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Exploring peripheral biomarkers of response to simvastatin supplementation in schizophrenia

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ABSTRACT

Schizophrenia is one of the most debilitating mental disorders, and its diagnosis and treatment present significant challenges. Several clinical trials have previously evaluated the effectiveness of simvastatin, a lipid-lowering medication, as a novel add-on treatment for schizophrenia. However, treatment effects varied highly between patients and over time. In the present study, we aimed to identify biomarkers of response to simvastatin in recent-onset schizophrenia patients. To this end, we profiled relevant immune and metabolic markers in patient blood samples collected in a previous clinical trial (ClinicalTrials.gov: NCT01999309) before simvastatin add-on treatment was initiated. Analysed sample types included serum, plasma, resting-state peripheral blood mononuclear cells (PBMCs), as well as PBMC samples treated *ex vivo* with immune stimulants and simvastatin. Associations between the blood readouts and clinical endpoints were evaluated using multivariable linear regression. This revealed that changes in insulin receptor (IR) levels induced in B-cells by *ex vivo* simvastatin treatment inversely correlated with *in vivo* effects on cognition at the primary endpoint of 12 months, as measured using the Brief Assessment of Cognition in Schizophrenia scale total score (standardised $\beta \pm SE = -0.75 \pm 0.16$, $P = 2.2 \times 10^{-4}$, $Q = 0.029$; $n = 21$ patients). This correlation was not observed in the placebo group ($\beta \pm SE = 0.62 \pm 0.39$, $P = 0.17$, $Q = 0.49$; $n = 14$ patients). The candidate biomarker explained 53.4 % of the variation in cognitive outcomes after simvastatin supplementation. Despite the small sample size, these findings suggest a possible interaction between the insulin signalling pathway and cognitive effects during simvastatin therapy. They also point to opportunities for personalized schizophrenia treatment through patient stratification.

1. Introduction

Schizophrenia is a psychiatric disorder affecting approximately 20 million people globally (Charlson et al., 2018). It is characterized by a complex clinical presentation, including positive, negative, cognitive, and mood symptoms. The underlying neurobiological background of the disease is poorly understood, and medications have limited efficacy, especially in treating cognitive and negative symptoms (Nielsen et al., 2015). Additionally, antipsychotic treatment often causes debilitating side effects contributing to the significantly higher mortality rates in schizophrenia patients (Laursen et al., 2014). Therefore, clinical research efforts have been focusing on trialling add-on treatments and identifying biomarkers to improve diagnostic procedures and therapeutic strategies.

A recent double-blind placebo-controlled trial conducted at the University Medical Center Utrecht (UMCU) and the University Medical Center Groningen (UMCG) evaluated the effectiveness of simvastatin as add-on medication to antipsychotic treatment in schizophrenia

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(Sommer et al., 2021). Simvastatin belongs to a class of medications known as statins, which have lipid-lowering and anti-inflammatory properties (Kim et al., 2019). Previous reports have suggested associations between hyperlipidemia and immune system dysfunction in the pathophysiology of schizophrenia (Correll and Kane, 2014; Miller et al., 2014; Najjar and Pearlman, 2015; Wysokiński et al., 2015). Additionally, metabolic syndrome and dyslipidemia are highly prevalent side effects of antipsychotic medication (Pillinger et al., 2020). In turn, simvastatin treatment has been observed to attenuate brain deterioration in mice (Fawzy Fahim et al., 2019), and protect from cognitive impairment in people (Iadecola and Parikh, 2019). Specifically in schizophrenia patients, simvastatin has also been shown to lower the levels of inflammation markers (Francesconi et al., 2019). Hence, simvastatin was hypothesized to be a promising add-on treatment for schizophrenia due to its ability to cross the blood-brain barrier, the anti-inflammatory effects on the cerebrum, and its lipid-lowering action (Kim et al., 2019). Although the trial found no overall effectiveness of simvastatin on symptom severity or cognition after 12 months of treatment compared to placebo, treatment effects varied highly between patients and over time. This prompted the present biomarker analysis to investigate biological patient subgroups in relation to treatment outcomes.

The identification of schizophrenia biomarkers has the potential to aid the diagnosis and inform on prognosis and effective treatment of the disease (Perkovic et al., 2017). *Ex vivo* studies conducted on peripheral blood mononuclear cells (PBMCs) have shown functional PBMC responses to neuropsychiatric medications at known and emerging drug targets (Gladkevich et al., 2004; Lago et al., 2019, 2021a). These studies provide proof-of-concept support for the use of PBMCs as biomarkers and drug discovery cell models in schizophrenia (Lago et al., 2019). More specifically, recent work has shown that metabolic markers from peripheral blood cells of neuropsychiatric spectrum patients correlated with treatment efficacy and side effects (Lago et al., 2021b).

In the current work, we leveraged the previously collected evidence on the predictive validity of metabolic biomarkers in PBMCs from neuropsychiatric patients to expand the original analysis of data collected in the clinical trial of simvastatin add-on treatment (Sommer et al., 2021). We hypothesized that one or more biomarkers from a selected panel could be used to predict simvastatin treatment response in the given patient cohort, therefore helping elucidate the biological mechanisms underlying treatment effects. Pre-treatment levels of serum, plasma and PBMC analytes were measured using multiplexed immunoassays and flow cytometry, and their association with clinical outcomes was evaluated using linear regression, accounting for relevant covariates and adjusting for multiple comparisons.

2. Methods

2.1. Study population

Samples in the present study were collected as part of the double-blind placebo-controlled clinical trial of simvastatin supplementation in schizophrenia (ClinicalTrials.gov identifier: NCT01999309) (Begemann et al., 2015; Sommer et al., 2021), which aimed to evaluate the utility of adjunctive simvastatin in improving psychotic and cognitive symptoms of schizophrenia. The study population included 119 participants between 18 and 50 years old who had been diagnosed with a schizophrenia spectrum disorder according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (American Psychiatric Association, 2000; Jablensky, 2010), with the first emergence of a psychotic episode occurring no longer than three years prior to the trial. The trial was conducted at the UMCU and UMCG in the Netherlands. Participants were recruited between 11/2013 and 2/2019. Further details of the trial, such as patient inclusion and exclusion criteria, and power calculations, can be found in a previously published report (Begemann et al., 2015).

2.2. Clinical procedures

Participants were randomised to 40 mg/day simvastatin ($n = 61$) or placebo ($n = 58$), both taken in the evening. The 40 mg dose of simvastatin is within the recommended initial simvastatin dosage (NHS, 2022). Both staff and patients were blinded to treatment allocation. The primary treatment outcomes were assessed at 12 months using the Positive and Negative Syndrome Scale (PANSS) and the Brief Assessment of Cognition in Schizophrenia (BACS). Secondary outcomes were evaluated at 1, 3, 6, 9, 12, and 24 months, and included PANSS subscale scores (positive, negative, and general psychopathology symptoms), the Global Assessment of Functioning (GAF) scale scores, and the depressive symptom severity ratings obtained using the Calgary Depression Scale for Schizophrenia (CDSS).

2.3. Sample preparation

Blood samples were prepared by the biobank at the UMCU. Only samples from participants who consented to international material and data transfer were analysed in the present study. This equated to 21 simvastatin- and 14 placebo-treated participants. Serum samples were prepared from whole blood collected into 9 ml serum separator tubes. Samples were allowed to clot for 1 h, and centrifuged for 10 min at 2000*g*. The supernatants were aliquoted and frozen at −80 °C. Plasma samples were prepared from whole blood collected into 3 ml lithium heparin tubes according to the UMCU standard operating procedures.

For PBMC isolation, blood was collected into 9 ml sodium heparin tubes and processed within 24 h. The blood was diluted with phosphate buffered saline (PBS) at a 1:2 ratio. The diluted blood was transferred onto Ficoll-Paque and centrifuged for 20 min at 1000*g* at room temperature in swing-out buckets. The resulting plasma layer was removed to prevent contamination of the enriched PBMCs with platelets, and the PBMC layer was harvested. The collected PBMCs were washed twice with 10 ml of PBS at 250 g for 10 min. Last, the resulting cell pellets were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium with 1 % penicillin-streptomycin and 10 % dimethyl sulfoxide (DMSO), and cryopreserved in liquid nitrogen.

2.4. Laboratory procedures

2.4.1. Serum and plasma measurements

Serum and plasma analytes were included from amongst key pro-inflammatory proteins, cytokines, chemokines, metabolic markers, hormones, growth factors, and acute phase reactants (Calza et al., 2012; Hu et al., 2009; Schwarz et al., 2012). Target analytes in serum included apolipoproteins (Apo) A1, A2, B, C2, and E (ProcartaPlex Human Apolipoprotein Panel 5-plex, Thermo Fisher, EPX050–15818-901); interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-a) (Human Magnetic Luminex Performance Assay, High Sensitivity Cytokine A, R&D Systems, LHSCM000); and serpin E1, chemokine ligand-2 (CCL-2), leptin, adiponectin, C-reactive protein (CRP), resistin, and complement factor D (Human Obesity Luminex Performance Assay, R&D Systems, LOBM000). The analytes were measured on a Luminex MAGPIX multiplexed immunoassay platform (Luminex Corporation). Analyses were conducted according to the instructions provided by assay manufacturers. All samples were analysed in duplicates, and three quality control samples were included in each assay plate. Samples were assayed at optimized dilutions and analyte concentrations were calculated from 5-point logistic standard curves. Analytes in plasma were measured according to standard operating procedures at UMCU, and included CRP, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, non-high-density lipoprotein (non-HDL) cholesterol, total cholesterol, glucose, and triglycerides.

2.4.2. Cell culture

PBMCs were thawed at 37 °C in sterile conditions and washed with

complete RPMI (cRPMI) medium (RPMI-1640 with sodium bicarbonate (Sigma-Aldrich), 10 % foetal bovine serum (Life Technologies), 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies), and 2 mM L-alanyl-L-glutamine dipeptide (Life Technologies)) with 20 μg/ml deoxyribonuclease (DNAse; Sigma-Aldrich). After counting, the cells were resuspended at 1×10^6 cells/ml in the cRPMI medium with DNAse, strained through a 40 μm strainer, and seeded into 96-well polypropylene plates (Starlab) at 0.2 ml/well. The cells were then rested for 24 h at 5 % CO_2 and 37 °C.

The next day, PBMCs from each patient were pelleted and seeded in four different conditions to evaluate their functional responses. The conditions encompassed unstimulated and stimulated cells ± simvastatin. Immune stimulation was achieved with 1 μg/ml of staphylococcal enterotoxin B (SEB), 1 μg/ml of anti-CD28, and 0.1 μg/ml of lipopolysaccharides (LPS), in order to observe increased metabolic activity and proliferation in PBMCs. Simvastatin treatment was tested at two concentrations, including physiological (10 nM) concentration representing serum levels of simvastatin at 40 mg intake, and experimental (10 μM) levels (Björkhem-Bergman et al., 2011). PBMCs were cultured in the cRPMI medium with the stimulants and/or the drug for the following 72 h at 5 % CO_2 and 37 °C. The incubation time of 72 h was determined based on previous literature and pilot experiments, to allow sufficient time for inducing changes in protein expression. PBS and DMSO were used at corresponding amounts as the negative control condition.

2.4.3. PBMC epitope staining

Relevant epitopes were selected based on previous research on PBMC subtypes and functional PBMC responses in schizophrenia, including target epitopes in the lipid metabolism, glucose transportation and oxidative stress pathways, which are known to mediate the effects of simvastatin (Drexhage et al., 2011; Lago et al., 2021b). PBMC staining was carried out according to Lago et al., 2021b. For staining, PBMCs were washed and resuspended in FACS buffer (PBS with 0.5 % bovine serum albumin (Sigma-Aldrich)) with 20 % human Fc receptor binding inhibitor (eBioscience). The cells were incubated at room temperature for 20 min to allow non-specific antibody binding. Next, staining was carried out in a total volume of 90 μl per sample, with 0.5 μl of anti-human CD3 (UCHT1) PE-Cy7 (eBioscience), 0.5 μl of anti-human CD4 (SK3) PerCP-eFluor 710 (eBioscience), 0.5 μl of anti-human CD8 (SK1) APC-eFluor 780 (eBioscience), and 0.3 μl of anti-human CD14 (MφP9) V500 (BD Biosciences). For samples stained for metabolic markers, 10 μl of anti-human glucose transporter 1 (GLUT1, clone 202915) FITC (R&D Systems), 20 μl of anti-human insulin receptor (IR, or CD220, clone 3B6/IR) PE (BD Biosciences), and 2.5 μl of anti-human fatty acid translocase (CD36, clone NL07) eFluor660 (Thermo Fisher) were added as per manufacturers' instructions. Equivalent volumes of FACS buffer were added to wells with unstained control samples. Cells were then incubated in the dark for 45 min at room temperature, and washed twice with FACS buffer. For acquisition, the cells were resuspended in 150 μl of FACS buffer with 1 μM DAPI (Sigma-Aldrich) and stored at 4 °C until analysis.

2.4.4. Flow cytometry

PBMCs were acquired using FACSVerse flow cytometer (BD Biosciences) with 405, 488, and 640 nm laser excitations at a mean flow rate of 2 μl/s. Quality control and standardisation of photomultiplier tube detector voltages throughout multiple experimental runs was performed using Multicolor Cytometer Setup and Tracking beads (BD Biosciences). Fluorescence compensation was carried out using anti-mouse IgGκ antibody capture beads (Bangs Laboratories) stained separately with anti-human CD3 (UCHT1) PE-Cy7, anti-human CD4 (SK3) PerCP-eFluor 710, anti-human CD8 (SK1) APC-eFluor 780, anti-human CD14 (MφP9) V500, anti-human GLUT1 (202915) FITC, anti-human CD220 (3B6/IR) PE, and anti-human CD36 (NL07) eFluor660, together with single-stain PBMC controls stained with DAPI.

2.5. Data analysis

2.5.1. Clinical data processing

Data processing and statistical analysis were performed using R v.4.0.5. Clinical and demographic characteristics were compared between the placebo and simvastatin groups using the 'tableone' package. Statistical significance was determined using the Mann-Whitney *U* test for continuous variables, and the Fisher's exact test for categorical variables (Pearson's chi-squared test in comparisons with more than two groups). Similarly, baseline analyte levels were compared between the placebo and simvastatin groups using the Mann-Whitney U test to assess potential pre-treatment biases. Total BACS scores were calculated adjusting for age and sex based on US population BACS component score norms (Keefe et al., 2008). Selection bias *versus* the original clinical trial cohort was evaluated using a *Z*-test for continuous variables and Pearson's chi-squared test for categorical variables.

2.5.2. Serum data processing

Raw serum data processing was conducted using the xPONENT software 4.1 (Luminex Corporation). Of the 14 serum analytes, four were excluded from the analysis due to >30 % of the values falling outside the linear range of the assays. The excluded analytes comprised adiponectin, CRP, complement factor D, and serpin E1. For the remaining 10 analytes, values outside the linear range of the assays (1.6 % of all values) were replaced with the concentrations of the lowest and the highest standard, respectively. Data points with a high (>50 %) coefficient of variation (CV) between replicate measurements were excluded. The average CV (± standard deviation) for the remaining samples was 6.6 ± 11.7 %. Batch effects caused by analysing samples in multiple plates and over multiple days were evaluated using the Kruskal-Wallis test, and individual plate effects were assessed using the Spearman's rank correlation test. The respective batch effects were removed using Z-factor scaling and linear regression.

2.5.3. PBMC data processing

The raw flow cytometry data were processed using FlowJo v.10.8 (Tree Star). Only PBMC samples with a viability of 60 % or more, and a minimum of 100 live cells per PBMC subtype, were analysed. Metabolic marker expression was evaluated using stain indexes, calculated for each epitope and PBMC subtype as the ratio of the mean MFI (median fluorescence intensity) of the antibody-stained and unstained control samples. Markers with low staining, *i.e.*, those with a stain index below 2, were removed. Epitope expression was evaluated in both resting state (*i.e.*, unstimulated and untreated) and in response to *ex vivo* immune stimulation and/or treatment with simvastatin. PBMC response to the immune stimulation was calculated for each sample by dividing epitope MFIs in the stimulated condition by the MFIs in the unstimulated condition, without simvastatin. Similarly, the *ex vivo* response to simvastatin was calculated for each sample as the MFI at given simvastatin concentration (*i.e.*, 10 nM or 10 μM) divided by the MFI at 0 μM simvastatin, separately in the unstimulated and stimulated condition. Additionally, the analysis included PBMC subtype frequencies and their ratios.

2.5.4. Treatment response prediction

The association of pre-treatment levels of serum, plasma, and PBMC readouts with clinical outcomes was evaluated using stepwise linear regression with forward feature selection and backward elimination to prevent overfitting. Clinical outcomes were calculated by subtracting baseline scores from follow-up scores. The analysis was adjusted for the optional covariates including age, sex, body mass index (BMI), and frequently used antipsychotics (olanzapine, aripiprazole, and clozapine). Additionally, baseline psychopathology scores were fixed within the respective models to account for regression to the mean effects when predicting longitudinal outcomes (Barnett et al., 2005). The analysis was carried out separately in the simvastatin and placebo groups. The

resulting *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure (shown as Q values). The significance threshold was set to $Q < 0.05$, and all tests were two-tailed. Standardised correlation coefficients were obtained from scaled predictor and outcome values in regression analysis.

3. Results

3.1. Demographic and clinical data comparison

The demographic and clinical characteristics of the study participants at baseline is summarised in Table 1, and baseline analyte levels for both the simvastatin and placebo groups are shown in Supplementary Table 1. The assessment of the selection bias *versus* the original clinical trial cohort is shown in Supplementary Table 2. Patients in the simvastatin ($n = 21$) and placebo ($n = 14$) groups were matched for most of the evaluated metrics except PANSS positive scores and diastolic blood pressure. On average, participants in the simvastatin group had 4.5-point higher PANSS positive scores ($P = 0.014$) and 8 mmHg higher diastolic blood pressure ($P = 0.036$) compared to the placebo group.

3.2. Serum and plasma analytes

To evaluate whether it is possible to predict response to simvastatin in schizophrenia using patient blood samples, pre-treatment blood parameters were correlated with post-treatment drug efficacy *in vivo*. In serum and plasma, a total of 17 markers were assessed for their association with 2 primary and 29 secondary endpoints. Twenty-seven nominally significant ($P < 0.05$) associations were identified in the serum and plasma data after adjusting for covariates. However, none of them remained significant after adjusting for multiple comparisons. A summary of the correlation between serum and plasma analytes and simvastatin efficacy is presented in Supplementary Fig. 1.

3.3. PBMC biomarkers

PBMC measurements comprised resting-state measurements, functional PBMC responses to *ex vivo* immune stimulation and treatment with simvastatin, and cell subtype frequencies. Amongst the 147 measured PBMC parameters, only one association was statistically significant after adjusting for covariates and multiple comparisons (Fig. 1 and Supplementary Fig. 1). The *ex vivo* effect of 10 μM simvastatin on IR expression in $CD3^-$ cells (largely B and natural killer (NK) cells; labelled 'B-cells') in unstimulated condition was negatively correlated with changes in total BACS scores at the primary endpoint of the trial of 12 months (standardised $\beta \pm SE = -0.75 \pm 0.16$, $P = 2.2 \times 10^{-4}$, $Q = 0.029$, adjusted for baseline BACS scores and clozapine use; Fig. 2A). The readout explained 53.4 % of variation in the cognitive outcomes of simvastatin treatment. A consistent, however not significant, effect was observed for B-cells treated with 10 nM concentration of simvastatin ($\beta = -0.55 \pm 0.21$, $P = 0.019$, $Q = 0.24$, 27.6 % of variance explained; Fig. 2C).

3.4. PBMC biomarker specificity

To evaluate whether the identified candidate biomarker was specific to simvastatin treatment effects and not related to non-specific changes in cognition, a parallel analysis was performed in the placebo group. No significant associations were observed in the placebo group between the *ex vivo* simvastatin effects on B-cell IR expression and 12-month changes in cognitive functioning. Results from the placebo group are shown in Fig. 2B and D.

Table 1
Demographic and clinical characteristics of patients in the simvastatin and placebo groups at baseline. BACS – Brief Assessment of Cognition in Schizophrenia scale, CDSS – Calgary Depression Scale for Schizophrenia, GAF – Global Assessment of Functioning scale, IQR – interquartile range, PANSS – Positive and Negative Syndrome Scale. [1)] Determined based on the Childhood Trauma Questionnaire question "Do you believe that you have been physically, emotionally, or sexually abused?". [2)] Calculated based on US BACS component score norms (Keefe et al., 2008). [3)] Only medications used by >1 participant are shown. [4)] Determined according to Jin and Benyshek (2013) (Jin and Benyshek, 2013). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Characteristic	Placebo	Simvastatin	*P*-value	Missing
	n = 14	n = 21		(%)
Sex, no. (%)				
Female	4 (29)	4 (19)	0.805	0
Male	10 (71)	17 (81)		
Age, median years, [IQR]	26.5 [22.0,33.5]	25.0 [24.0,28.0]	0.625	0
Highest level of education, no. (%)				
College	9 (64)	9 (43)	0.378	3.2
Primary	1 (7)	1 (5)		
Secondary	4 (29)	11 (52)		
University	0 (0)	0 (0)		
Nationality, no. (%)				
Iran	0 (0)	1 (5)	1	
Netherlands	14 (100)	20 (95)		
Duration of illness, years, median [IQR]	1.0 [0.0,1.0]	1.0 [1.0,2.0]	0.131	0
Childhood abuse, No. (%)[1)]	6 (43)	8 (38)	1	1.6
Rating scales, median, [IQR]				
PANSS positive	9.5 [7.5,12.8]	14.0 [11.0,20.0]	0.014*	0
PANSS negative	14.0 [12.2,19.0]	15.0 [11.0,17.0]	0.866	0
PANSS general	28.0 [26.0,34.8]	30.0 [27.0,33.0]	0.448	0
PANSS total	55.0 [47.5,65.0]	59.0 [46.0,73.0]	0.296	0
GAF	57.5 [55.0,64.2]	51.0 [45.8,62.0]	0.127	1.6
CDSS	1.0 [0.0,6.0]	3.0 [1.0,4.0]	0.329	1.6
BACS Total[2)]	−1.4 [−2.6,-1.0]	−1.4 [−2.7,-0.3]	0.686	0
Medication, no. (%)[3)]				
Aripiprazole	4 (29)	5 (24)	1	0
Clozapine	0 (0)	4 (19)	0.233	0
Haloperidol	2 (14)	1 (5)	0.712	0
Lorazepam	1 (7)	2 (10)	1	0
Methylphenidate	1 (7)	1 (5)	1	0
Olanzapine	3 (21)	5 (24)	1	0
Paliperidone	0 (0)	4 (19)	0.233	0
Quetiapine	3 (21)	2 (10)	0.622	0
Alcohol consumption, no. (%)	9 (64)	18 (86)	0.285	3.2
Portions/week, median [IQR]	1.0 [0.0,2.0]	2.0 [1.0,5.0]	0.065	4.8
Recreational drug use, no. (%)	4 (29)	5 (24)	1	3.2
Smoking, no. (%)	10 (71)	14 (67)	1	4.8

(continued on next page)

Table 1 (*continued*)

Characteristic	Placebo	Simvastatin	*P*-value	Missing
	n = 14	n = 21		(%)
Cigarettes/week, median [IQR]	7.5 [0.8,13.8]	10.0 [0.0,14.0]	0.891	4.8
Coffee consumption, no. (%)	9 (64)	18 (86)	0.285	3.2
Cups/week, median [IQR]	1.5 [0.0,2.0]	2.0 [1.0,4.0]	0.122	6.5
Clinical characteristics, median [IQR]				
Systolic blood pressure, mmHg	121.0 [117.5,125.8]	127.0 [116.0,135.0]	0.428	1.6
Diastolic blood pressure, mmHg	72.0 [70.0,76.0]	80.0 [73.0,83.0]	0.036*	1.6
Weight, kg	68.5 [65.2,86.2]	80.0 [69.0,90.0]	0.095	1.6
Height, cm	176.5 [169.8,180.8]	183.0 [175.0,185.0]	0.125	1.6
Body mass index, kg/m^2	22.5 [21.3,25.7]	24.6 [20.6,26.9]	0.625	1.6
Waist circumference, cm	87.0 [83.0,96.0]	91.0 [86.0,100.0]	0.202	3.2
Metabolic syndrome, no. (%)[4)]	1 (7)	1 (5)	1	0
Non-fasting samples, no. (%)	0 (0)	3 (14)	0.202	0

4. Discussion

4.1. Overview

The current study was a follow-up to the clinical trial of simvastatin supplementation in patients with recent-onset schizophrenia, with the goal of identifying biomarkers predicting treatment outcomes. To this end, 164 serum, plasma, and PBMC measurements were collected from 35 schizophrenia patients. PBMC markers included resting-state measurements, and functional PBMC responses to *ex vivo* immune stimulation and treatment with simvastatin. Linear regression was used to evaluate the association of analyte levels against the clinical endpoints of the trial. Following adjustment for multiple comparisons, expression levels of insulin receptor (IR) on B-cells after exposure to simvastatin *ex vivo* was identified as a predictor of cognitive outcomes after 12 months of simvastatin treatment. The observed association of increased IR levels on simvastatin-treated B-cells with cognitive outcomes exclusively in simvastatin-treated patients suggests a connection between response to simvastatin, insulin signalling, and B-cell activity.

4.2. Role for insulin signalling

The correlation between the *ex vivo* effects of simvastatin on B-cell IR expression and *in vivo* effects on cognition suggests a connection between insulin signalling and the cognitive effects of simvastatin. Although the current analysis evaluated only protein levels and not protein function, the observed increase in IR in response to simvastatin may indicate higher activity of the IR signalling pathways (Aleem et al., 2011). This would render the cells more sensitive to stimulation by insulin, or alternatively, could indicate an upregulation of IR due to decreased insulin receptor sensitivity or activity. Ample evidence exists linking insulin signalling to schizophrenia and cognitive outcomes (Guest, 2019; Kong et al., 2018; Tomasik et al., 2019; Willmann et al., 2020). Insulin resistance in the brain has been linked with worse cognitive outcomes in numerous reports (Kong et al., 2018; Ma et al., 2015; Spinelli et al., 2019), and schizophrenia patients are known to be predisposed towards insulin resistance (Tomasik et al., 2019). Additionally, while statins have favourable metabolic effects, they may also cause insulin resistance through impacting IR signalling pathways (Bell et al., 2014; Brault et al., 2014; Kain et al., 2015). It has been suggested that statins can reduce insulin signal transduction through inhibiting relevant signalling events (Kain et al., 2015). Therefore, the IR upregulation by simvastatin observed in patients with lower cognitive outcomes in the present study may represent a compensatory mechanism countering these effects. While the current results are limited to peripheral immune system cells, simvastatin-induced changes in insulin signalling may extend to other cell types, including brain tissue, whereby they could precipitate cognitive alterations. Interestingly, previous studies reported significant correlations between peripheral insulin sensitivity and insulin signalling in the brain, specifically in areas concerned with memory and executive function (Heni et al., 2014, 2015, 2017; Kullmann et al., 2016). Thus, peripheral markers may be reflecting causal changes in insulin signalling in the brain. Previous evidence from samples of patients with Alzheimer's disease and murine models showed that insulin resistance in neurons may lead to neuronal damage (Moloney et al., 2010; Moroz et al., 2008). Additionally, peripheral insulin resistance was found to increase amyloid beta and inflammatory cytokines (interleukin-6 and tumour necrosis factor alpha) concentrations in the brain (Craft, 2005; Watson and Craft, 2003), which could induce neuronal loss, amyloid beta plaques, and neurofibrillary tangles (Fishel et al., 2005). In the context of the current study, the putative causal relationship between altered IR expression profiles and cognitive changes is speculative, in the absence of experimental evidence validating that peripheral B-cell IR levels correlate with neuronal cellular effects leading to cognitive changes. Further research is warranted to investigate the effect of simvastatin on IR signalling, and its possible causative links with changes in cognitive functioning.

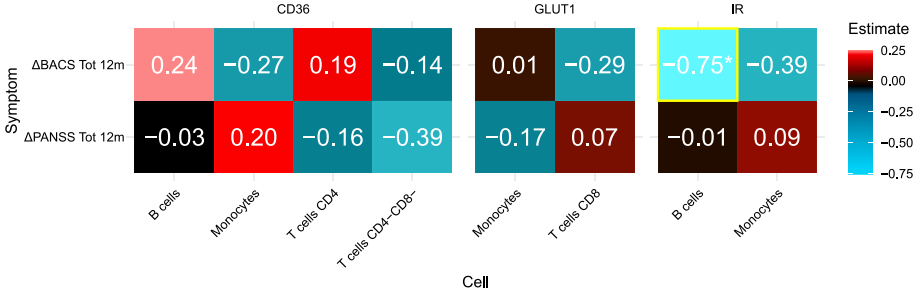

Fig. 1. Association between the *ex vivo* 10 μM simvastatin effect on metabolic marker expression in PBMC subtypes, and the *in vivo* efficacy of simvastatin on the primary endpoints of the trial. Heatmap shows standardised correlation coefficients, with negative estimates shown in blue and positive estimates shown in red. Epitopes with stain index below two were excluded from the analysis and are not shown. Complete results from PBMC analysis are included in Supplementary Fig. 1. BACS – Brief Assessment Cognition Schizophrenia, CD – cluster of differentiation, CD36– fatty acid translocase, GLUT1 – glucose transporter 1, IR – insulin receptor, m – months, PANSS – Positive and Negative Syndrome Scale, PBMC – peripheral blood mononuclear cells, Tot – total. * Q < 0.05 (yellow border). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

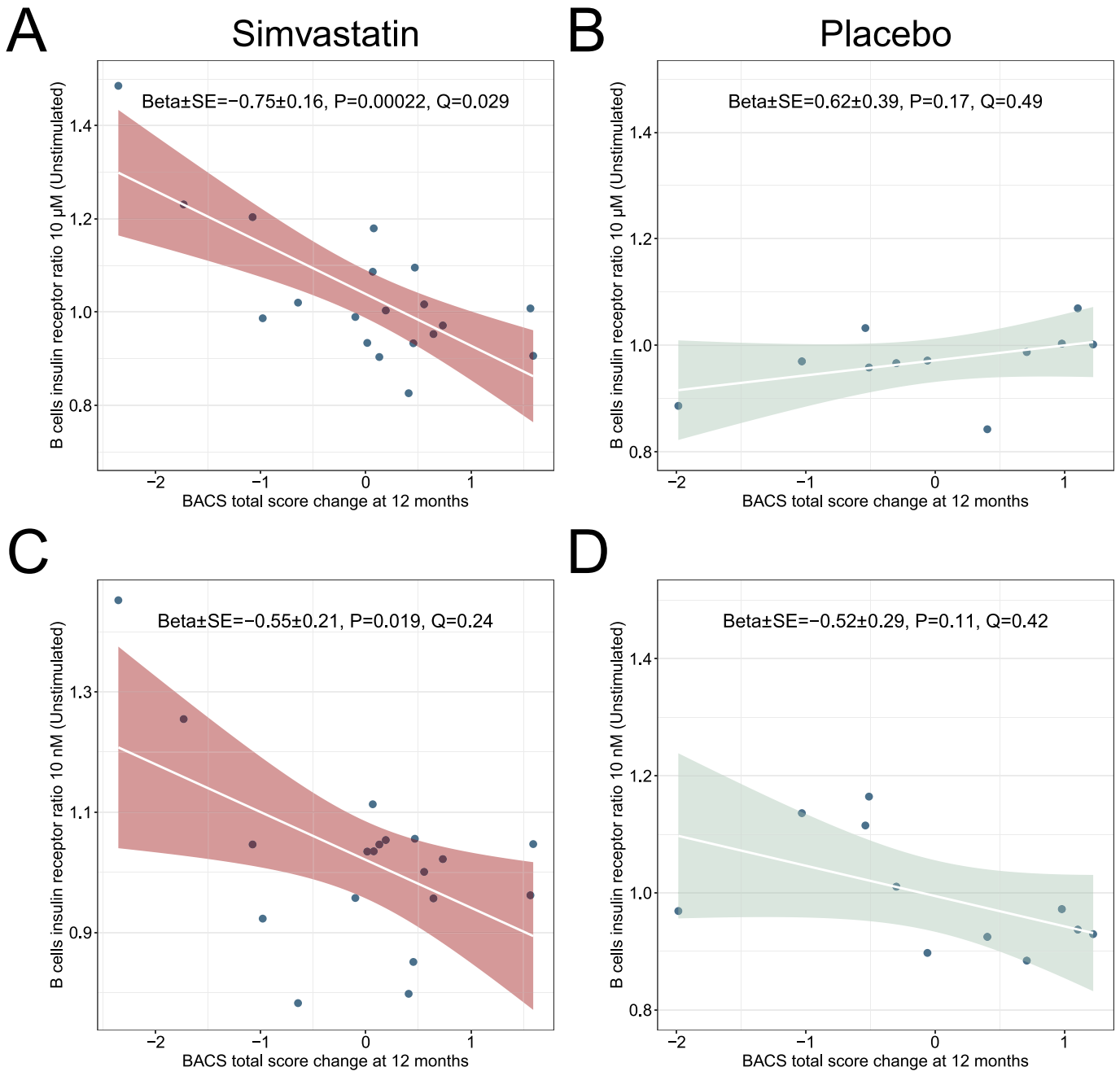

Fig. 2. Predictive biomarker of response to simvastatin in schizophrenia. Scatterplots show the association between *ex vivo* simvastatin effect on B-cell IR expression in unstimulated condition (Y axis) and *in vivo* changes in cognition after 12 months of treatment measured using the BACS scale (X axis). Shown are results from the simvastatin ($n = 21$; A and C) and placebo ($n = 14$; B and D) groups. Changes in B-cell IR expression were measured in response to experimental (10 µM; A and B) and physiological (10 nM; C and D) simvastatin concentration. Effect ratios were calculated by dividing epitope MFI in treated condition by epitope MFI in untreated condition. The model was adjusted for baseline BACS scores. Other relevant covariates (age, sex, BMI, treatment with olanzapine, aripiprazole, and clozapine) were selected using stepwise linear regression. White line represents the fitted multivariable linear regression model, and shaded areas represent 95 % confidence intervals. BACS – Brief Assessment Cognition Schizophrenia, BMI – body mass index, IR – insulin receptor, MFI – median fluorescence intensity, SE – standard error.

4.3. B-cell involvement

Increased expression of the IR may result not only in altered energy metabolism including glycogenesis, but as insulin is a weak growth factor, also in increased proliferation (Aleem et al., 2011). This suggests that simvastatin could be inducing increased B-cell activity by altering the cells' metabolic profile. However, previous evidence on the anti-inflammatory properties of statins showed decreased $CD3^-$ cell activation (Shimabukuro-Vornhagen et al., 2010; Tanaka et al., 2006). More specifically, simvastatin led to a dose-dependent inhibition of B-cell activation and proliferation following stimulation *via* CD40 (Shimabukuro-Vornhagen et al., 2010). To the best of our knowledge, the B-cell insulin signalling pathway has not been investigated in relation to B-cell activation and disorders characterized by cognitive impairment. Further research is required to determine whether the present finding of increased IR expression on B-cells in response to simvastatin relates to a specific role for B-cells in the immunoinflammatory landscape leading to a loss of cognitive function. This is of great interest, as multiple reports show evidence of B-cell alterations in schizophrenia (Steiner et al., 2010) and indicate a role for B-cells in schizophrenia pathogenesis (van

Mierlo et al., 2019). Additionally, B-cells have been previously identified to have a causal effect on cognitive impairment in multiple disorders. For instance, B-cell removal therapy has been shown to cause cognitive symptom improvements in models for stroke (Doyle et al., 2015), Alzheimer's disease (Kim et al., 2021), and multiple sclerosis (Ahn et al., 2021). Thus, better understanding of how insulin signalling, cognitive decline, and B-cells interact with each other is crucial.

4.4. Research and clinical implications

The present findings corroborate the importance of investigating functional PBMC biomarkers in schizophrenia. The analysis revealed potential links between metabolic and immune players, schizophrenia, and cognitive function. Functional PBMC biomarkers are not commonly analysed in biomarker discovery studies due to the challenges of use and handling. However, they provide additional insights into drug mechanism of action at the cellular level. Therefore, the present results suggest that despite the challenges, biomarker discovery research could benefit from incorporating functional readouts alongside other analyte measurements, due to the additional insight and targets they provide.

Our findings may translate more widely to investigations evaluating cognitive effects of simvastatin. Currently, statins are ranked amongst the most highly prescribed medications in the UK and the US (Audi et al., 2018; Fuentes et al., 2018). With prescription trends increasing over time (O'Keeffe et al., 2016), it becomes increasingly important to identify potential vectors of their cognitive outcomes. It is pertinent to acknowledge that the cognitive implications of statin therapy are inconsistent and disputed, with both protective and adverse effects reported (Schultz et al., 2018; Tan et al., 2019). With regard to adverse effects, previous reports have shown that treatment with statins may negatively affect the myelination process (Klopfleisch et al., 2008; Miron et al., 2009), with brain demyelination found to be a feature of mild cognitive impairment, Alzheimer's disease, and vascular dementia (Bouhrara et al., 2018). Simvastatin has previously been found to induce demyelination in the brain through decreased cholesterol levels that prevent myelin sheath formation (Schultz et al., 2018).

Considering the inconclusive findings pertaining to cognitive implications of statin therapy, it is vital to investigate the potential long-term effects of simvastatin treatment in longitudinal trials focusing on cognitive endpoints. In this context, implementing PBMC biomarker analysis into future clinical research could provide further insights into functional drug effects, cell pathways involved, and potential strategies for patient stratification. Additionally, larger sample sizes would enable a more detailed biomarker correlation analysis, as well as facilitate investigations into the multivariate biomarker patterns for predicting clinical outcomes and aiding patient stratification. Potential biomarkers identified with this approach could facilitate the prescription of statins to patients with high predispositions to favourable metabolic outcomes while reducing the risk of side effects.

4.5. Limitations

First, the sample size of the analysed clinical trial dataset was limited, and the number of measured variables was high, resulting in the multiple hypothesis testing problem. Second, the patient population in the trial was not drug-naïve, introducing the possibility of analyte levels being affected by medication. Validation in larger, drug-naïve patient cohorts is essential to confirm the relevance of these findings. Third, the candidate biomarker identified in the present study is not viable as a standalone biomarker, as live cell measurements are high-cost and slow to complete. Transition to a faster and more scalable method, such as the TruCulture® blood stimulation system (Myriad RBM) (Brunet et al., 2016), is necessary for practical applications. Fourth, while the cluster of differentiation (CD) markers employed in the current study were chosen to optimize detection of majority cell populations within the multiplexing capacity of the instrumentation used, each CD combination has cell subpopulations which may vary in epitope expression. Fifth, the insulin-related conclusions cannot be confirmed due to a lack of insulin measurement data, and the results are based on interpreting *in vivo* results from *in vitro* experiments, which do not necessarily translate to similar effects in patients. Further studies are therefore required to employ deeper immunophenotyping techniques relating epitope expression to individual cell subtypes. Finally, due to unavailability of some data, the analysis was not controlled for certain factors that could have affected the levels of inflammatory markers and clinical outcomes, such as cardiometabolic conditions.

4.6. Conclusions

We found that changes in IR levels in B-cells treated *ex vivo* with simvastatin could predict cognitive symptom changes in schizophrenia patients treated with the drug. This finding suggests the utility of exploring PBMC signalling epitopes in the prediction of drug efficacy in schizophrenia. While the observed cellular-level changes associated with treatment efficacy are not necessarily causative, cellular readouts hold promise in enabling patient stratification and prioritising cell types and signalling pathways for new targeted therapies. In this study, insulin signalling and B-cell function are called into question in relation to simvastatin efficacy and cognitive outcomes. Further research into the mechanism could provide a causal explanation for the potential cognitive effects of simvastatin. If further validated, a biomarker test could be developed to inform the prescription of simvastatin to patients most likely to benefit from it in terms of cognitive outcomes.

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CRediT authorship contribution statement

Jihan K. Zaki: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Santiago G. Lago:** Data curation, Methodology, Writing – review & editing. **Benedetta Spadaro:** Writing – review & editing. **Nitin Rustogi:** Data curation, Methodology, Writing – review & editing. **Shiral S. Gangadin:** Data curation, Methodology, Writing – review & editing. **Jiri Benacek:** Data curation, Writing – review & editing. **Hemmo A. Drexhage:** Conceptualization, Methodology, Writing – review & editing. **Lot D. de Witte:** Conceptualization, Methodology, Writing – review & editing. **René S. Kahn:** Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing. **Iris E. C. Sommer:** Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing. **Sabine Bahn:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **Jakub Tomasik:** Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

SB is a director of Psynova Neurotech Ltd. and Psyomics Ltd. and has financial interests in Psyomics Ltd. The other authors have no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.schres.2024.02.011.

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