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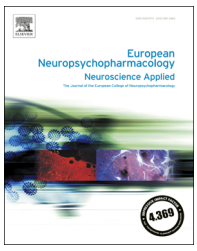

Biomarkers for drug development in early psychosis: Current issues and promising directions

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Abstract
A major goal of current research in schizophrenia is to understand the biology underlying onset and early progression and to develop interventions that modify these processes. Biomarkers can play a critical role in identifying disease state, factors contributing to underlying progression, as well as predicting and monitoring response to treatment. Once biomarker-based therapeutics are established, biomarkers can guide treatment selection. It is increasingly clear that a wide range of potential biomarkers should be examined in schizophrenia, given the large number of genetic and environmental factors that have been identified as risk factors. New models for analysis of biomarkers are needed that represent the central nervous system as a highly complex, dynamic, and interactive system. Many tools are available with which to study relevant brain chemistry, but most are indirect measures and represent only a small fraction of the potential etiologic factors contributing to the molecular, structural and functional components of schizophrenia. This review represents the work of the International Society for CNS Clinical Trials and Methodology (ISCTM) Biomarkers Working Group. It discusses advantages and disadvantages of different categories of biomarkers and provides a summary of evidence that biomarkers representing inflammation, oxidative stress, endocannabinoids, glucocorticoid, and biogenic amines systems are dysregulated and potentially interactive in early phase schizophrenia. As has been recently demonstrated in several neurodevelopmental

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and neurodegenerative disorders, a multi-modal, longitudinal strategy involving a diverse array of biomarkers and new approaches to statistical modeling are needed to improve early interventions based on the fuller understanding.

1. Definitions and potential roles of biomarkers

As defined by an NIH working group, a biomarker "is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to an intervention"(BDWG, 2001). Biomarkers can be used to make a diagnosis, to stage an illness, to predict diagnostic conversion or estimate prognosis, or to predict and monitor clinical response to treatment (FDA, 2014). Efforts to identify biomarkers that differentiate individuals with schizophrenia from healthy controls or other psychiatric disorders are hampered by the heterogeneity of the phenotype and the diverse assortment of genes and environmental factors that have been associated with the illness. It may be more productive to identify subgroups of individuals who share biological patterns in common that may predict preferential response to targeted treatments rather than attempt to find a "schizophrenia biomarker". In order to guide treatment, biomarkers should reflect disease mechanisms that are relevant to the selection of therapeutic options. Some schizophrenia risk genes and environmental factors are involved early in brain development and may produce alterations in cellular differentiation and connectivity. If measured early enough, biomarkers for these factors might identify individuals at risk in order to modify or halt progression of the aberrant neurodevelopmental process. For example, in mouse models of maternal immune activation, interventions directed at maternal inflammatory cytokines protect against neurodevelopmental abnormalities in their offspring (Ito et al., 2010; Pang et al., 2005). Other risk genes and environmental factors are primarily involved in the biochemistry of signaling pathways; treatments for these individuals may target nodes or hubs in these pathways which can restore a more functional equilibrium. Examples include treatments that reduce inflammation or stress if these factors are found to exacerbate symptoms, or treatments that correct molecular deficits produced by genetic variants, such as folate supplementation in individuals with the MTHFR risk allele (Roffman et al., 2013) or drugs that decrease COMT activity (e.g., tolcapone) in Val158Val COMT polymorphism subjects (Apud et al., 2007). The symptoms of schizophrenia reflect a convergence of diverse etiologic factors upon common pathways or networks which can also be identified by biomarkers and can be targeted pharmacologically, as in the case of dopamine D2 receptor antagonism, which improves psychotic symptoms in most individuals regardless of etiology. However, to substantially improve current outcomes it will be necessary to develop a personalized medicine approach that identifies an individual's relevant genetic and environmental risk factors and the resulting biochemical, functional or structural pathology within a neurodevelopmental context and thereby target individualized therapies.

The FDA in their recent guidance on Drug Development Tools (DDT; Qualification Process for Drug Development Tools; (FDA, 2014)) identifies two separate processes necessary to develop novel biomarkers for drug development. The first step is analytical validation in which the characteristics and performance of assays are quantitatively described, including accuracy, precision, reproducibility, linearity, specificity and sensitivity. The second step defines how the biomarker should be used in the context of clinical management or drug development and includes the biomarker's purpose, its boundaries, the conditions of qualified use, and its interpretation. The largest hurdle is to amass enough clinical data to describe the use and its restrictions (i.e., qualification) in the patient setting. In the clinical research literature, statistically significant differences in mean values of analytes or imaging findings between target populations and controls groups are often interpreted as potential biomarkers. However, the predictive value of a biomarker depends on many factors, including generalizability across clinical populations, the reliability of the biomarker based on elements of sample collection, sample preparation and assay method, the sensitivity and specificity of the biomarker for the specific target population and on the relative prevalence of the target population. Due to issues of poor reliability and generalizability, many promising biomarkers have failed replication and for those biomarkers that have successfully achieved replication, overlap in values between groups may be great enough to make the biomarker of little or no clinical value. Very few publications provide the positive predictive value (PPV), i.e. the ratio of the number of true or accurate positive predictions / total number of positive test predictions (both true and false).

When evaluating peripheral biomarkers, it can be difficult to determine whether they reflect active pathologic factors that may serve as targets for intervention. For example, biomarkers reflecting environmental factors that elevate risk in the perinatal period, such as inflammatory cytokines or folate deficiency, may continue to correlate with disease characteristics in adulthood (Meyer, 2013; Roffman et al., 2011). It is not clear whether these biomarkers identify factors that in adulthood are actively contributing to the illness and can be targeted by therapeutic approaches or are factors that disrupted early brain development and are no longer promising targets for treatment. Even if such biomarkers are no longer drug targets they may be useful in enriching a clinical trial sample by removing unwanted variability. In addition, biomarkers may reflect secondary effects of the illness or treatment rather than etiologic factors. For example, cigarette smoking, metabolic syndrome, insomnia and the stress of psychosis can

elevate inflammatory markers which, although associated with the illness, may not play an etiological role in symptom expression (Fawzi et al., 2011; O'Connor et al., 2009).

In the past, models for schizophrenia focused on single molecules, such as dopamine or glutamate; the specificity of these models largely restricted the breadth of biomarker candidates. In contrast, recent estimates point to as many as 6000 to 12,000 single nucleotide polymorphisms (SNPs) that may contribute to risk for schizophrenia (Andreassen et al., 2014; Ripke et al., 2013). The focus has accordingly shifted from individual molecules to pathways as a means of organizing the vast amount of risk data. Relevant biochemical pathways can best be understood as participants in highly interactive networks or "webs" that tend to maintain equilibrium in the face of environmental stress while maintaining plasticity to respond to environmental challenge (Fienberg et al., 1998). Due to the high degree of interactivity, individual components within a network may influence many other factors and, in turn be regulated by multiple factors. A single biomarker may be abnormal because of multiple modulatory inputs and hence may not represent a primary etiologic factor nor a treatment target as it may merely reflect rather than influence a more fundamental dysregulation of a larger network. On the other hand, single genes such as microRNAs can affect the functioning of hundreds of target genes (Delalle et al., 2014; Potkin et al., 2010; van Erp et al., 2014). More complex models are required to represent these highly interactive patterns and to identify sources of disequilibrium that may be contributing to the illness. New multivariate approaches have been developed to identify causal biomarkers from high-throughput genetic data and may be applied to this problem (Alekseyenko et al., 2011). Studies employing genomics, transcriptomics, proteomics, lipidomics and methylomics (Aberg et al., 2014; English et al., 2011; Prabakaran et al., 2004) identify large numbers of potential biochemical risk factors in a black box approach unconstrained by existing models. It is also possible to sample representative pathway biomarkers to assess key factors, such as inflammation, oxidative stress, hypercortisolemia, growth factors and methylation that can be used to identify disease-related dysregulation of fundamental processes that influence a broad range of relevant biochemical interactions. In addition, points of convergence that are more proximal to symptom generation can be studied as a link between key networks and symptoms. One example is the AKT/mTor pathway that links multiple environmental inputs, such as stress and inflammation, to neuronal plasticity and cell survival and may mediate some effects of antipsychotic medication (Bowling et al., 2014).

The identification of illness-related biomarkers can be confounded by medication treatment, social factors, and other modifying genes. Studying subjects at genetic or clinical high-risk for developing schizophrenia (e.g., non-ill relatives or at-risk adolescents) can avoid the confounds of medication and disease status. Non-ill high-risk subjects may have the genetic vulnerability, but the vulnerability has been insufficient to produce the disease, possibly because of the lack of additional genetic or environmental influences or the presence of compensatory mechanisms. Studies of adolescents at high-risk based on prodromal symptoms or familial risk have the advantage of longitudinal follow-up so that disease- and risk- related events can be separated. However, such studies require many years to complete and considerable resources. One approach to addressing these limitations is to study individuals with schizotypal personality disorder, a disorder within the schizophrenia spectrum. Converging data support a continuum from severe chronic schizophrenia to the attenuated schizophrenia-like traits of schizotypal personality disorder, sharing genetic, brain and psychophysiologic biomarkers (Siever and Davis, 2004). Individuals with schizotypal personality disorder may be an ideal population for biomarker discovery and testing of novel treatments targeted at those biomarkers (Rosell et al., 2015).

DNA, blood, CSF and imaging-based biomarkers all provide information which may differ in relevance according to the particular question that is addressed. Increasingly, combinations of peripheral, genetic and imaging biomarkers are successfully testing hypotheses about relationships between biological mechanisms, structural and functional brain imaging changes, and neurodevelopment which may lead to therapeutic interventions (Mondelli et al., 2011; Pagliaccio et al., 2014; Roffman et al., 2013). This is in contrast to studies in oncology that have tended to find that a focus on the single biomarker most proximal to the illness, e.g., gene expression, may be more predictive than a multimodal analysis (Ray et al., 2014). One promising example of a multimodal approach is the North American Prodrome Longitudinal Study (NAPLS) Consortium, which is examining multimodal imaging and peripheral biomarkers to understand factors contributing to progression from prodrome to schizophrenia (Cannon et al., 2014). Another successful example of an integrated approach to biomarkers is the Alzheimer's Disease Neuroimaging Initiative (ADNI) which has combined clinical measures, imaging, genetics, and CSF and blood omics biomarkers in the longitudinal analysis of individuals at risk for developing Alzheimer's Disease (Weiner et al., 2013). In this paper we will provide an overview of current biomarkers for clinical characterization and monitoring of drug treatment in early stage schizophrenia and make recommendations based on existing models. While these biomarkers may be relevant to other treatment modalities, including cognitive remediation, neuromodulation and psychosocial interventions, we have focused on biomarkers for drug development (Figure 1). In addition, whereas post-mortem brain markers may contribute to identification of pathways for drug development, this review focuses on biomarkers that are potentially available in clinical practice.

2. Categories of biomarkers

2.1. Brain imaging

There are many strategies for using imaging-based biomarkers for drug development and for clinical decision making. Early in the process of drug development, PET ligand occupancy studies can establish target engagement to verify bioavailability and guide dose finding (e.g., defining the dose range for lurasidone with 18F-fallypride PET) (Potkin et al., 2014). In a recent example, Umbricht and colleagues (Martin-Facklam et al., 2013; Umbricht et al., 2014) used

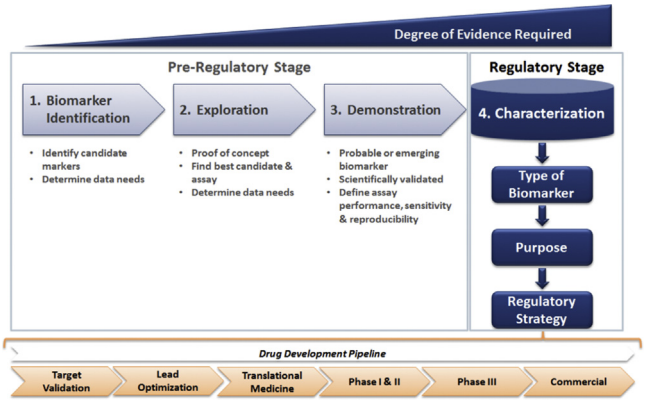

Figure 1 Graphic description of the continuum of biomarker development. The determination of the type of biomarker pertains to a specific category, such as diagnostic, prognostic, predictive, pharmacodynamics, surrogate endpoint, etc. Determining the purpose of the biomarker, helps define the specific regulatory pathway, which in turn informs the Regulatory strategy for a medical device or a drug development tool.

PET occupancy studies and CSF measures of glycine concentrations to establish the range of therapeutic doses for clinical trials of the GlyT1 inhibitor, bitopertin. In the absence of a specific PET ligand, functional MRI and electrophysiological approaches can be used to identify alterations in brain function that may serve as more sensitive assays than behavioral observation for identifying CNS drug effects. Such measurable brain effects are arguably more proximal to the etiological cause than symptoms that are influenced by many factors, including environmental context. Measurable changes in biomarkers associated with treatment, if sufficiently sensitive and specific, can allow smaller sample sizes in clinical trials or can make possible studies of shorter duration if the biomarker predicts longer-term outcome. This approach has been used in other areas of medicine, e.g., hypercholesterolemia and blood pressure as biomarkers for long-term cardiovascular consequences such as myocardial infarction. Additionally biomarkers can be used to enrich samples in clinical trials by eliminating subjects with symptoms that do not vary, e.g., prodromal subjects who, based on biomarkers, are not predicted to progress to schizophrenia. Structural MRI and diffusion tensor imaging have been used to monitor changes in volume and integrity of brain components which may predict the long-term course for trials of treatments that employ neuroprotection or facilitate neuroplasticity (Eack et al., 2009). Similarly, changes in connectivity measured by resting state fMRI correlations may identify alterations in plasticity associated with cognitive enhancement (Díaz-Caneja et al., 2015; Wang et al., 2014). MR spectroscopy provides estimates of concentrations of molecules relevant to drug treatment, and has produced promising findings related to neuronal integrity (NAA), glutamate signaling (Glx), GABA, and oxidative stress (glutathione) (Monin et al., 2014; Salavati et al., 2015). Poor reproducibility of single voxel MRS has been problematic for biomarker research; however, by summing the phase-aligned and frequency-aligned spectra from a larger brain volume or using stronger magnets, intra-individual coefficients of variation can be reduced below 10% for most analytes (Kirov et al., 2012). Taken together, imaging approaches provide the most direct brain measures to classify patients and evaluate drug effects but of these modalities, only PET and MRS provide information about biochemical pathways that mediate pharmacologic treatment response. For the limited number of molecules for which PET ligands are available, PET remains the gold standard biomarker.

Structural neuroimaging is the most studied brain endophenotype in psychiatry and offers the opportunity to understand the anatomy of schizophrenia, but does not provide sufficient information regarding the mechanisms of the observed pathology. Functional brain imaging (fMRI, MRS, and FDG PET) represents a more direct measure of the functional consequences of genetic risk, although abnormalities of brain structure and function are often related. As genetic risk genes do not operate in isolation, genetic pathways and/or abnormal circuitry may be closer to the underlying causal mechanisms than the abnormal structure or function of any single anatomical brain area. Circuitry dysfunction may infer greater risk than the individual components of the circuitry. Similarly, pathways may infer greater risk than the individual genes that comprise the pathway. Resting state activity is measured in a non-task condition, e.g., at rest, and can be used to identify networks of co-activated brain areas. A recent analysis demonstrated a significant association between copy number deletion burden, a known risk factor for schizophrenia, and disordered resting state functional connectivity, poor cognitive performance, and changes in regional brain volume (Martin et al., 2014). Brain imaging can also clarify the brain effects of candidate risk genes. For example, Walton and colleagues (Walton et al., 2013) found that neurogranin SNPs that have been linked to schizophrenia predicted cortical thickness and abnormal patterns of activation during a working memory task, thereby linking specific functional and morphometric imaging biomarkers with neurogranin genetic risk, a possible etiological mechanism. Initiatives such as the Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia (CNTRICS) aim to identify new biomarkers including neuroimaging to measure cognitive deficits and their response to new therapies, including cognitive remediation, social cognitive training and intranasal oxytocin (Millan and Bales, 2013; Perez-Rodriguez et al., 2014).

Brain imaging can be used as a quantitative trait (QT) to discover unanticipated risk genes for schizophrenia. In a sample of less than 200 subjects, dorsolateral prefrontal cortex fMRI activation was measured during a working memory task and used as a QT to identify unanticipated risk SNPs for schizophrenia including TNIK, a regulator of glutamate and AMPA transmission (Potkin et al., 2009). A similar quantitative imaging genetics strategy identified microRNA-137 as an unanticipated risk factor for schizophrenia which was confirmed in large case-control studies and described as an “etiological” mechanism for schizophrenia (Potkin et al., 2010; Ripke et al., 2011). Gene regulatory networks can be inferred from expression profiles, regulatory motifs, and micro-RNAs and used to determine which group of genes or pathways is over-enriched in relation to a diagnostic disorder or cognitive dysfunction.

2.2. Cerebrospinal fluid

Although infrequently studied in schizophrenia, cerebrospinal fluid (CSF) has been a highly informative source of biomarkers in Alzheimer's disease and neuroinflammatory disorders (Aluise et al., 2008; Meeker et al., 2011). Because CSF readily transverses gap junctions to fill spaces between brain cells, it contains solutes secreted from brain cells, exosomes, and cell membrane fragments (Johanson et al., 2008; Delalle et al., 2014). CSF is produced within the cerebral ventricles by the choroid plexus which filters blood at a rate of approximately 0.4 ml/min, replacing the full volume of CSF every 6 h. In addition, some blood solutes are found in low concentrations in CSF as a result of passive diffusion, facilitated transport and active transport. Functioning like the kidney, the choroid plexus maintains homeostasis of the brain's extracellular fluid by providing micronutrients, vitamins, peptides, nucleosides and growth factors to brain cells; ageing and illness, including oxidative stress, may compromise this function (Johanson et al., 2008). Animal studies suggest that the CSF provides the portal of entry and pathway for circulation of peripheral immune cells in response to inflammation (Schmitt et al., 2012). CSF biomarkers thus may be informative by identifying invading immune cells, inflammatory cytokines and pathogens, disease-associated extracellular proteins, alterations in choroid plexus secretory patterns, and diffusion from blood, particularly if the blood brain barrier has been breached. Transthyretin, which is synthesized primarily by the choroid plexus, serves as a biomarker for choroid plexus function, whereas albumin, which is not synthesized in the CNS, serves as a marker for blood brain barrier integrity. A large decrease in CSF tranthyretin levels was found in two samples of first episode schizophrenia subjects compared to healthy and psychiatric controls (Huang et al., 2006); this evidence for impaired choroid plexus function is all the more impressive given that schizophrenia subjects had much higher rates of cigarette smoking– nicotine has been shown to stimulate tranthyretin secretion in rats (Li et al., 2000). Studies of the ratio of albumin in CSF and blood have indicated that as many as 30% of schizophrenia patients have a significant disruption of the blood brain barrier (BBB) (Muller and Ackenheil, 1995); increased permeability of the BBB has been linked to inflammatory markers and negative symptoms in patients (Schwarz et al., 1998) and to first and second generation antipsychotics in rat studies (Ben-Shachar et al., 1994). Schizophrenia patients have disrupted sleep spindles and slow wave sleep (Manoach et al., 2014). Sleep is related to clearance of a beta and other toxic substances (Xie et al., 2013). Neural cells are highly sensitive to their chemical environments and waste products must be efficiently removed from brain interstitial space into the blood. This “glymphatic transport” can affect both CSF and blood biomarker measures (Plog et al., 2015).

2.3. Blood markers

The brain is unique among organs in its sequestration from the circulating blood and so measurements of constituents of blood may poorly reflect relevant brain concentrations. The separation of capillaries from extracellular fluid in brain is accomplished by tight junctions between endothelial cells and by the choroid plexus which separates CSF from blood. The BBB protects the brain from bacterial infection and from circulating antibodies while also preventing the passage of neuroactive substances from blood that would otherwise disrupt brain signaling. Sequestration of relevant biochemical factors can occur at a cellular level as well; for example, neither kynurenic acid or quinolinic acid cross the blood brain barrier—kynurenic acid, which modulates NMDA receptor activity, is only synthesized in astrocytes, whereas potentially neurotoxic products of the kynurenic pathway are only found in microglia where they are used in immune defense (Schwarcz et al., 2012). Blood contains thousands of proteins and peptides which vary in concentration by a factor of 10^{15}. Albumin and IgG comprise 80% of proteins in blood; the roughly 20 proteins that are found in the highest concentrations must be removed to allow assay of lower concentration proteins that would otherwise be masked (Aluise et al., 2008). Preparation of blood samples, which varies according to the biomarker of interest, is also critical to ensure valid assays and reliability between studies (Luque-Garcia and Neubert, 2007). Inconsistencies in the clinical conditions under which samples are obtained, in their preparation, and in analytic methods have contributed to the frequent failures to replicate blood biomarkers; an effort is underway to standardize these factors (Poste et al., 2014). Plasma and serum have relative advantages and disadvantages; plasma requires an anticoagulant that may affect some analytes (Hosnijeh et al., 2010); in contrast, during preparation of serum samples, coagulation may release substances, such as BDNF and inflammatory cytokines, from platelets and other cells, thus altering the proteome. However, in general, sensitivity of serum is higher than plasma whereas reproducibility of plasma is greater (Yu et al., 2011). Best studied is BDNF, for which plasma and whole blood concentrations have been found to correlate reasonably well with brain levels (r=0.6–0.7) in rats and pigs (Klein et al., 2011) and with CSF in humans (r=0.5) (Pillai et al., 2010). The composition of blood may be highly influenced by diet, medications, activity and diseases of peripheral organs, glymphatic and blood brain barrier integrity can be compromised by inflammation and other disease processes, further complicating blood-based biomarkers. All of these factors make establishing reliability and validity of blood biomarkers a challenge.

3. Treatment-related genetic markers

Functional polymorphisms of target receptors and drug metabolizing enzymes can greatly influence pharmacokinetic and pharmacodynamic relationships for a given drug. Functional polymorphisms of drug metabolizing enzymes including CYP2D6, CYP2C9, CYP2C19 can result in large variations in the systemic exposure of the drug with a given dose. For example, poor CYP2D6 metabolizers can develop toxic levels of antipsychotic drugs (e.g., aripiprazole, iloperidone, thioridazine, brexpiprazole), while ultra-rapid metabolic status is associated with sub-therapeutic levels at recommended doses (Drozda et al., 2014). Genetic biomarkers may also predict adverse drug events. Dopamine

3 receptor (DRD3) variants have been associated with the development of tardive dyskinesia (TD) following antipsychotic treatment (Shamy et al., 2011). Genetic variants have also been associated with antipsychotic-induced weight gain (e.g., HTR2C (Kao and Muller, 2013); the strongest association appears to be between the melanocortin 4 receptor (MC4R) gene (rs489693) and second-generation antipsychotic-induced extreme weight gain (Malhotra et al., 2012). A strong association between genetic variation in HLA-DQB1 (6672 G > C) and risk for clozapine-induced agranulocytosis has also been identified (Athanasiou et al., 2011). Examples of genetic biomarkers predicting therapeutic response to antipsychotics include genetic variation in the serotonin 2 A receptor (HTR2A) and the dopamine 2 receptor (DRD2) (Zhang and Malhotra, 2011). In addition, combinations of risk genes may enhance prediction, as in the example of six risk genotypes (SNPs in NPAS3, XKR4, TNR, GRIA4, GFRA2, and NUDT9P1) that when combined into four approximately equal-sized groups highly predicted response to iloperidone but not ziprasidone in a discovery sample (Volpi et al., 2009). Such approaches may have important clinical relevance for drug development in early psychosis by decreasing subject heterogeneity and could lead to a precision medicine approach in which treatment is targeted to subgroups of patients based on genetic predictors of response and side effects.

Gene expression in peripheral lymphocytes has been utilized as a marker for gene expression in brain, although the relationship between the two is not well-established. It has been estimated that roughly 50% of schizophrenia candidate genes are expressed in both blood and brain and that the median correlation for gene expression between blood and brain is 0.5 (Sullivan et al., 2006). Comparison of blood and brain gene expression from separate groups of patients and controls found that 6 of 123 putative peripheral gene expression biomarkers were also differentially expressed in brains of individuals with schizophrenia (Glatt et al., 2005). Even within the brain, different cell types differ markedly in gene expression, as was recently demonstrated by the comparison of pyramidal neurons and parvalbumin-positive interneurons in superior temporal cortex in schizophrenia versus healthy controls (Pietersen et al., 2014a, 2014b). In addition, abnormalities in gene expression differ markedly by brain region, as demonstrated by oligodendrocyte-related genes (Haroutunian et al., 2007). Studies of peripheral blood mononuclear cell (PBMC) gene expression in medication-naïve schizophrenia patients have implicated pathways involving AKT1 related to inflammation and neurodevelopment, many of which normalized with antipsychotic treatment, although the direction of abnormal baseline expression and change has not been consistent between studies (Kumarasinghe et al., 2013; van Beveren et al., 2012). Gene expression in PBMCs may be informative to the extent that they reflect the interactive influence of the full genome and these cells share many receptors in common with CNS cells, particularly with microglia. Additionally, gene expression may not be a fully reliable indicator of protein expression or protein activity. For many molecules that are relevant to brain development and function, intermediate factors, such as AMPK and mTOR, regulate the translation from gene expression to protein synthesis based on the cell's energy status (Mihaylova and Shaw, 2011). For kinases like AKT, phosphorylation status is more relevant than protein concentration (Franke, 2008). Despite these many theoretical limitations, PBMC expression of inflammatory and growth factor genes predicted hippocampal volume in early schizophrenia (Mondelli et al., 2011). Another approach to clarifying the relationship between genotype and gene expression is the analysis of DNA methylation which provides information about early environmental or inherited factors that influence gene expression. Analysis of the DNA methylome from whole blood in schizophrenia participants revealed altered methylation of multiple genes, including genes involved in hypoxia, infection and microRNA regulated networks linked to neuronal differentiation and dopamine receptor expression (Aberg et al., 2014). In peripheral lymphocytes, gene expression of enzymes involved in methylation and demethylation are also altered in schizophrenia samples versus controls (Auta et al., 2013). In addition, microRNA, which also modulates gene expression, may also be dysregulated in schizophrenia (Potkin et al., 2010; Zhang et al., 2014) although findings in PBMCs have not been consistent between studies (Delalle et al., 2014).

4. Factors implicated in early schizophrenia by biomarkers

4.1. Inflammation

It has been estimated that 40% of genes linked to schizophrenia are involved in the brain's response to infection (Carter, 2009). For example, one of the strongest linkages is with the major histocompatibility complex which mediates several aspects of immune response (2014). Among the best established environmental risk factors for schizophrenia are exposure *in utero* to maternal infection, traumatic stress and pre-eclampsia—all conditions known to release inflammatory cytokines (Gilmore and Jarskog, 1997). Mice exposed to maternal immune activation (MIA) exhibit many structural and functional deficits associated with schizophrenia along with increased expression of genes associated with hypoxia and cell death and decreased expression of genes associated with neurodevelopment—particularly with the migration of GABAergic interneurons (Oskvig et al., 2012). Studies in mice have identified alterations in neuronal migration and synaptic function in MIA exposed offspring, in addition to microglial priming and dysregulation of immune function (Knuesel et al., 2014). Alterations in pro-inflammatory and anti-inflammatory cytokine levels in offspring differ between brain regions and by developmental stage and did not correlate with blood cytokine levels (Garay et al., 2013). When applied to schizophrenia this model suggests that early neurodevelopmental exposure to inflammation may alter the regulation of CNS neuroinflammatory components but these changes may not be detected reliably by assay of standard peripheral biomarkers. The identification of a biomarker signature that would identify individuals with an early developmental history of MIA potentially could guide the clinical application of preventive interventions derived from this animal model (Zhu et al., 2014).

There are several hypotheses to explain the association between onset of illness and inflammation (Meyer, 2013). As a result of early exposure to inflammation, microglia may be "primed" and release excessive levels of inflammatory cytokines in response to infection or stress in young adulthood. The excessive neuroinflammatory response may disrupt glutamatergic signaling, diminish parvalbumin (PV) expression in interneurons, reduce neuroplasticity and promote neuronal injury or death. Priming of microglia may result from interferon gamma (IFNg) exposure which shifts cells towards the release of pro-inflammatory cytokines (Bsibsi et al., 2014). If the inflammatory process is peripheral, tumor necrosis factor-alpha (TNFα crosses the blood brain barrier and stimulates release of inflammatory cytokines in brain; in addition, IL-6 increases permeability of the blood brain barrier (Brett et al., 1995). As a compensatory response to early inflammatory exposure, anti-inflammatory or neuroprotective mechanisms, such as the shunting of tryptophan metabolism towards kynurenic acid (KYNA) production, may contribute to pathological antagonism of NMDA receptor transmission (Schwarcz et al., 2012). It is also possible that alterations in inflammatory markers are a consequence, rather than cause, of illness onset since the psychological stress associated with psychosis might produce an elevation of inflammatory markers.

The most direct marker for CNS inflammation is provided by PET ligands targeting the mitochondrial translocator protein (TSPO, aka peripheral benzodiazepine receptor protein [PBR]), which is expressed by activated microglia. Binding of the TSPO ligand, [11(C)]PBR28, was shown to increase by 62% following systemic infection with e. coli in nonhuman primates; this increase in activated microglia significantly correlated with elevations in serum concentrations of the inflammatory cytokines, Il-1b and IL-6 (Hannestad et al., 2012). Studies with first and second generation TSPO ligands in schizophrenia have produced inconsistent evidence for microglial activation (Doorduin et al., 2008; Kenk et al., 2014; Takano et al., 2010; van Berckel et al., 2008); evidence for neuroinflammation was strongest for early stage patients and most prominent in the hippocampus. The poor selectivity of TSPO binding limited interpretation of results from the first generation of ligands (e.g., 11C-PK11195) and genetically-determined differences in TSPO binding affinity complicates interpretation of newer ligands such as 18F FEPPA (Owen et al., 2012). In addition, microglial activation may not always signify a neurotoxic process since microglia are also involved in neurogenesis, pruning, and can play a neuroprotective role (Chen et al., 2014; Wake et al., 2013).

Blood and CSF markers in schizophrenia suggest a mixed picture early in the course of illness of both inflammatory and anti-inflammatory markers, which may reflect dysregulated immune activation and compensatory neuroprotective responses to chronic inflammation (Drexhage et al., 2011; Freudenreich et al., 2010). Inflammatory cytokines in blood are elevated in acute psychosis along with elevated CSF concentrations of the neuroprotective NMDA antagonist, kynurenic acid (Linderholm et al., 2012), the anti-inflammatory endogenous cannabinoid, anandamide (Leweke et al., 2007) and the glial-secreted molecule, S100B which has concentration-dependent neuroprotective or neurotoxic activity (Aleksovska et al., 2014; Yelmo-Cruz et al., 2013). Of the cytokines that are reliably measured in CSF (IL-6, IL-8 & IL-1B), only IL-1B was elevated in CSF of first episode patients (Soderlund et al., 2009). IL-1B is released by activated microglia under conditions of inflammation and can be released in response to psychological stress (O'Connor et al., 2009). IL-1B stimulates inflammatory pathways, increases DA and cortisol levels (Song et al., 2007) and increases kynurenic acid levels by inducing the TDO (tryptophan 2,3-dioxygenase) enzyme (Lavebratt et al., 2014). IL-1B also inhibits human hippocampal neurogenesis (Zunszain et al., 2012). In chronic patients, PBMC gene expression studies suggest a shift from Type I inflammatory response to a Type 2 anti-inflammatory states (Freudenreich et al., 2010). In a recent meta-analysis, Miller and colleagues found that inflammatory cytokines IL-1B, IL-6, and TGF-beta are elevated in blood during episodes of acute illness, whereas elevation of IL-12, IFN-gamma, TNF-alpha, and sIL-2R may be trait markers (Miller et al., 2011). In serum samples of medication-naïve first episode psychosis subjects, IL-1B, IL-6, TNFα were found to decrease with risperidone treatment (Song et al., 2014). In a sample of subjects from the NAPLS study of individuals at high risk for conversion from prodrome to schizophrenia, blood levels of inflammatory cytokines predicted gray matter loss and transition to schizophrenia (Cannon et al., 2014). In summary, inflammatory markers in blood and IL-1B in CSF are elevated in acute, early psychosis, possibly reflecting immune cell "priming" in utero followed by a "second hit" in young adulthood which may be an infectious process or psychological stress. While it may not be clear whether inflammation is a cause or consequence of psychosis, it interacts with multiple transmitters and molecular networks implicated in schizophrenia and inflammatory biomarkers appear to be predictive of illness course.

4.2. Cannabinoids

The endocannabinoid system also links inflammation, cortisol release and stress to schizophrenia and modulates glutamate, dopamine and GABA transmission (Pistis et al., 2002). The cannabinoid CB1 and CB2 receptors are found in peripheral and CNS stress-responsive circuits and immune cells and may be viewed as a primary homeostatic mechanism (Bioque et al., 2013). PET studies using the [^{18}F]MK-9470 CB1 ligand found upregulation of CB1 receptors in the mesocorticolimbic regions in drug-naïve schizophrenia patients which inversely correlated with negative symptoms and depression (Ceccarini et al., 2013). It is not clear whether these alterations in CB1 receptors represent a pathological mechanism or a compensation in response to dopamine dysregulation. In contrast, CB2 knockout mice display behaviors consistent with schizophrenia (Ortega-Alvaro et al., 2011).

The endogenous cannabinoid receptor agonist, anandamide, reduces inflammation by blocking microglial activation and stimulates neurogenesis. Anandamide concentrations in CSF were increased 10-fold in medication-naive first episode patients who were not heavy cannabis abusers (lifetime use $\leq$ 5 times) and inversely correlated with psychotic symptoms (Leweke et al., 2007). In contrast, medication-naïve first episode schizophrenia subjects who were heavy cannabis users

(lifetime use>20 times) did not exhibit elevation of CSF anandamide concentrations, even if they had been abstinent from marijuana for several weeks. Elevated concentrations of CSF anandamide were also found in individuals who met criteria for ultra-high risk—among this group, lower CSF levels of anandamide predicted progression to schizophrenia (Koethe et al., 2009). While CSF anandamide appears to be a promising biomarker, serum anandamide concentrations failed to differentiate groups (Leweke et al., 2007). In PBMCs, mRNA for the endocannabinoid synthesizing enzymes, N-acyl phosphatidlyethanolamine phospholipase (NAPE) and diacylglycerol lipase (DAGL) were decreased and mRNA for the endocannabinoid degrading enzymes, fatty acid amide hydrolase (FAAH) and monacylglycerol lipase (MAGL) were increased (Bioque et al., 2013). FAAH expression inversely correlated with total symptom scores (Bioque et al., 2013). Cannabidiol, an endogenous low-affinity agonist at cannabinoid receptors, elevates anandamide levels by blocking degradation by FAAH, and was shown in one study to have potent antipsychotic effects, comparable to the antipsychotic, amisulpiride (Leweke et al., 2012). Cannabidiol reduces release of Th1 pro-inflammatory cytokines and increases release of Th2 anti-inflammatory cytokines. In summary, CSF anandamide is elevated in first episode patients with psychosis, shifts the immune response from pro-inflammatory to anti-inflammatory, decreases cortisol release, and is associated with reduced symptom expression. Potential biomarkers include CSF anandamide concentrations and PBMC expression of NAPE, FAAH and MAGL.

4.3. Glutamate

The ketamine model of schizophrenia posits decreased activation of glutamatergic NMDA receptors on inhibitory GABAergic interneurons resulting in excessive glutamate release from pyramidal neurons and disruption of gamma oscillatory synchrony (Lisman et al., 2008; Olney and Farber, 1995). In mice, NMDA antagonists produce an inflammatory response which increases oxidative stress and decreases PV (parvalbumin) expression of inhibitory GABAergic interneurons (Behrens and Sejnowski, 2009). Glutamate concentrations are elevated in CSF and blood in medication-free schizophrenia patients (Beckmann and Gattaz, 2002) and elevated levels of glutamate measured by MRS have been associated with hippocampal volume loss (Kraguljac et al., 2013). Serum glutamate levels increase with antipsychotic treatment (Beckmann and Gattaz, 2002; Evins et al., 1997; Goff et al., 2002) and were found to increase following a switch from haloperidol to olanzapine (Goff et al., 2002). Olanzapine-associated elevation in cingulate cortex glutamate and glutamine (Glx) levels predicted improvement of negative symptoms (Goff et al., 2002). In healthy subjects, CSF and serum glutamate concentrations were significantly correlated (r=0.67) (Alfredsson et al., 1988). Kynurenic acid (KYNA) a metabolite of tryptophan, and an NMDA antagonist, has been found to be elevated in CSF of patients with schizophrenia (Linderholm et al., 2012) and bipolar patients with psychotic features (Olsson et al., 2010). As an antagonist at the glycine site of the NMDA receptor, KYNA is neuroprotective against glutamatergic excitotoxicty but may contribute to psychosis and cognitive impairment. Because KYNA does not cross the blood brain barrier, blood levels are not informative; however kynurenine is actively transported into the brain and metabolized by astrocytes to KYNA (Javitt, 2014). Plasma concentrations of tryptophan and its metabolites did not differ between first episode patients and healthy controls (Condray et al., 2011). In summary, dysregulation of glutamate signaling is linked to inflammation and symptoms of schizophrenia; kynurenic acid in particular may play a role in symptom production in schizophrenia but because it does not cross the blood brain barrier, only CSF levels appear to be informative.

4.4. Oxidative stress

Oxidative and nitrosative stress result from an imbalance between release of reactive oxygen and nitrogen species and a deficiency in endogenous antioxidants. The brain is particularly vulnerable because of its high utilization of oxygen and the presence of redox-active metals. Oxidative stress can damage lipid membranes, proteins and DNA while also dysregulating redox-sensitive processes such as NMDA receptor activation, cell differentiation and protein synthesis. Oxidative stress also impairs mitochondrial function, oligodendrogia-mediated myelination and expression of PV in interneurons. Deficits in antioxidant capacity may result from genetic vulnerability, or may result from early developmental exposure to maternal immune activation. In addition, synthesis of the endogenous antioxidant, reduced glutathione (GSH), requires the trans-sulfuration pathway which is dependent on folate. GSH, glutathione disulfide (GSSG) and glutathionylated proteins (PSSG) are sensitive and reliable markers of oxidative stress but blood samples require pretreatment to prevent oxidation (Rossi et al., 2006). Thioredoxin and thioredoxin reductase are antioxidants that are released from neurons, glial and the choroid plexus in the presence of oxidative stress (Silva-Adaya et al., 2014) and are involved in cell survival, cell proliferation and differentiation. Serum thioredoxin levels were elevated in medication naïve FEP patients compared to healthy controls and levels significantly correlated with psychosis, whereas levels were not elevated in chronic patients suggesting an inflammatory process associated with onset of schizophrenia (Zhang et al., 2009). In medication naïve childhood onset FEP, red blood cell total GSH significantly predicted brain volume loss over a two-year follow-up (Fraguas et al., 2012). In antipsychotic-naïve FEP patients, baseline blood measures of antioxidant status and of oxidative stress significantly predicted cognitive functioning after 6 months of treatment (Martinez-Cengotitabengoa et al., 2012).

4.5. Cortisol

Glucocorticoids are released by the hypothalamus in response to stress and act on glucocorticoid and mineralocorticoid receptors to rapidly modulate neuronal activation while also modulating gene expression (Groeneweg et al., 2011). Glucocorticoids act on dopamine pathways to modify stress-related behavior and cognitive function (McEwen, 2013). Early exposure to stress may produce epigenetic changes to ventral tegmental dopamine neuron firing relevant to psychosis (Niwa et al., 2013; Pruessner et al., 2004).

Acute stress potentiates prefrontal cortical glutamatergic signaling and enhances working memory, whereas chronic stress or glucocorticoid treatment produces reversible neuronal atrophy in prefrontal cortex and hippocampus and loss of neurogenesis (Sousa and Almeida, 2012). The prefrontal cortex in adolescence is particularly sensitive to chronic stress (Yuen et al., 2012). In humans, low parental care is associated with increased ventral striatal dopamine release in response to stress, but not in subjects with high parental care; salivary cortisol concentrations positively correlated with striatal dopamine release under stress (Pruessner et al., 2004). Studies of salivary and blood cortisol levels in first episode psychosis have generally found hypercortisolemia which normalizes with antipsychotic treatment and does not consistently correlate with symptoms (Karanikas et al., 2014; Mondelli et al., 2010). Salivary cortisol levels have predicted decreased hippocampal volume in first episode schizophrenia (Mondelli et al., 2011) and PBMC expression of glucocorticoid receptor genes has also predicted hippocampal volume and response of depression to treatment (Cattaneo et al., 2013).

4.6. Methylation

Epigenetic factors influence DNA methylation of genes involved in neurodevelopment, including BDNF and the glucocorticoid receptor. Two enzymes involved in DNA methylation and demethylation pathways, DNA-methyltransferase (DNMT1) and ten-eleven translocator-1 methylcytosine deoxygenase (TET1), are increased in corticolimbic structures in schizophrenia brain. Lymphocyte gene expression of these enzymes is similarly elevated in first episode and chronic patients, but failed to correlate with symptoms in chronic patients who were treated with antipsychotics (Auta et al., 2013). BDNF gene expression, which is regulated by methylation/demethylation pathways, significantly negatively correlated with lymphocyte expression of DNMT1 (Auta et al., 2013). A third trimester dietary deficiency of folate, which is the primary source of methyl groups, is associated with increased risk of neurodevelopmental deficits, including schizophrenia. Serum folate concentrations are highly correlated with CSF concentrations ($r=0.7$) across a typical range of concentrations, but at high serum concentrations achieved with folate supplementation, CSF concentrations plateau (Obeid et al., 2007). In patients with schizophrenia, low serum folate concentrations in combination with genotypes associated with a reduced capacity to absorb and convert dietary folate to the methyl-donor (e.g., SAM, S-adenosylmethionine) predict negative symptoms and their response to folate supplementation (Roffman et al., 2011, 2013). A deficit of methylation capacity might also impair antioxidant defenses by decreasing glutathione synthesis, impair NMDA receptor transmission by elevating homocysteine levels, and disrupt synthesis of neurotransmitters and DNA (LaSalle, 2011).

5. Recommendations

For a molecular characterization of illness onset and progression that could guide pharmacologic interventions, direct longitudinal measurement of gene expression and protein concentrations within individual cell types and across brain regions would be ideal to construct a dynamic, interactive model. However, in clinical practice we are left with very indirect measures from blood, imaging, and possibly CSF. One cannot assume that peripheral blood biomarkers reflect CNS concentrations as the relationship between peripheral blood and CNS concentrations can be quite variable. The ultimate predictive value of models based on these markers remains to be established, although current findings with inflammatory markers in schizophrenia have been quite promising. The emerging technology of induced pluripotent stem cells (IPS), derived from peripheral somatic cells of patients with schizophrenia, opens a promising new avenue to explore disease mechanisms of schizophrenia. IPS cells provide a viable source of human central nervous system cell lines – including neurons – for biomarker discovery and early testing of target engagement, mechanism and response to medications (Brennand et al., 2011).

Given the large inter-individual variability in biomarker patterns, in blood brain permeability, and in genetic variants associated with risk, longitudinal studies examining within subject change in large samples, employing genotyping along with CSF, PET and MRS measures, may be most informative to develop predictive models for drug development. Peripheral biomarkers can be identified which best correlate with these more direct measures of brain biochemistry. For such efforts to be successful, close attention must be paid to subject selection, standardization of conditions under which biomarkers are obtained, methods of sample preparation, and analytic methods. In addition, new methods for data analysis must be employed to derive and compare the multiple permutations of combinations of variables that represent multiple pathways to illness onset and progression.

Finally, an important question is the appropriate clinical role for biomarker and genetic testing in clinical practice. This is especially relevant since such testing is becoming increasingly available from commercial vendors. There are important social, privacy, policy, and ethical issues involved in the widespread adoption of such testing, including the need to train clinicians on their meaning and appropriate use.

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Drs.Goff and Potkin wrote the first drafts of the manuscript.All authors contributed to and have approved the final manuscript. The manuscript reflects the International society for CNS Clinical Trials and Methodology (ISCTM) Biomarkers Working Group's deliberations over the past two years.

Conflict of interest

In the past three years, Steven Potkin has received grant support, funding, honoraria, or has been a paid consultant to the following companies that conducted scientific or medical research and/or marketed medications related to psychiatric and

neurodegenerative disorders: Alkermes, Amgen, Eli Lilly, FORUM Pharmaceuticals, Genentech, Janssen Pharmaceutical, Lundbeck, Merck, Novartis, Otsuka, Sunovion, Roche, Takeda Pharmaceuticals International, Takeda Global Research and Development, and Toyama Pharmaceuticals. He has also received grant support, funding, or been a consultant to the following funding agencies, universities and university affiliates, as well as professional organizations that conduct medical or scientific research related to psychiatric and neurodegenerative disorders: NIAAA, NIBIB, NIH/NCRR, University of Southern California, University of California San Francisco, University of California San Diego, Baylor College of Medicine, American Psychiatric Association, Alzheimer's Association. David Crandall is an employee of Sunovion Pharmaceuticals. All other authors have no conflicts of interest to disclose.

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