to the low rate of transition from ARMS to psychosis of about30% [91]. Finally, itis important to note that the establishment of biomarkers may not only be of value in the prediction of schizophrenia, but also in the assessment of treatment response, long-term outcome and clinical phenotypes [6]. Pathway analysis software is currently widely used to look for statistically significant pathways in high-throughput molecular measurements. The obvious limitation to pathway analysis programs such as IPA and STRING (results shown in Supplementary Table 2), for the aim of this review specifically, is their rudimentary knowledge bases. This is true of proteomics data in that new protein functions, and interactions, are being discovered at an extremely fast rate. However, as the number, and indeed type, of functional annotations increase, in parallel with technological advances, the utility of pathway analysis and confidence in results will likely improve [92,93]. In conclusion, despite the fact that high-throughput blood biomarker research in schizophrenia is still in its infancy, studies are beginning to yield consistent findings, in terms of individual protein and pathways. These valuable findings provide an opportunity for the early identification and treatment of these debilitating disorders, and thus lead to better patient outcomes. Mass spectrometry and multiplex immunoassay technologies and platforms are advancing rapidly, and this will no doubt lead to a more accurate, sensitive, reproducible and comprehensive biological signature for schizophrenia.

Key issues

● The field of blood biomarker discovery in schizophrenia is beginning to yield consistent candidates.

● There are limitations associated with using first-onset drug naïve schizophrenia patients; but a shift in focus to at-risk subjects may overcome someof these,whilst enabling earlier clinical intervention and potentially prophylactic treatment.

● Multiplex immunoassays have produced more replicated findings due to their superior sensitivity over current discovery proteomics.

● Proteomic technologies are however rapidly advancing.

● Sensitivity issues associated with proteomic acquisition methods are being overcome by modern targeted approaches (SRM/PRM).

● Ultimately, it may soon be possible to combine the superior specificity of discovery mass spectrometry with the sensitivity of targeted approaches.

● Data-independent acquisition shows great potential for reproducible discovery and 'pseudo-targeted' workflows; for biomarker development or high-throughput validation.

● Currently, issues with DIA include statistical handling of data; in particular, for discovery experiments.
● Pathways implicated include innate and adaptive immune system communication, the acute-phase response, coagulation, glucocorticoid receptor signalling, LXR/RXR signalling and FXR/RXR signalling.

● These processes may converge on synaptic plasticity and in this way contribute to the neuropathology of schizophrenia.

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Declaration of interest

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