



Full-length Article

Peripheral lymphocyte signaling pathway deficiencies predict treatment response in first-onset drug-naïve schizophrenia



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ABSTRACT

Despite being a major cause of disability worldwide, the pathophysiology of schizophrenia and molecular basis of treatment response heterogeneity continue to be unresolved. Recent evidence suggests that multiple aspects of pathophysiology, including genetic risk factors, converge on key cell signaling pathways and that exploration of peripheral blood cells might represent a practical window into cell signaling alterations in the disease state. We employed multiplexed phospho-specific flow cytometry to examine cell signaling epitope expression in peripheral blood mononuclear cell (PBMC) subtypes in drug-naïve schizophrenia patients ($n = 49$) relative to controls ($n = 61$) and relate these changes to serum immune response proteins, schizophrenia polygenic risk scores and clinical effects of treatment, including drug response and side effects, over the longitudinal course of antipsychotic treatment. This revealed both previously characterized (Akt1) and novel cell signaling epitopes (IRF-7 (pS477/pS479), CrkL (pY207), Stat3 (pS727), Stat3 (pY705) and Stat5 (pY694)) across PBMC subtypes which were associated with schizophrenia at disease onset, and correlated with type I interferon-related serum molecules CD40 and CXCL11. Alterations in Akt1 and IRF-7 (pS477/pS479) were additionally associated with polygenic risk of schizophrenia. Finally, changes in Akt1, IRF-7 (pS477/pS479) and Stat3 (pS727) predicted development of metabolic and cardiovascular side effects following antipsychotic treatment, while IRF-7 (pS477/pS479) and Stat3 (pS727) predicted early improvements in general psychopathology scores measured using the Brief Psychiatric Rating Scale (BPRS). These findings suggest that peripheral blood cells can provide an accessible surrogate model for intracellular signaling alterations in schizophrenia and have the potential to stratify subgroups of patients with different clinical outcomes or a greater risk of developing metabolic and cardiovascular side effects following antipsychotic therapy.

1. Introduction

Schizophrenia is a major debilitating neuropsychiatric disorder which affects approximately 1% of the population worldwide (Kahn et al., 2015). Major risk factors such as genetic background, urbanicity, migration status and substance abuse are associated with even greater prevalence in specific communities (Kahn et al., 2015). Life expectancy

is reduced by 10–20 years and unemployment rates reach up to 80–90% (Owen et al., 2016). The majority of the patient population does not respond fully to available treatments, with treatment resistance and refractory symptom subdomains, such as negative and cognitive symptoms, being persistent problems which critically affect long-term patient outcomes (Leucht et al., 2013; Zhu et al., 2017; Schooler et al., 2015). Moreover, severe side effects of pharmacological treatment such as

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extrapyramidal symptoms, sedation, weight gain and increased risk of metabolic syndrome and cardiovascular disease severely affect patient quality of life and lead to high rates of treatment cessation (Kahn et al., 2015; Leucht et al., 2013; Lally and MacCabe, 2015). Although considerable progress towards elucidating the basis of schizophrenia has been made in recent years, a lack of pathophysiological understanding, disease heterogeneity, incomplete characterization of the molecular mechanisms of existing drugs and a paucity of relevant preclinical models have stymied the search for improved clinical treatments (Marder et al., 2011; Agid et al., 2007; Millan et al., 2016). Moreover, the need to accurately predict treatment response and side effect profiles for individual patients, at critical early stages in the disease course, is an imperative concern for optimizing the clinical use of existing drugs and improving the outcomes of emerging clinical trials (Millan et al., 2016; Crespo-Facorro et al., 2013; Tomasik et al., 2012; Schennach et al., 2012; Crespo-Facorro et al., 2019; Lago and Bahn, 2019).

Much of the research conducted into the pathophysiology of schizophrenia and identification of drug targets has focused on synaptic monoamine (e.g. dopamine D2 and 5-hydroxytryptamine 2) and glutamate (e.g. N-methyl-D-aspartate) receptors and their relative contributions to abnormal neuronal circuitry (Carpenter and Davis, 2012; Moreno et al., 2016; Moghaddam and Javitt, 2012; Beaulieu, 2012). However, relatively little attention has been given to the intracellular signaling cascades on which these and other disease-associated receptor families converge. Recent evidence suggest the polymorphisms in cell signaling proteins, such as Akt, Stat, PLC, PKC and GSK-3 subtypes, are associated with an increased risk of schizophrenia and related neuropsychiatric disorders (Ripke et al., 2014; Stahl et al., 2019; Beurel et al., 2015). Moreover, studies in animal models involving transgenic editing of key cell signaling epitopes have shown multiple behavioral phenotypes reminiscent of neuropsychiatric symptoms, such as depressive and manic-like features following an alanine mutation of the GSK-3 β (pS9) regulatory site in animal models of bipolar disorder (Yang et al., 2010) and schizophrenia-like features following PLC isotype knockdown in animal models of psychotic disorders (Yang et al., 2016; Yang et al., 2017). Importantly, alterations in cell signaling proteins in the form of genetic polymorphisms, tissue-specific expression patterns and changes in activation profiles, have been associated with disease status, symptom severity and therapeutic efficacy across a range of neuropsychiatric disorders (Beurel et al., 2015; Li and Jope, 2010; Emamian, 2012; Pan et al., 2011; Pandey et al., 2015). In light of the renewed interest in intracellular kinases as the third major class of drug targets within the human genome after ion channels and G-protein-coupled receptors, with a wealth of well characterized preclinical compounds (Santos et al., 2016), these data suggest that exploration of intracellular signaling proteins in schizophrenia and related neuropsychiatric disorders might provide fertile ground for identification of a new generation of therapeutic drugs and treatment response predictors. Lithium, used in the treatment of bipolar disorder and thought to act by inhibiting GSK-3 β and disrupting the Akt/ β -arrestin 2/protein phosphatase 2A signaling complex, is a notable example (Beurel et al., 2015; Pan et al., 2011).

Neuropsychiatric disorders are increasingly recognized as systemic disorders with parallel manifestations in the brain and peripheral tissues. In this respect, accessible peripheral tissues have recently been shown to provide a potential wealth of physiologically relevant cellular models, such as induced pluripotent stem cells (iPSCs) derived from skin fibroblasts or peripheral blood mononuclear cell (PBMC) subtypes, in which to explore disease-specific alterations in cell signaling behavior (Brennan et al., 2011; Lago et al., 2019, 2021; Mertens et al., 2015). PBMC subtypes express many cell signaling proteins downstream of central nervous system (CNS) receptors which are implicated in neuropsychiatric disorders (Gladkevich et al., 2004) and PBMC subtype-specific gene expression enhancers have recently been shown to be enriched among schizophrenia risk loci (Ripke et al., 2014). Moreover, subsets of cell signaling alterations, for example decreased expression of Akt1 have been reported in both *post mortem* brain and PBMCs from

schizophrenia patients (Emamian et al., 2004). Importantly, the application of high-content single-cell screening technologies to PBMCs from neuropsychiatric patients has revealed cell signaling targets which support novel drug discovery and *ex vivo* treatment response prediction in schizophrenia (Lago et al., 2019) in addition to alterations in cell signaling network connectivity which differentiate closely related disorders across the neuropsychiatric spectrum (Lago et al., 2020). Furthermore, evidence that peripheral immune cell alterations can induce functional changes in the CNS and corresponding behavioral symptomatology suggests that PBMCs may plausibly be involved in underlying pathophysiological mechanisms (Dantzer et al., 2008). Finally, given the likelihood of systemic changes in the cell signaling environment following chronic administration of neuropsychiatric medication, there is an urgent need to assess cell signaling changes in drug-naïve first-onset patient cohorts and relate these findings not only to drug efficacy, but also to key side effect profiles.

In the present work, we aim to explore PBMC cell signaling alterations in schizophrenia in a comprehensive cohort of first-onset drug-naïve schizophrenia patients and controls and relate their potential utility to the prediction of clinical treatment response and side effects. We focus on a mechanistically diverse set of signaling protein epitopes ($n = 11$) spanning key cell signaling pathways, including Akt/GSK-3 β , MAPK/ERK, JAK/STAT, IRF, RTK/integrin and T/B cell receptor (TCR/BCR) signaling, which have previously shown either altered expression in drug-naïve schizophrenia relative to controls, altered expression over the longitudinal course of antipsychotic therapy or altered functional network connectivity in previous studies (Lago et al., 2019, 2020). Moreover, to explore the functional characteristics of these signaling proteins we focus on key phosphorylation sites which determine their activity status using phospho-site specific flow cytometry (Krutzik et al., 2008) and explore the heterogeneity of these phospho-epitopes across different PBMC subtypes ($n = 3$) using multiplexed immunophenotyping. Additionally, we profile 43 immune response proteins in serum of the same individuals using a multiplexed immunoassay platform to characterize immune pathways associated with the identified cell signaling deficiencies. Finally, we cross-reference individual signaling alterations with schizophrenia polygenic risk scores for each individual, derived from Illumina PsychArray sequencing of primary risk SNPs which were identified in schizophrenia genome-wide association studies (GWAS) (Ripke et al., 2014). Taken together, this generates a matrix of 33 unique signaling epitope - cell subtype combinations with which to probe the peripheral signaling environment in schizophrenia and prioritize these changes relative the clinical course of the disease and its genetic context.

2. Materials and methods

2.1. Clinical sample recruitment

PBMCs from first-onset antipsychotic drug-naïve schizophrenia donors ($n = 49$) and matched typical controls ($n = 61$) were collected at the University Hospital Marqués de Valdecilla, Santander, Spain; **Table S1**). Schizophrenia samples were collected as part of the longitudinal First Episode Psychosis Clinical Program 10 (PAFIP10) study. Control samples were collected in parallel from healthy subjects, without psychiatric illness, from the same catchment area, through public advertisements, and assessed at the same time points. Blood samples for laboratory analyses were obtained after an overnight fast. Control as well as schizophrenia groups were matched for sex, smoking, alcohol consumption and cannabis use ($P > 0.05$). Quality control (QC) samples ($n = 10$) from a single typical control donor were collected at the University of Cambridge, Cambridge, UK. The medical faculty ethical committees responsible for the respective sample collection sites approved the study protocols. Informed consent was given in writing by all participants and clinical investigations were conducted according to the Declaration of Helsinki (World Medical Association, 2013).

Diagnoses of neuropsychiatric pathology were conducted by experienced psychiatrists and were based on the Diagnostic and Statistical Manual of Mental Disorders-IV-Text Revision (DSM-IV-TR) (American Psychiatric Association, 2000). The severity of symptoms in schizophrenia was measured using the Brief Psychiatric Rating Scale (BPRS) (Leucht et al., 2005), the Scale for the Assessment of Positive Symptoms (SAPS) and the Scale for the Assessment of Negative Symptoms (SANS) (Andreasen, 1989). Measurements of serum biochemistry, including cholesterol, high-density lipoprotein, low-density lipoprotein, glucose, insulin and triglycerides, and body mass index (BMI) were conducted as part of the routine clinical follow-up. Follow-up data was collected at baseline, 6 weeks, 3 months and 1 year for BPRS, SAPS and SANS and at baseline, 3 months and 1 year for BMI and serum biochemistry measurements. The exclusion criteria for patients and controls included: age below 18 years old, additional neuropsychiatric diagnoses, other neurological conditions including epilepsy, mental retardation, multiple sclerosis, immune/autoimmune conditions, infectious disease, metabolic conditions including diabetes, obesity, cardiovascular disease, hepatic and renal insufficiency, gastrointestinal conditions, endocrine conditions including hypo-/hyperthyroidism and hypo-/hypercortisolism, respiratory diseases, cancer, severe trauma, substance abuse including psychotropic drugs and alcohol, somatic medication for non-CNS indications with CNS side effects and somatic medication affecting the immune system including glucocorticoids, anti-inflammatory/immuno-modulatory drugs and antibiotics.

2.2. PBMC isolation and culture

PBMCs were prepared within 4 h from blood collected into 7.5 mL acid citrate dextrose solution A tubes (BD Biosciences; clinical samples) or sodium heparin tubes (BD Biosciences; QC samples). Whole blood was pelleted at 500 g for 10 min to remove platelet-rich plasma, diluted 1:1 with Dulbecco's phosphate-buffered saline solution (PBS; Sigma-Aldrich) and centrifuged over Ficoll-Paque PLUS (GE Healthcare) at 750 g for 20 min at 23 °C. PBMCs were extracted from the interphase, washed three times with PBS at 300 g for 10 min and cryopreserved at 5×10^6 cells/ml in heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich) containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich). For cell culture, PBMCs were thawed at 37 °C and resuspended in sterile conditions in complete Roswell Park Memorial Institute (RPMI) media with deoxyribonuclease (DNase) (RPMI-1640 with sodium bicarbonate (Sigma-Aldrich), 10% FBS (Life Technologies), 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies), 2 mM L-alanyl-L-glutamine dipeptide (Life Technologies) and 20 µg/ml DNase (Sigma-Aldrich)). The cells were counted using a Coulter Counter (Beckman Coulter), pelleted, resuspended at 0.4×10^6 cells/ml and strained via a 40 µm cell strainer (Corning). The cells were rested for 24 h at 37 °C/5% CO₂ in 96-well polypropylene plates (Starlab).

2.3. Intracellular staining of cell signaling epitopes in PBMC subsets

A Biomek NX liquid handler (Beckman Coulter) was used for staining steps. PBMCs were pelleted and resuspended in complete RPMI media without penicillin–streptomycin at 0.4×10^6 cells/ml. Cells were fixed for 10 min at 37 °C using paraformaldehyde (Sigma-Aldrich) in PBS at a final concentration of 1.6%. Fixed cells were washed with PBS and permeabilized in 100 µl ice-cold methanol (Fisher Chemical) for 20 min at to allow penetration of antibodies against intra-cellular epitopes. 100 µl/well of ice-cold PBS was added to the suspension of cells in methanol and incubated in the dark for 30 min at 2 °C. The cells were washed four times in ice cold FACS buffer (PBS with bovine serum albumin 0.5% (Sigma-Aldrich)) and resuspended at 0.5×10^6 cells/ml in FACS buffer for staining. The suspension of fixed-permeabilized cells was stained in a total volume of 90 µl/well. Immunophenotyping was conducted with 0.03 µl of anti-human CD3 (UCHT1) PE-Cy7 (eBioscience) and 0.03 µl of anti-human CD4 (SK3) PerCP-eFluor 710 (eBioscience) and 7.5 µl

human Fc receptor-binding inhibitor (eBioscience). Staining of intracellular signaling epitopes was conducted using fluorescently-conjugated anti-human antibodies including Akt1 (55/PKBα/Akt) Alexa Fluor (AF) 488, Akt (pS473) (M89-61) AF647, CrkL (pY207) (K30-391.50.80) AF488, ERK1/2 (pT202/pY204) (20A) AF647, IRF – 7 (pS477/pS479) (K47-671) AF488, Pyk2 (pY402) (L68-1256.272) Phycoerythrin (PE), Src (pY418) (K98-37) AF488, Stat3 (M59-50) PE, Stat3 (pS727) (49/p-Stat3) AF488, Stat3 (pY705) (4/P-STAT3) AF647 and Stat5 (pY694) (47/Stat5 (pY694)) AF647 (BD Biosciences) as per the manufacturer's instructions. Antibodies against intracellular epitopes were used in groups of up to three antibodies per plex. For the majority of samples (n = 60), intracellular staining panels were as follows: 1) Akt (pS473) AF647 and Akt1 AF488, 2) Stat3 (pS727) AF488, Stat3 PE and Stat3 (pY705) AF647, 3) Src (pY418) AF488, Pyk2 (pY402) PE and Stat5 (pY694) AF647, 4) CrkL (pY207) AF488, 5) ERK1/2 (pT202/pY204) AF647 and 6) IRF-7 (pS477/pS479) AF488. For a subset of samples from a different experimental run (n = 50), CrkL (pY207) AF488, ERK1/2 (pT202/pY204) AF647, IRF-7 (pS477/pS479) AF488 and Stat5 (pY694) AF647 were each run as part of plexes with other intracellular antibodies. An equivalent volume of FACS buffer was added to unstained control wells per sample. Samples were incubated with antibodies for 45 min in the dark at room temperature. The cells were washed twice and resuspended in FACS buffer at 0.3×10^6 cells/ml for acquisition.

2.4. Data acquisition using flow cytometry

PBMC cell suspensions were acquired using an eight color FACSVerser flow cytometer (BD Biosciences) with 405, 488 and 640 nm laser excitation at an average flow rate of 2 µl/sec. On average (\pm standard deviation), 4353 ± 3074 events were acquired from each sample, corresponding to $15 \pm 11\%$ cell recovery from cell culture. This included 306 ± 339 CD3- cells (largely B and natural killer (NK) cells; labelled 'B cells'), 1089 ± 1013 CD4 + T cells, and 606 ± 575 CD4- T cells. Multicolor Cytometer Setup and Tracking beads (BD Biosciences) were used for quality control and standardization of photomultiplier tube detector voltages across multiple experimental runs. Fluorescence compensation was conducted using anti-mouse IgGκ antibody capture beads (Bangs Laboratories) labelled separately with anti-human CD3 (UCHT1) PE-Cy7, anti-human CD4 (SK3) PerCP-eFluor 710 or relevant fluorescently-conjugated intracellular signaling antibodies.

2.5. Serum sample collection

Serum samples were prepared following a standard operating procedure. Blood samples were collected between 8 am and 12 pm into S-Monovette 7.5 mL serum tubes (Sarstedt; Numbrecht, Germany). Samples were then allowed to clot at room temperature for 2 h and centrifuged at 4000 g for 5 min. The resulting supernatants were stored at –80 °C in low protein-binding polypropylene tubes.

2.6. Multiplexed immunoassay analysis of immune response proteins in serum

Levels of 43 immune response proteins were measured in 300 µl of serum in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory (Myriad RBM Inc.; Austin, TX, USA) as part of a custom (225analytes) Human DiscoveryMAP® 250 + v. 3.0 multiplexed immunoassay panel. Samples were analyzed at optimized dilutions and raw intensity measurements were converted into absolute protein concentrations using duplicate 8-point standard curves. Assay performance was evaluated using quality control samples at low, medium, and high concentrations for each analyte. Sample analysis was randomized to minimize bias due to measurement-related effects.

2.7. Genotype analysis and calculation of polygenic risk scores

All patients were genotyped using the Illumina Infinium PsychArray Bead Chip and imputed using the standard SHAPEIT2/IMPUTE2 pipeline (Howie et al., 2009; Delaneau et al., 2013). PLINK 1.9 (Chang et al., 2015) was used for the calculation of Polygenic Risk Scores (PRS) using methodologies described elsewhere (Wray et al., 2014). Briefly, based on the results of the SCZ-PGC GWAS (Ripke et al., 2014) and using a P value threshold = 1, PRS were calculated by multiplying the imputation dosage for each risk allele by the effect size for each genetic variant in the discovery schizophrenia GWAS. A total number of 113,282 independent variants were included. The resulting values were summed up in an additive fashion obtaining an individual estimate of the genetic load for schizophrenia risk in each subject. A P value threshold = 1 was used in order to account for the extensive polygenicity of this trait and the evidence that genetic risk variants that are not genome-wide significant ($P > 5 \times 10^{-8}$) also contribute to schizophrenia risk (Smeland et al., 2020).

2.8. Statistical data analysis

Flow cytometry data was analyzed in FCS 3.0 file format using Flow Jo v.10.0.8 software (Tree Star). Statistical analysis was conducted using R v.3.6.1 software (R Core Team) (R Core Team, 2017). PBMC samples in which the lymphocyte gate contained <20% of events, measured by forward scatter (FSC-A)/side scatter (SSC-A), were excluded from further analysis. Experimental variables including positional effects within and across 96-well plates, sample viability, cell counts, experimental day, clinical group and sample source were investigated using principal component analysis (Fig. S1). Batch effects in median fluorescence intensities (MFIs) caused by different experimental runs were normalized for each intracellular signaling marker and PBMC subtype using median scaling based on control samples from each experimental run. Serum multiplexed immunoassay data were filtered to exclude analytes with >30% of values missing. All the remaining missing values in the data set were due to measurements being below the lowest limit of quantitation, and were replaced with half the minimum measured value, consistent with previous work (Tomasik et al., 2020; Tomasik et al., 2016). Because the immunoassay analysis was part of a large international project, batch effects caused by analyzing serum samples from multiple cohorts and clinical centers interspersed across multiple experimental plates were removed using an empirical Bayes framework, ComBat (R package 'sva'). Matching of clinical groups was conducted using the *t*-test for continuous variables or the Fisher's exact test (chi-squared test for more than two groups) for categorical variables. All statistical tests were two-sided, unless specified otherwise. P values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure (shown as Q values). Samples with missing data were excluded from analysis.

The stain index of each antibody, per PBMC subtype, was calculated for each clinical group individually as the weighted average of the ratio of the median MFI of the antibody-stained sample divided by the median MFI of the corresponding unstained control across experimental batches. For comparison of intracellular signaling marker expression across clinical groups, only signaling markers with a median stain index greater than four in both of the comparison groups were used for analysis (i.e., 23 of the 33 measured readouts). Likewise, for each PBMC cell subtype only samples with a minimum of 200 live cells in the PBMC cell subtype gate were included in analysis. Filtering the data for viability and cell counts resulted in excluding 22.3% of all data points. Association of intracellular signaling marker expression in each PBMC subset (or serum protein levels) with clinical group status was investigated using the analysis of covariance (ANCOVA) F-test ($P < 0.05$) after adjusting for covariates. Optional covariates, age, sex and BMI, were selected in a stepwise procedure for each marker and PBMC subtype (or serum analyte) separately using Bayesian Information Criterion. Box plots of

disease-associated nodes show interquartile range with the median (horizontal line) and the minimum and maximum values (whiskers), excluding outliers (dots). Association of the differential epitope expression with age at onset of psychosis and duration of untreated psychosis was evaluated using Spearman's rank correlation test.

Associations of intracellular signaling nodes significantly linked to schizophrenia with serum immune molecules were evaluated using multivariable linear regression. Optional covariates, age, sex, BMI and clinical group status, were selected in a stepwise procedure, separately for each analyte, using Bayesian Information Criterion. Interaction between the clinical group status and serum analyte levels was evaluated by additionally including an interaction term in the model. Associations of intracellular signaling markers, significantly linked to schizophrenia, with schizophrenia polygenic risk score (PRS) and clinical measures of symptom improvement (Δ BPRS, Δ SAPS and Δ SANS) or side effects (Δ BMI, Δ cholesterol, Δ HDL, Δ LDL, Δ glucose, Δ insulin and Δ triglycerides) were investigated using multivariable linear regression. Changes (Δ) in symptoms or side effects were calculated as scores at follow-up time point – scores at baseline (0 weeks) time point. Optional covariates, age, sex and BMI, in addition to baseline measures (BPRS, SAPS, SANS, BMI, cholesterol, HDL, LDL, glucose, insulin and triglycerides) and treatment drug when predicting response to treatment, were selected in a stepwise procedure for each marker and PBMC subtype separately using Bayesian Information Criterion. In sensitivity analyses, data on initial drug dose, medication switching status at given time point, and an interaction with the initial medication were additionally included as optional covariates. Drug doses were converted to chlorpromazine equivalent units for analysis using R package chlorpromazineR (Gardner et al., 2010).

Data was visualized using Flow Jo, R software and Adobe Illustrator (Adobe Systems).

3. Results

3.1. Identification of basal signaling alterations in drug-naïve schizophrenia

To explore peripheral markers of intracellular signaling alterations in schizophrenia, we compared the expression of previously identified cell signaling epitopes ($n = 11$) (Lago et al., 2019, 2020), including nine phosphorylation sites and two total protein sites, in different PBMC subtypes, including CD4 + T cells (T helper, T follicular helper and T regulatory cells), CD4- T cells (largely T cytotoxic cells) and CD3- cells (largely B and natural killer (NK) cells; labelled 'B cells'), from individuals with first-onset antipsychotic drug-naïve schizophrenia ($n = 49$) and unaffected typical controls ($n = 61$; Table S1 and Fig. S2 and S3). This provided a total of 33 signaling epitope-cell subtype combinations, or 'nodes', measured in each clinical sample. The results revealed a significant (ANCOVA $P < 0.05$, $Q < 0.05$; stain index threshold > 4) downregulation of seven epitopes distributed across five signaling proteins in schizophrenia, including Akt1, Akt (pS473), IRF-7 (pS477/pS479), CrkL (pY207), Stat3 (pS727), Stat3 (pY705) and Stat5 (pY694) (Fig. 1 and Table S2). Most epitopes, with the exception of CrkL (pY207) and Stat3 (pY705) were altered in two or more of the cell subtypes. There were no significant associations between the differential epitope expression and age at onset of psychosis or the duration of untreated psychosis ($P > 0.07$).

3.2. Association of altered signaling nodes with serum immune response proteins

To identify relevant immune signaling pathways, we correlated cell signaling nodes significantly linked to schizophrenia with 43 serum immune response proteins. This analysis revealed significant ($Q < 0.05$, multivariable linear regression) negative correlations between Stat3 (pY705) in CD4 + T cells and CD40 antigen (standardized regression

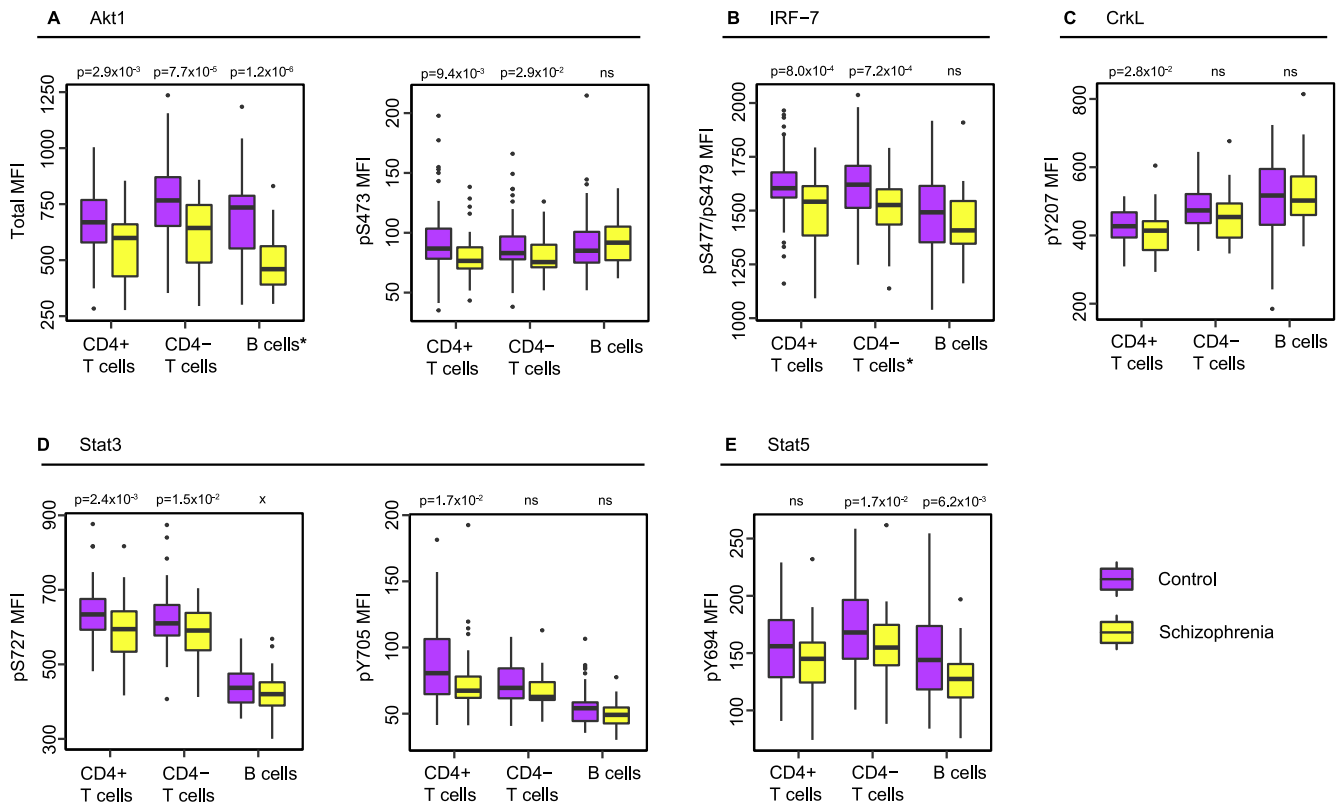


Fig. 1. Intracellular signaling alterations in peripheral blood mononuclear cell (PBMC) subtypes in drug-naïve schizophrenia patients. Shows intracellular signaling epitopes on Akt1 (A), IRF-7 (B), CrkL (C), Stat3 (D) and Stat5 (E) proteins (phosphorylation sites inset marked with ‘p’) and PBMC cell subtypes with a significant association ($P < 0.05$ ANCOVA F-test, Q (Benjamini-Hochberg) < 0.05) to first-episode antipsychotic-naïve schizophrenia ($n = 49$) relative to typical controls ($n = 61$) in at least one of the cell populations. Plots show median fluorescence intensity (MFI) of epitope expression (y axis) per clinical group and cell subtype (x axis). ‘Total MFI’ in panel A refers to non-phosphorylated Akt1 MFI. Optional covariates included age, gender and body mass index. Box plots show interquartile range with the median (horizontal line) and minimum and maximum values (whiskers), excluding outliers (dots), and P values for association with schizophrenia (above). Asterisks next to cell population labels denote epitopes which additionally showed significant associations ($P < 0.05$, multivariable linear regression) to schizophrenia polygenic risk scores (Ripke et al., 2014). Akt - protein kinase B, IRF-7 - interferon regulatory factor 7, CrkL - Crk-like protein, ns - not significant, Stat3 - signal transducer and activator of transcription 3 and Stat5 - signal transducer and activator of transcription 5, x - readout excluded due to low staining (stain index below four).

coefficient (β_{std}) = -0.41 , $Q = 0.035$), CrkL (pY207) in CD4 + T cells and interferon-inducible T-cell alpha chemoattractant (CXCL11; β_{std} = -0.36 , $Q = 0.041$), and IRF-7 (pS477/pS479) in CD4- T cells and CD40 antigen (β_{std} = -0.39 , $Q = 0.041$; Fig. 2 and Table S3). In addition, the CD40 antigen was also significantly upregulated in schizophrenia (fold change = 1.31, $Q = 1.40 \times 10^{-7}$, ANCOVA F-test; Table S4). No significant interactions were detected between clinical group status and the association of intracellular signaling nodes with circulating immune response proteins after adjusting for multiple comparisons (interaction $Q \geq 0.05$, multivariable linear regression).

3.3. Association of altered signaling epitopes with schizophrenia polygenic risk scores

We explored whether altered expression of intracellular signaling epitopes was associated with polygenic risk of schizophrenia across the clinical groups. This showed a significant (ANCOVA $P < 0.05$) association between schizophrenia polygenic risk score and the expression of total Akt1 protein in B cells (ANCOVA $P = 0.047$) and IRF-7 (pS477/pS479) phosphorylation status in CD4- T cells (ANCOVA $P = 0.016$).

3.4. Prediction of clinical response and side effects in schizophrenia using intracellular signaling epitopes

To assess the clinical relevance of peripheral signaling alterations in schizophrenia, we explored the potential of the most significantly

altered cell signaling nodes from the group comparison (ANCOVA $Q < 0.02$; Table S2) to predict the response to antipsychotic treatment, defined as changes in the Brief Psychiatric Rating Scale (Δ BPRS), Scale for the Assessment of Positive Symptoms (Δ SAPS) and Scale for the Assessment of Negative Symptoms (Δ SANS) scores, in addition to the principal side effect of weight gain, defined by changes in body mass index (Δ BMI), and markers of metabolic syndrome, including changes in serum insulin, glucose, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and cholesterol concentrations, in a longitudinal follow-up of first-episode antipsychotic-naïve schizophrenia patients, treated initially with antipsychotic drugs aripiprazole ($n = 25$) or risperidone ($n = 24$; Table S1). This revealed that baseline expression of total Akt1 in B cells predicted changes in serum insulin concentrations (Δ insulin; $P_{3months} = 0.032$, $\beta_{3months} = -0.010$; $P_{1year} = 0.011$, $\beta_{1year} = -0.051$), Stat3 (pS727) phosphorylation status in CD4 + T cells predicted improvements in general psychopathology scores (Δ BPRS; $P_{6weeks} = 0.021$, $\beta_{6weeks} = 0.033$) and the side effect of weight gain (Δ BMI; $P_{3months} = 0.033$, $\beta_{3months} = -0.007$) and IRF-7 (pS477/pS479) phosphorylation status in CD4- and CD4 + T cells predicted improvements in general psychopathology scores (Δ BPRS; $P_{6weeks} = 0.011$, $\beta_{6weeks} = 0.022$) and changes in serum HDL (Δ HDL; $P_{1year} = 0.002$, $\beta_{1year} = -0.081$) and cholesterol (Δ cholesterol; $P_{1year} = 0.032$, $\beta_{1year} = 0.049$) concentrations, respectively (Fig. 3 and Table S5). In sensitivity analyses, these associations did not differ significantly between the aripiprazole and risperidone baseline treatment groups (P interaction > 0.05). Furthermore, drug dose and medication switching status at 3

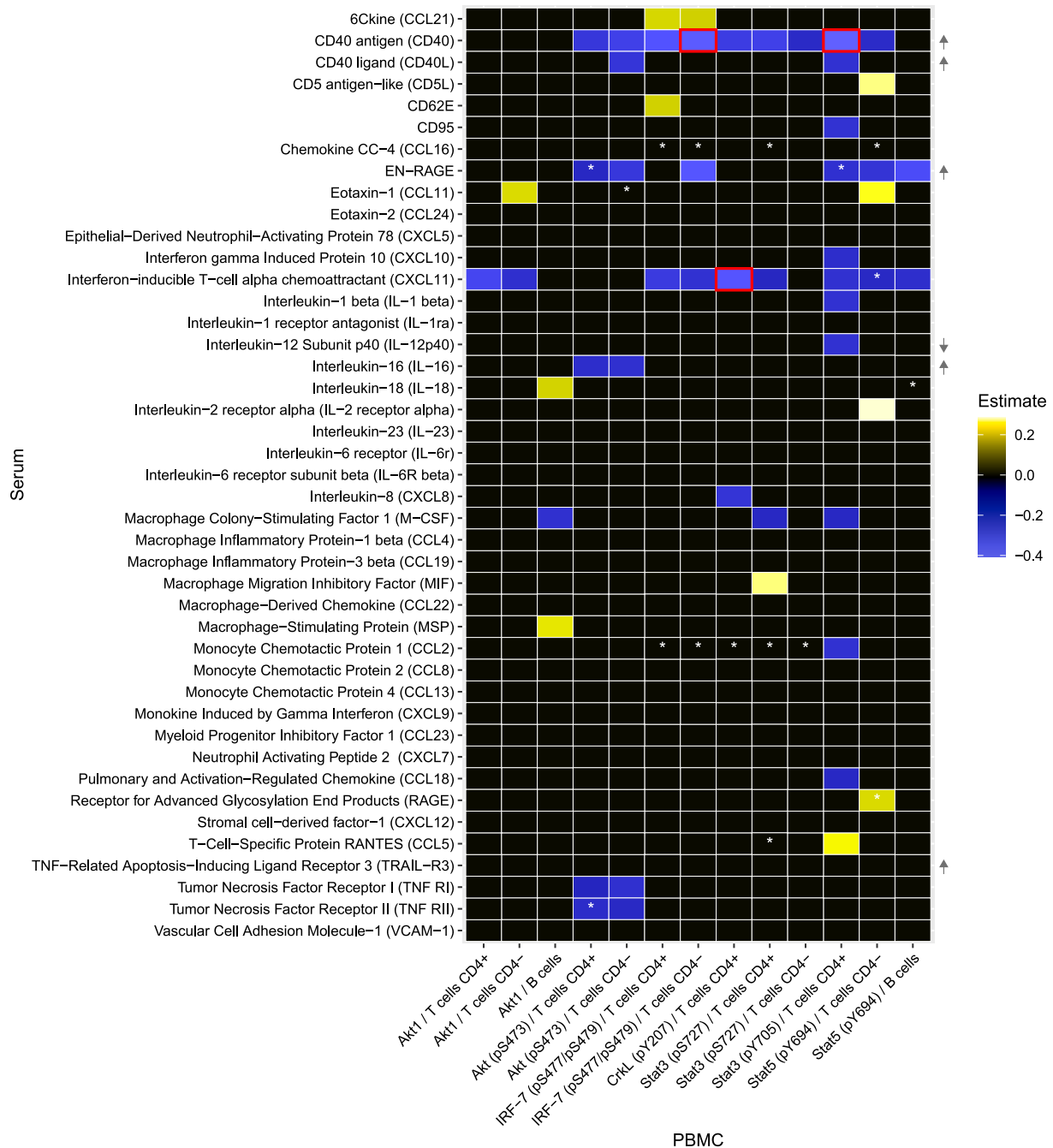


Fig. 2. Association of intracellular signaling epitopes, significantly linked to schizophrenia, with serum immune response proteins. Shown are significant ($P < 0.05$, multivariable linear regression) standardized estimates for the association between intracellular signaling nodes (x axis) and circulating immune response proteins (y axis). Associations which were significant after Benjamini-Hochberg correction ($Q < 0.05$, multivariable linear regression) are framed in red. Significant interactions ($P < 0.05$, multivariable linear regression) between clinical group status and the association of intracellular signaling nodes with circulating immune response proteins are indicated with asterisks (*). Serum proteins significantly up- or down-regulated in schizophrenia ($Q < 0.05$, ANCOVA F-test) are marked with up (↑) and down (↓) arrows, respectively. PBMC – peripheral blood mononuclear cells.

months were selected only for one association, i.e., when predicting change in insulin levels after 3 months from baseline Akt1 expression in B cells (Fig. 3A). Nonetheless, this association remained significant after additionally adjusting for the two covariates ($P = 0.035$, $\beta = -0.009 \pm 0.004$). Neither drug dose nor medication switching status were selected for any of the other associations.

4. Discussion

The present results expand the scope of previous findings of cell

signaling alterations in peripheral tissues in schizophrenia and suggest that these alterations are observable in first-onset drug-naïve samples before the onset of pharmacological treatment. The association between expression of total Akt1 protein in B cells and IRF-7 (pS477/pS479) phosphorylation in CD4- T cells with schizophrenia polygenic risk scores highlights a potential genetic basis for these findings.

Decreased Akt1 protein in B cells is consistent with work by [Emanian et al. \(2004\)](#) which showed a decrease in Akt1 protein level and the phosphorylation of its substrate GSK-3 β (pS9) in B lymphoblastoid cell lines and frontal cortex of *post-mortem* brains from schizophrenia

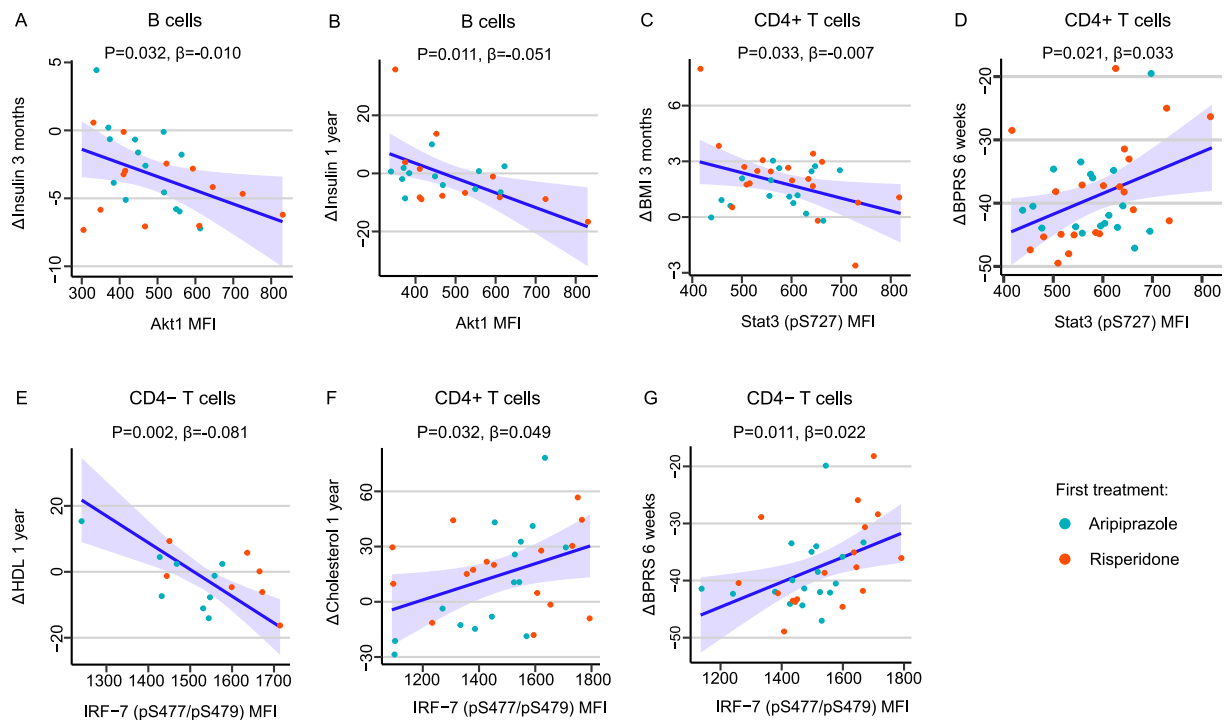


Fig. 3. Prediction of antipsychotic efficacy and side effects using PBMC intracellular signaling markers associated with schizophrenia. Prediction of changes (Δ) in clinical symptoms, measured using the Brief Psychiatric Rating Scale (BPRS), the side effects of weight gain, measured using body mass index (BMI), and markers of metabolic syndrome (insulin, high-density lipoprotein (HDL) and cholesterol) in a longitudinal follow-up of first-episode antipsychotic-naïve schizophrenia (SCZ) patients, treated initially with antipsychotic drugs aripiprazole ($n = 25$, blue) or risperidone ($n = 24$, red), using expression of intracellular signaling epitopes Akt1 (A,B), Stat3 (pS727) (C,D) and IRF-7 (pS477/pS479) (E-G). Plots shows significant marginal effects ($P < 0.05$, multivariable linear regression), with 95% confidence intervals and estimates (β), and individual values adjusted for optional covariates (baseline value, age, sex, BMI and treatment status). Only epitopes significant ($P < 0.05$ ANCOVA F-test, $Q < 0.02$ Benjamini-Hochberg) in the clinical group comparison are shown.

patients relative to controls. Reduced Akt1 levels also correlated to putative disease haplotypes in the Akt1 gene. Associations of Akt1 haplotypes with schizophrenia have subsequently been confirmed across different ethnic backgrounds (Xu et al., 2007; Thiselton et al., 2008; Schwab et al., 2005; Ikeda et al., 2004; Bajestan et al., 2006) in addition to the association of other Akt isoforms in GWAS analyses (Ripke et al., 2014). Notably, some studies have associated Akt1 risk haplotypes with alterations in executive function and fMRI responses during attentional processing (Blasi et al., 2011; Tan et al., 2008). The present results extended these findings to suggest that Akt1 reductions are common to multiple PBMC subtypes, including CD4+ and CD4- T cells, albeit with moderate phenotypes relative to B cells, supporting the notion that alterations in Akt1 expression may extend across multiple tissue types in schizophrenia.

Akt1 is a signal transducer for a range of monoamine receptors in the brain, including dopamine and 5-hydroxytryptamine receptors, which have been linked to schizophrenia and the mechanisms of action of antipsychotic medications (Beaulieu, 2012). Concurrent with this hypothesis, are findings that antipsychotic treatments might induce changes in the expression and phosphorylation of Akt1 and downstream signaling partners (Beaulieu, 2012; Emamian, 2012; Jope and Roh, 2006; Lago et al., 2019; Molteni et al., 2009). Thus, the implication of altered Akt1 in the present study might extend beyond an associated disease biomarker to a target of antipsychotic efficacy. Interestingly, changes in Akt1 have been observed with antipsychotics that predominantly treat positive symptoms, such as haloperidol, suggesting that the relevance of the target to negative symptomatology remains to be examined.

The finding of altered IRF-7 (pS477/pS479) phosphorylation status in CD4- cells is intriguing in the context of viral hypotheses of schizophrenia. IRF-7 is responsible for transducing signals from pathogen-associated molecular pattern (PAMP) receptors, including Toll-like

receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors, to induce the transcription of the type I interferon (IFN) cytokines, including IFN α and IFN β , which stimulate both innate and adaptive immune responses against viral and bacterial pathogens (Ning et al., 2011). In line with this, we observed a significant negative correlation between IRF-7 (pS477/pS479) phosphorylation in CD4- cells and serum levels of a co-stimulatory protein CD40, which acts as a key enhancer of type I IFN responses (Li et al., 2020; de Silva et al., 2020). Importantly, constitutive expression of IRF-7 in immune cells is responsible for priming immune responses and subsequently feedback amplification of IFN α and IFN β production, highlighting IRF-7 as a key regulator of both early and late immune responses (Ning et al., 2011). This is relevant in the case of schizophrenia given hypotheses that viral infection during the third trimester of pregnancy could explain an increased risk of developing schizophrenia as reported for babies born during late winter and spring (Kahn et al., 2015; Fatemi et al., 2012; Yolken and Torrey, 1995). Furthermore in animal models of schizophrenia, peripheral administration of the viral mimetic Poly I:C during the peripubertal period increased expression of IRF-7 in the hippocampal dentate gyrus and resulted in behavioral deficits in prepulse inhibition (Lukasz et al., 2013). Interestingly a similar functional imprint on brain IRF-7 expression was also observed following adolescent social isolation, another risk factor for schizophrenia (Lukasz et al., 2013). Reduced phosphorylation observed at the IRF-7 pS477/pS479 activation epitope in the present study might suggest a lower constitutive priming of T cells and consequently an altered response to viral infection. It is notable that associations between polygenic risk of schizophrenia and altered IRF-7 pS477/pS479 expression were specific to the CD4- T cell compartment, comprised predominantly of cytotoxic T cells, which are responsible for eliminating virally infected cells. The findings are also notable given previously reported alterations in the NF- κ B p65 pathway in different immune cell subsets in schizophrenia and their possible

association to negative symptomatology (Lago et al., 2020). Thought to have evolved side-by-side in the mammalian nervous system, NF- κ B p65 and IRF-7 share many of the same signaling pathways and engage in considerable functional crosstalk during regulation of pathogenic immune responses (Ning et al., 2011; Iwanaszko and Kimmel, 2015). Although viral hypotheses of schizophrenia require further data and direct evidence of viral RNA transcripts in schizophrenia *post mortem* brains is lacking (Tomasik et al., 2018), the present results suggest that maladaptive responses to viral exposure mediated by IRF-7 at key developmental stages might warrant further investigation in schizophrenia.

Although not associated to cumulative schizophrenia genetic risk, as defined by polygenic risk scores, other signaling epitopes, identified in the case-control comparison, might offer potential insights into plausible signaling alterations in schizophrenia. CrkL is one of the key genes within the schizophrenia-associated 22q11.2 microdeletion locus, which affects embryonic retinoic acid homeostasis, reelin signaling and neuronal migration (Moon et al., 2006; Guris et al., 2006; D'Arcangelo, 2014; Li et al., 2013), and has been reported in studies of copy number variations (CNVs) (Luo et al., 2014) and exome sequencing (Fromer et al., 2014; Purcell et al., 2014; Iossifov et al., 2012). Moreover, its expression is regulated by top GWAS candidate micro RNA-137 (Ripke et al., 2014) in human neural progenitor cells (Hill et al., 2014). Importantly, CrkL (pY207) phosphorylation status has been reported to be increased following antipsychotic administration in PBMCs *ex vivo* and after longitudinal clinical follow up of antipsychotic-treated schizophrenia patients *in vivo* (Lago et al., 2019). Reduced CrkL (pY207) phosphorylation in CD4 + T cells from schizophrenia patients in the present study therefore raises the possibility that baseline alterations in this epitope might be normalized following successful clinical treatment. Moreover, reports that multi-target efficacies likely underlie the clinical efficacy of antipsychotic medications (Roth et al., 2004) suggests that targets such as CrkL might warrant further investigation.

Reduced phosphorylation of Stat3 (pS727), Stat3 (pY705) and Stat5 (pY694) across different PBMC subsets is relevant in the context of meta-analyses in first-episode schizophrenia patients which suggest elevated levels of cytokines such as interleukin (IL)-6, IL-12, IL-2R and IFN- γ (Miller et al., 2011) which signal through the aforementioned Stat transcription factors. Present reductions in the phosphorylation status of these epitopes could therefore represent a desensitization of these signaling proteins as a compensatory mechanism. Alternatively, they could reflect intrinsic cellular phenotypes, which drive altered cytokine secretion in schizophrenia. Interestingly, serum from first-onset antipsychotic drug-naïve schizophrenia patients has also been shown to induce decreases in Stat3 (pY705) phosphorylation in SV40 human microglial cells suggesting that the effects of circulating factors on proinflammatory cell signaling networks may extend to immune cells in the brain (van Rees et al., 2018). Concurrent with this hypothesis are reports that elevated circulating cytokines might be associated to cognitive impairments in subgroups of schizophrenia patients (Fillman et al., 2015). Several JAK-STAT signaling modulators (e.g. minocycline, withaferin A and pravastatin) have been tested in schizophrenia clinical trials (Lago and Bahn, 2019; van Rees et al., 2018; Miklossy et al., 2013).

Functional cross-talk between several of the signaling motifs identified in schizophrenia in the present study, for example between IRF-7 and JAK-STAT pathways (Ning et al., 2011) or CrkL-Stat5 heterodimers, is relevant in terms of type I IFN responses (Fish et al., 1999). The potential involvement of type I IFN signaling in schizophrenia is supported by the observed correlation between Stat3 (pY705), CrkL (pY207) and IRF-7 (pS477/pS479) epitopes and type I IFN signaling molecules in serum, CD40 and CXCL11 (Li et al., 2020; de Silva et al., 2020; Sandhya Rani et al., 1996), while no association was observed for IFN type II analytes, such as IFN- γ induced protein 10 (CXCL10) or monokine induced by gamma interferon (CXCL9). However, the fact that CD40 and CXCL11 can also be induced by other pathways suggests that further work, correlating the observed changes to IFN type I-specific genes and

molecules (e.g. MxA, IFI3, IFI44 and IFI-44L) (Busse et al., 2020; Ekholm et al., 2016), is warranted to determine the specificity of the signaling alterations. Taken together, this suggests that there is further scope for high-content ligand-based interrogation of cell signaling networks to determine functional alterations in immune responses in schizophrenia.

Exploration of the predictive value of schizophrenia-associated cell signaling epitopes to determine clinical response and side effect profiles over the course of antipsychotic treatment revealed several interesting features. The finding that Akt1 in B cells predicted increases in serum insulin concentrations at three month and one year time points suggests that these signaling abnormalities may underlie metabolic side effects of treatment. This is consistent with the fact that Akt1 and related isoforms are hub proteins in the insulin receptor signaling pathway (Rowland et al., 2011; Guo, 2014) and that *Akt* isoform knockouts in animal models lead to insulin resistance (Chen et al., 2009; Hay, 2011). Insulin resistance and metabolic syndrome have principally been considered side effects of atypical antipsychotic medication (Vancampfort et al., 2015; Vestri et al., 2007; Kroeze et al., 2003; Zhao et al., 2006). However, recent data suggests that metabolic abnormalities, such as abnormal glucose metabolism in the brain and peripheral tissues (Holden and Mooney, 1994; Herberth et al., 2011; Meyer and Stahl, 2009; Pillinger et al., 2017) and elevated circulating insulin and insulin-related peptides (Guest et al., 2011; Guest et al., 2010; van Beveren et al., 2014), are present at disease onset in schizophrenia and may predispose individuals to the metabolic interactions of these drugs (Herberth et al., 2011; Guest et al., 2010; van Beveren et al., 2014; Schwarz et al., 2014; Tomasik et al., 2019). The present Akt1 findings are in line with previous findings in the same drug-naïve schizophrenia cohort, showing dysregulated expression of the insulin receptor and glucose 1 transporter (GLUT1) in PBMC subtypes (Lago et al., 2021) and a relationship between polygenic risk of schizophrenia and insulin resistance at disease onset (Tomasik et al., 2019). The fact that lower levels of Akt1 expression at baseline were associated with larger insulin increases during treatment raises the possibility that the peripheral signaling environment may provide fertile grounds for predictive biomarkers of antipsychotic side effects. Likewise, deficiencies in Stat3 (pS727) phosphorylation at baseline predicted the major side effect of weight gain (increased BMI) at three months. This is consistent with the role of Stat3 in leptin signaling (Jiang et al., 2008) and reports that STAT3 knockout mice show obese phenotypes (Cui et al., 2004).

The association of baseline IRF-7 (pS477/pS479) phosphorylation in PBMC subsets with serum measures of lipid homeostasis, including changes in cholesterol and HDL at one-year follow-up, raises the possibility that this signaling marker might relate to antipsychotic-induced hyperlipidemia. Interestingly, IRF-7 has been shown to regulate cholesterol efflux and homeostasis in macrophages, in addition to mediating the formation of atherosclerotic plaques via Toll-like receptor signaling (Sorrentino et al., 2010; Senatus et al., 2020). Moreover, the proinflammatory effects of advanced glycosylation end-products (AGEs), which have been suggested to impair vascular repair mechanisms in schizophrenia, involve IRF-7 signaling downstream of the AGE receptor (Senatus et al., 2020; Kouidrat et al., 2015). This suggests that abnormalities in IRF-7 might plausibly mediate cross-talk between key facets of antipsychotic-induced metabolic syndrome, hyperglycemia and hyperlipidemia, and long-term cardiovascular complications in schizophrenia (Khasawneh and Shankar, 2014; Correll et al., 2014). Exploration of IRF-7 phosphorylation in myeloid cells (e.g. monocytes and macrophages) in relation to long-term cardiovascular parameters in schizophrenia might be warranted in this respect.

The association of baseline signaling abnormalities in IRF-7 (pS477/pS479) and Stat3 (pS727) with changes in general psychopathology as measured by BPRS at six weeks suggests that, in addition to the prediction of side effects, peripheral cell signaling abnormalities might have the potential to predict clinical efficacy. The data also suggests that patients with larger signaling deficiencies are more likely to show greater improvement in BPRS. This is concurrent with reports that the

activation status of key cell signaling epitopes in PBMCs is linked to the severity of neuropsychiatric symptoms (Yang et al., 2010) and might predict neuropsychiatric drug efficacy *ex vivo* (Lago et al., 2019). However, these readouts were not linked to changes in positive or negative symptoms as measured by SAPS and SANS subscales, suggesting that validation in larger cohorts may be necessary to understand which symptom subdomains relate to individual signaling abnormalities across PBMC subsets.

Taken together, these findings suggest that subgroups of peripheral cell signaling epitopes are associated with drug-naïve schizophrenia at baseline and may show utility for the prediction of clinically relevant side effects and drug efficacy in schizophrenia. This is important as metabolic and cardiovascular side effects of current medications severely affect patient quality of life, medication adherence and long-term disease outcomes (Correll et al., 2014; Vancampfort et al., 2014). Moreover, patient-specific accessible cellular readouts may improve the drug discovery pipeline (Lago and Bahn, 2019) by facilitating patient stratification, as seen in clinical trials for treatment-resistant major depressive disorder (Raison et al., 2013), or enabling early intervention, shown to be important in clinical trials for schizophrenia (Zheng et al., 2017). Finally, by associating peripheral signaling abnormalities with genetic risk of schizophrenia, the present data helps to prioritize signaling pathways that may constitute novel targets for neuropsychiatric treatments and adjunctive medications.

While the current findings provide novel insights into the peripheral cell signaling environment in schizophrenia, there are several limitations to consider with respect to future work. First, although obtaining large numbers and sufficient volumes of clinically well-characterized drug-naïve neuropsychiatric patient samples for cellular analysis remains a significant challenge both logistically and in terms of cost, validation in larger patient cohorts is essential to confirm the clinical relevance of these findings. At the same time, larger sample volumes would allow a wider range of cell signaling epitopes to be measured, beyond the targeted panel applied in the present study, and facilitate exploration of the relationships between epitopes and normalization of phosphorylation status to total protein levels. This also applies to immunophenotyping. The cluster or differentiation (CD) markers in the present study are designed to optimize detection of majority cell populations for a limited clinical sample within the multiplexing capacity of the instrument. Thus, while results for CD4 + T cells, CD4– T cells and B cells focus on the most prevalent populations, each of these CD combinations has subpopulations of cells which may vary in epitope expression. For example, the CD3– population, comprised primarily of B cells, contains a functionally distinct minority of NK cells. Further studies, which allow deeper immunophenotyping, are warranted to determine whether observed changes in intracellular marker expression relate to changes in cell subtype composition, as has been suggested for Th1 vs. Th2 T helper cell ratios in schizophrenia (Potvin et al., 2008). Likewise, other relevant PBMC types such as monocytes were not analyzed in this study due to low yields and spectral compatibility. In these respects, advances in the multiplexing capacity of approaches such as mass cytometry (Levine et al., 2015; Amir et al., 2013) allow more signaling epitopes to be measured in parallel with more extensive immunophenotyping to determine the most relevant cell subtypes within a patient sample, in addition to facilitating empirical reconstruction of downstream signaling effects. Second, polygenic risk scores based on GWAS data currently only account for up to 7% of disease liability in schizophrenia (Kahn et al., 2015; Ripke et al., 2014). Thus, while they are a relevant means to prioritize cell signaling targets relative to what is known about common genetic variation, such strategies will have to be re-evaluated as the genetic map of schizophrenia evolves, including rare *de novo* mutations (Fromer et al., 2014) and copy number variants (Kushima et al., 2016). In this respect, twin and family studies in which genetic risk of schizophrenia is estimated to reach up to 64–81% (Lichtenstein et al., 2009) may provide further insights into cell signaling abnormalities which are not currently indexed by polygenic

risk scores. Moreover, comparison of cell signaling profiles in patient cohorts with high versus low genetic loading are essential to determine the effects of environmental risk factors such as social adversity and chronic stress on the cell signaling repertoire. Finally, while the present work discusses possible biological interpretations for cell signaling abnormalities, observations of disease association do not necessarily imply that they are causative in the disease process. In this respect, definition of tissue specificity using neuronal lineages or brain organoids derived from patient induced pluripotent stem cells (iPSCs) (Lago et al., 2021), mechanistic dissection using counter screening techniques such as small interference RNA (siRNA) or CRISPR-Cas9 genome editing and a better understanding of how peripheral abnormalities can influence CNS symptoms and vice versa will facilitate the prioritization of biomarkers and novel treatment targets.

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Author contributions

S.B., S.G.L., and J.T. conceived the study and designed experiments. B.C.-F. and P.S.-P. collected PBMC samples and were responsible for clinical diagnoses. B.C.-F., J.V.-B. and S.P. provided the genotyping data and selected sample metadata. S.G.L., J.T., G.F.v.R. and N.R. conducted the experiments. S.G.L. analyzed the raw data and J.T. matched the study groups for demographic variables. J.T. conducted the statistical analysis. S.G.L., J.T. and S.B. prepared the manuscript.

Declaration of Competing Interest

J.T. was consultant for Psynova Neurotech Ltd. until April 2016. S.L. was part funded by Psynova Neurotech Ltd. until October 2015. S.B. is a director of Psynova Neurotech Ltd. and Psyomics Ltd. The remaining authors declare that they have no conflict of interest.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the [Supplementary Materials](#). Additional data related to this paper may be requested from the authors. Materials including clinical samples provided under material transfer agreements can be requested pending availability, scientific review, and a completed material transfer agreement from Department of Medicine and Psychiatry, University of Cantabria, Santander, Spain (B.C.-F.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2022.03.016>.

- Zhao, Z., Ksiezak-Reding, H., Riggio, S., Haroutunian, V., Pasinetti, G.M., 2006. Insulin receptor deficits in schizophrenia and in cellular and animal models of insulin receptor dysfunction. *Schizophr. Res.* 84, 1–14.
- Zheng, W., Bin Cai, D., Yang, X.H., Ungvari, G.S., Ng, C.H., Müller, N., Ning, Y.P., Xiang, Y.T., 2017. Adjunctive celecoxib for schizophrenia: a meta-analysis of randomized, double-blind, placebo-controlled trials. *J. Psychiatr. Res.* 92, 139–146.
- Zhu, Y., Li, C., Huhn, M., Rothe, P., Krause, M., Bighelli, I., Schneider-Thoma, J., Leucht, S., 2017. How well do patients with a first episode of schizophrenia respond to antipsychotics: A systematic review and meta-analysis. *Eur. Neuropsychopharmacol.* 27, 835–844.