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Inflammatory monocyte gene signature predicts beneficial within group effect of simvastatin in patients with schizophrenia spectrum disorders in a secondary analysis of a randomized controlled trial

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ABSTRACT

Immune dysregulation has been reported in schizophrenia spectrum disorders (SSD). In the past decade, several trials using anti-inflammatory agents for treatment of SSD have been completed, with so far limited success. One such anti-inflammatory agent used is simvastatin. A recent, large-scale, randomized controlled trial with simvastatin augmentation failed to show improvement in the predefined primary outcome. However, baseline inflammatory profiles were not taken into account. Here we employed a data-driven clustering approach to investigate whether patients with an inflammatory monocyte gene signature respond better to add-on simvastatin treatment than those without such a signature, over a treatment period of 2 years. In 61 patients (60 randomized, 1:1 placebo:simvastatin) and healthy controls, a previously validated monocyte gene expression signature was assessed using quantitative polymerase chain reaction. Resulting delta cycle threshold values were used to identify patient clusters. Two major patient clusters with either up- or downregulated pro-inflammatory factors were detected. Linear mixed models showed a significant three-way interaction between the inflammatory cluster, treatment, and time for psychotic symptoms. Only patients treated with simvastatin who were in the inflammatory group, showed a consistent improvement: symptom severity gradually decreased after 3 months and reached significance after 12 and 24 months compared to baseline ($p.adj<0.05$). The effects were small, and overall between-group effects were not significant. Here, we show that patient stratification based on inflammatory gene expression might be useful to select appropriate treatment augmentation for patients with SSD, highlighting the need for precision medicine approaches. Our findings corroborate the results of the primary analyses, showing that in the overall group, simvastatin was not effective; however, at the individual level the treatment might make a difference.

1. Introduction

In 2016, 20.9 million patients worldwide were affected by schizophrenia (SCZ) (Charlson et al., 2018). SCZ is associated with a shortened life expectancy and an increased financial burden for the health care systems in Europe (De Hert et al., 2011; Weye et al., 2021). Despite this burden, new treatment options are still missing. Treatment with current medication, mainly second-generation antipsychotics, is associated with an inadequate response: only approximately one in seven patients meets the criteria for recovery after treatment (Jääskeläinen et al., 2013). A recent meta-analysis showed that SCZ was associated with a weighted average of 14.5 potential life years lost (Hjorthøj et al., 2017). The high number of years lived with disability, the shortened life expectancy of SCZ patients, and the low recovery rates demand exploration of novel,

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improved therapeutic approaches.

Considering psychosis as a multi-systemic disease and targeting different aspects of the disease might help in finding these new options. In particular, an important role for the immune system in both the progression and the development of symptoms has been suggested. Evidence comes from clinical observations such as a higher prevalence of autoimmune diseases in SCZ and a higher number of infections requiring hospitalization (Benros et al., 2011) but also from studies with biological outcomes. In particular, studies assessing antibody and cytokine profiles have shown various abnormalities in patients with SCZ (Fraguas et al., 2019; Frydecka et al., 2018; Dunleavy et al., 2022). Another convincing source of evidence for impaired immune system function comes from studies assessing immune cells in the brain and the periphery, mainly pointing to overexpression of genes related to inflammatory processes and abnormal expression of adaptive T-cell subsets (Saetre et al., 2007; Cai et al., 2020; Volk et al., 2015; Akkouh et al., 2020; Schlaaff et al., 2020; Drexhage et al., 2010, 2011a; Counotte et al., 2018). In the past decade, these reported abnormalities have prompted researchers to investigate anti-inflammatory agents as add-on therapy in SCZ. However, results of the anti-inflammatory agents are varied and not as promising as might be expected: A recent meta-analysis by Jeppesen et al. (2020) showed that randomised-controlled studies investigating anti-inflammatory drugs (like non-steroidal anti-inflammatory drugs, minocycline, monoclonal antibodies and medication with potential anti-inflammatory mechanisms like neurosteroids, estrogens, and fatty acids) led to a small improvement in psychotic symptoms. But effects were minimal, only found in the smaller trials, and primarily anti-inflammatory drugs were not superior to the other drugs, questioning the mechanisms of action (Jeppesen et al., 2020). In line with these findings, our own team also found no effect of simvastatin after the pre-defined time point of a 12 month treatment period (Sommer et al., 2021). Simvastatin is a hydroxy-methylglutaryl coenzyme A reductase inhibitor that, among other things, lowers low-density lipoprotein cholesterol, triglycerides, and apolipoprotein B (Pedersen and Tobert, 2004). In this large randomized, placebo-controlled double-blinded trial in patients with early-stage schizophrenia spectrum disorder (SSD) it was hypothesized that by lowering cholesterol and cholesterol independent mechanisms (e.g., inhibition of isoprenoid synthesis through the mevalonate pathway (Stancu and Sima, 2001)), simvastatin acts as an anti-inflammatory drug.

In the age of precision medicine, it has become very clear that a one-size fits all treatment is a utopic approach to the treatment of psychiatric disorders. In fact, it was recently shown that regarding inflammation, around 40% of patients with SCZ show an altered inflammatory response pathway (Fillman et al., 2013), in reverse suggesting that ant-inflammatory treatment might potentially be used in vain for the remaining 60%. Therefore, stratification is a useful and necessary means to assess the efficacy of treatment in a given patient subgroup. Inflammatory proteins and cells have already been used for stratification in psychiatric disorders such as depression for several years (Raison et al., 2013; Nettis et al., 2021) and few studies have used stratification approaches to predict treatment in SSD as well (for an excellent review refer to (Bishop et al., 2022)). Indeed, results of the OPTIMISE study, the so-far largest trial investigating clinical subtypes, found an association of specific clinical subtypes and inflammation, which were also relevant for clinical outcomes 4 weeks later (Martinuzzi et al., 2019). Recently, the acute phase protein C-Reactive Protein (CRP) has been used as a single, prospective stratification marker for treatment with aspiring in SCZ (Weiser et al., 2021), but failed to find an effect. We are not aware of research using stratification of patients based on their inflammatory gene-expression profile in SSD to predict response to anti-inflammatory treatment trials. Given that simvastatin in the above trial was primarily used with the intention to decrease inflammation, but inflammatory profiles were not used for stratified analysis, we here set out to stratify patients in a secondary analysis of the simvastatin trial.

Several methods for stratification exist and the choice of stratification markers needs to be conducted carefully. We have previously established selected panels of genes that were initially identified in genome-wide association studies in monocytes and were related to inflammatory processes in schizophrenia and various other psychiatric patient groups, such as Major Depressive Disorder (MDD) (Drexhage et al., 2010, 2011b; Padmos et al., 2008). These identified gene panels were characterized by clusters of correlated genes involved in the production of pro-inflammatory cytokines (amongst others containing the genes IL1B, IL6, CCL20, and CXCL2) or in chemotaxis/adhesion and coagulation processes (amongst other genes CCL2, CCL7, SERPINB2). Determination of these monocyte gene clusters has the clear advantage to give information about several processes at hand in contrast to only using one protein (such as interleukin (IL)-6 or C-Reactive Protein (CRP). Since previously, a higher expression level of particularly the pro-inflammatory cytokine gene cluster was found in the monocytes of young, minimally medicated psychotic schizophrenia patients (Drexhage et al., 2010), a group similar to those patients described in the simvastatin trial by Sommer et al. (2021), this gene signature may provide useful for stratification.

In addition to the above-mentioned genes, we have established another cluster of genes in monocytes using a panel of genes characteristic of mitochondrial apoptosis and growth regulation (such as BAX, BCL10, EGR1, and EGR2) genes characteristic of monocytes/macrophages in atherosclerotic plaques (such as ABCA1/ABCG1, NR1H3, CD163, MRC1, MVK, IL-10) (Simon et al., 2021). Abnormal expression of these latter genes can point to a state of mitochondrial dysfunction and an abnormal pro-inflammatory state of normally anti-inflammatory athero-protective M(hb) macrophages (Simon et al., 2021). In healthy individuals, M(hb) macrophages pump out pro-inflammatory intracellular cholesterol (Chistiakov et al., 2015) yielding an anti-inflammatory profile while taking up heme from the environment via the CD163 receptor. These genes might thus be relevant for the anti-inflammatory effects of simvastatin. Since the above described monocytic genes are related to inflammation in SSD or may have direct relevance to the effects of simvastatin, we used this versatile panel of genes with valuable information on four major processes to determine the ideal gene panel for stratification.

Not only innate, but also adaptive immune cells play a major role in SCZ research (Tarantino et al., 2021; Sneeboer et al., 2020; Miller et al., 2013). Although we here aim to use a limited monocyte gene expression as a potential and easily accessible clinical tool for stratification, it is important to know if other compartments of the immune system are also affected. Therefore, as validation of the potential inflammatory activation found in monocytes, we chose to perform Fluorescence-activated cell sorting (FACS) analysis on blood lymphocytes, and measure levels of IL-6 and CRP to provide a more complete insight into inflammatory activation in SSD.

Depressive symptoms are very common in SSD patients and have a huge impact on their quality of life (Li et al., 2020) and several studies support the view that immune system alterations occur in at least a subgroup of patients with depression (Branchi et al., 2020; Lamers et al., 2019). Since the inflammatory status of patients also plays a role in the treatment responsiveness of depressed patients (Nettis et al., 2021), we also assessed whether in SSD, this inflammatory status could be associated with a favourable response to statins in terms of CDSS scores.

In this secondary analysis of the statin schizophrenia trial, we aim to investigate if patient stratification using monocyte inflammation-related gene expression predicts outcome for a) psychotic symptoms measured by the Positive and Negative Syndrome Scale (PANSS) and b) depressive symptoms measured by the Calgary Depression Scale for Schizophrenia (CDSS). We hypothesize that particularly patients with a clear upregulated inflammatory gene expression profile improve during statin treatment, whereas patients with downregulated gene expression do not benefit from simvastatin add-on treatment. Furthermore, given the literature on MDD and inflammation, we hypothesize that inflammatory profiles are correlated to depressive symptomatology in SSD.

2. Methods

This multicenter, randomized placebo-controlled trial was registered (ClinicalTrails.gov: NCT01999309; EudraCT-number:2013-000834-36) and approved by the ethical committee of the University Medical Center Utrecht (UMCU) in the Netherlands with the protocol number 13–249. Patients and controls provided written informed consent before study-related procedures. Inclusions for the study ran from November 2013 to February 2019. All study-related procedures and in-and exclusion criteria are described in detail in Begemann et al. (2015). In brief, 127 patients were included if they satisfied the diagnosis of either schizophrenia, schizoaffective, schizophreniform disorder (295.x), or psychotic disorder not otherwise specified (298.9), according to DSM-IV (Bell, 1994). The first psychotic episode occurred within 3 years before study inclusion. Importantly, patients were excluded if they met formal criteria for statin prescription (i.e. cholesterol value higher than 8 mmol/l), were treated with chronic anti-inflammatory medication (glucocorticosteroids/NSAIDS), had co-medication with interaction, or were pregnant/breast-feeding. Of these 127 patients, 119 were randomized. For the present report, only a subset of biological material was available for analysis (n = 61), see the flow diagram in the supplementary information for exact numbers (Supplementary Fig. 1). Patients who were enrolled in the study were randomized (1:1 n = 30 and n = 30) into two arms adding either simvastatin 40 mg or a placebo daily to their treatment as usual (antipsychotic medication). Symptom severity was assessed using the PANSS for psychotic symptoms (Kay et al., 1987) at baseline, 1, 3, 6, 9, 12, and 24 months, the CDSS (Addington et al., 1993) for depressive symptomatology at baseline, 6, 12, and 24 months. Additionally, general psychopathology was recorded using the Comprehensive Assessment of Symptoms and History interview (Andreasen et al., 1992).

2.1. Monocyte gene expression

Expression levels of monocyte genes were assessed using procedures that have been described in previous publications (Grosse et al., 2015; Schiweck et al., 2020). In brief, sodium heparinized peripheral blood samples were drawn and peripheral blood mononuclear cells (PBMCs) were isolated by low-density gradient centrifugation via Ficoll Paque, then frozen in 10% dimethylsulfoxide in liquid nitrogen. PBMCs were thawed and washed once with complete culture medium (RPMI-1640 culture medium plus 10% fetal calf serum (FCS) and penicillin/streptomycin). Mean recovery after thawing wFas 68% and viability 97%, as assessed by Trypan blue staining.

$CD14^+$ monocytes were isolated from these PBMCs by magnetic cell sorting (Miltenyi Biotec, B.V., Bergisch Gladbach, Germany) and ribonucleic acid (RNA) was isolated (RNeasy minikit; Qiagen, Hilden, Germany). 1 μg of RNA was reverse transcribed (High Capacity cDNA Reverse Transcription kit, Applied Biosystems) to obtain cDNA for quantitative-polymerase chain reaction (qPCR). qPCR was performed using TaqMan Gene expression assays (Applied Biosystems, see Supplementary Table 1). The expression levels of genes were determined using the cycle threshold (CT) method (Livak and Schmittgen, 2001). All values were normalized to the value of the housekeeping gene ABL1 (delta CT (dCT) values), which is a superior housekeeping gene for leukocytes (Beillard et al., 2003). CT values for ABL did not differ between groups.

2.2. Fluorescence-activated cell sorting (FACS)

FACS was used to determine percentages of T lymphocytes ($CD3^+$), T helper lymphocytes ($CD3^+CD4^+$), T cytotoxic lymphocytes ($CD3^+CD8^+$), natural killer cells ($CD3^-CD56^+$), B cells ($CD19^+$), and monocytes ($CD14^+$). A 7-color membrane staining was performed on 50.000 thawed noncultured PBMCs as described previously (Snijders et al., 2016; Grosse et al., 2016).

For the determination of T-helper cell subsets (Th1, Th2, Th17, Treg cells), 1×10^6 of defrosted PBMCs were stimulated for 4 h at 37 °C (5% CO2) in RPMI-1640 culture medium with 50 ng/ml phorbol 12-myristrate 13-acetate (PMA; Sigma Aldrich, St. Louis, MO, USA) and 1.0 μg/ml ionomycin (Sigma) in the presence of Golgistop (BD Biosciences). An 8-color (membrane and intracellular) staining was performed to determine percentages relative to total lymphocytes as described previously. T helper cell subsets were identified by their secreting cytokines: T helper (Th)1 ($CD3^+CD4^+IFN\gamma^+$), Th2 ($CD3^+CD4^+IL4^+$), Th17 ($CD3^+CD4^+IL17A^+$). T regulatory (T_{reg}) cells were identified by their transcription factor FOXP3 ($CD3^+CD4^+CD25^{hi}FOXP3^+$).

2.3. Serum IL-6 and CRP determination

Serum was prepared and stored at −80 °C in aliquots within 4 h after blood draw by the Central Biobank of the UMC Utrecht and UMC Groningen. High-sensitive C-reactive protein (hsCRP) was measured in these samples through the central diagnostic laboratory of the UMC Utrecht and Groningen using the Siemens Atellica™ Solution turbidimetric immunoassay. IL-6 levels were assessed using a high sensitivity assay. To this end, the Meso Scale Discovery S-PLEX Human IL-6 Kit (MSD Cat# K151B3S) was used according to the manufacturer's protocol. All samples were detected within the standard range of the assay.

2.4. Statistical analysis

Statistical analyses were performed in R, version 4.1.0 (R Core Team, 2022). For monocyte gene expression, raw CT values were expressed relative to the housekeeping gene ABL1 (delta CT). Following the method by Livak and Schmittgen (2001), the ABL1 corrected CT values were subsequently expressed relative to the average of the healthy control population, as delta CT values (ddCT). We opted to use ddCT values corrected for directionality in the analyses since they are approximately normally distributed. ddCT values of genes with missing data (HMOX, TNF, THBS, BAX, and BCL10 all <30% missings) were imputed using random forest imputation (R package *imputeMissings*) (Meire et al., 2016).

Monocyte gene cluster belonging was determined using the *NbClust* package (Charrad et al., 2014) with Euclidean distance as a distance measure, Ward.D2 as cluster method, a minimum of 2 and a maximum of 10 possible clusters, and alphaBeale set to 0.1. The *NbClust* function for cluster determination provides 30 available indices and determines the optimal number of clusters as chosen by the majority of indices. Visualization of cluster analysis was performed using the *Heatmap* function in the *ComplexHeatmap* package (Gu et al., 2016).

Continuous demographic characteristics between HC and patients were compared with the Wilcoxon rank sum test, ddCT gene expression values were compared using the two-sided Wilcoxon test for independent samples with mu = 0. Differences in IL-6, CRP, and T cell subsets between the identified patient clusters and controls were compared using Kruskal's Wallis Test followed by Dunn's test, if significant. Benjamini-Hochberg correction was used for False Discovery Rate correction where applicable. To test changes in PANSS and CDSS scores over time within the inflammation clusters and treatment conditions, linear Mixed Models with random intercepts were calculated using the *lme4* (Bates et al., 2014) and *afex* (Singmann et al., 2015) packages. For the linear mixed models, assumptions were tested by visual inspection of residual plots. Models included main effects, two-way, and a three-way interaction for timepoint, condition, and patient cluster. Age, sex, and baseline PANSS/CDSS scores were added as covariates in the respective models. Pairwise contrasts were computed using the *emmeans package* (Lenth et al., 2019). Results of pairwise comparisons and outcomes of gene differences calculated with Dunn's test were adjusted for FDR using the Benjamini-Hochberg Method. Finally, to assess relevant interactions between the selected genes, we created a Protein-Protein Interaction Network using the STRING App in Cytoscape (Szklarczyk et al., 2021;

Doncheva et al., 2019).

3. Results

Demographic information on patients and healthy controls are summarised in Table 1. Patients had a mean age of 26 years, were predominantly male (64%), and had a mean BMI of 24. Controls had a similar BMI, sex distribution, and age. Patients were more likely to be smokers ($X^2 = 14.10$, $p < 0.001$). One patient withdrew before randomization, leaving 60 patients with monocyte gene expression of which 30 (50%) received placebo and 30 (50%) received simvastatin treatment. Patients on placebo and simvastatin treatment did not differ concerning demographic baseline characteristics (see Supplementary Table 2). Medication and drug use are listed in Supplementary Tables 3 and 4

3.1. Pattern of gene expression in monocytes of SSD patients

Fig. 1 A shows a heatmap representing the correlation between monocytic genes, which could be divided into two gene clusters. As can be seen from the figure, the larger gene cluster could be subdivided into 3 different subclusters (1/blue, 2/red, 3/green) in line with clusters previously identified (Drexhage et al., 2010; Padmos et al., 2008; Vogels et al., 2017; Simon et al., 2021; Schiweck et al., 2020). The first cluster (blue) consists of genes coding for molecules involved in chemotaxis, motility and adhesion/coagulation processes related to the vessel wall (such as CCL2/7; NAB2).

The second cluster, in which the genes correlated the strongest, was a cluster composed of genes for the expression of well-known pro-inflammatory cytokines/chemokines or transcription regulators thereof, such as IL1B, CCL20, CXCL2, IL6, MAFF, and TNFAIP3 (in red). A similar cluster of predominantly pro-inflammatory genes has also been identified by us previously in monocytes of another series of schizophrenia patients (Drexhage et al., 2010), in active bipolar patients (Padmos et al., 2008; Vogels et al., 2017) and it also occurs in monocytes of major depressed patients with a history of childhood trauma (Simon et al., 2021; Schiweck et al., 2020). The core genes of this cluster are highly stable and include the genes IL-6, IL1 beta, TNFAIP3, and CCL20 (Drexhage et al., 2010; Simon et al., 2021; Schiweck et al., 2020).

Cluster 3 and 4 contain genes coding for molecules characteristic of M(hb) cells, which are anti-inflammatory athero-protective macrophages operative in the vessel walls. Some genes of this cluster are involved in pumping out intra-cellular cholesterol (ABCA1, ABCG1), others are involved in anti-inflammatory or cholesterol metabolism mechanisms, known of these cells (MRC1 and MVK). To further assess the relationship between the selected genes, we performed a string analysis in Cytoscape (Fig. 1B). It became evident that a strong interaction exists between the genes encoding inflammatory cytokines/chemokines (e.g., TNF, IL-B, IL-6, CXCL2, CCL2, IL-10), and genes regulating the inflammatory state such as HMOX1, MRC1, CD163 and ABCA1.

3.2. Difference in genes between patients with SSD versus healthy controls

We first explored the differences between HC and SSD. Normally distributed genes showed a clear upregulation of the genes characteristic of anti-inflammatory anti-atherogenic M(hb) cells in SSD, such as the cholesterol pump genes (ABCA1 and ABCG1), and the inflammation-regulating genes MRC1 and MVK (Table 2). These expression levels indicate the presence of higher level of M(hb)-like cells in the circulation of SSD patients, but are somewhat atypical since they showed signs of pro-inflammatory activity, (down-regulation in the expression of the gene for the anti-inflammatory HMOX-1 gene expression (p adj.<0.001; Table 2)). This could suggest that in schizophrenia, M(hb) monocyte heme anti-oxidation was reduced. See Table 2 for statistical comparison). Although several genes were either significantly up- or down-regulated (Fig. 2A indicates that some genes did not follow a normal distribution (e.g., TNFAIP3). In particular, the highly inter-correlated pro-inflammatory genes showed a high variability, and sometimes bimodal distribution, with very low and high expression levels in different patients (Fig. 2A.). The variability on the patient level is illustrated in Fig. 2B.

3.3. Stratification based on inflammatory genes

Since we hypothesized that the inflammatory state predicts treatment outcome, we focussed on the expression of the pro-inflammatory gene cluster (containing CCL20, IL6, TNFAIP3, IL1B, CXCL2, and MAFF) with the highest variability for stratification (Fig. 2C). The data-based cluster analysis revealed two groups: those with upregulated genes (representing a high inflammatory potential, n = 18) or those with a down-regulation of these genes (representing a low inflammatory potential, n = 43). Demographic differences between patients with an upregulated and downregulated profile including age, BMI, smoking status, childhood trauma, PANSS total score, or CDSS scores were not identified (see Table 3). Exact monocyte gene expression values for the "up- and down-regulated" patients can be found in Supplementary Table 5. As can be seen from this table, the two patient groups did not differ with regard to the level of M(hb)-like cells, in other words concerning the expression levels of NR1H3/LXRα, ABCA1, ABCG1, MVK, and MRC1.

3.4. Prediction of PANSS and CDSS scores by gene cluster

In the next step, we assessed whether the identified patient groups could be meaningful to predict treatment outcomes. For PANSS total scores, next to main effects for baseline PANSS score ($F = 212.63$, $df = 52.10$, $p < 0.001$), and age ($F = 6.06$, $df = 56.95$, $p = 0.017$), a significant three-way interaction occurred between treatment condition, time, and patient cluster ($F = 2.15$, $df = 257.39$, $p = 0.049$), according to the linear mixed model. Follow-up analyses revealed that "upregulated" patients who received simvastatin (n = 9), experienced a continuous reduction from month 3 onwards, with results reaching significance at

Table 1
Demographic data for patients with SSD and healthy controls. Na = not applicable, sd = standard deviation, BMI= Body Mass Index, PANSS= Positive and Negative Syndrome Scale, CDSS= Calgary Depression Scale for Schizophrenia. Childhood Trauma was assessed with the Jeugd Trauma Vragenlijst (JVT).

	N total	Schizophrenia		Healthy Controls				
		Mean	sd	N total	Mean	sd	test statistic	p-value
Age baseline	61	26.31	6.42	30	24.27	5.02	$F = 2.34$	0.130
BMI	61	23.99	3.88	28	24.02	4.53	$F = 0.00$	0.977
Sex female n (%)	61	16.00	26%	30	9	30%	$X^2 = 0.02$	0.897
Smoking, yes n,(%)	61	40.00	66%	29	6	21%	$X^2 = 14.10$	$p < 0.001$
Condition (0 = placebo)	60	30.00	50%	na	na	na	na	na
Illness_duration_years	61	1.31	1.04	na	na	na	na	na
PANSS total score	61	59.98	14.05	na	na	na	na	na
CDSS total score	61	3.21	3.74	na	na	na	na	na
Childhood Trauma	60	49.12	12.41	na	na	na	na	na

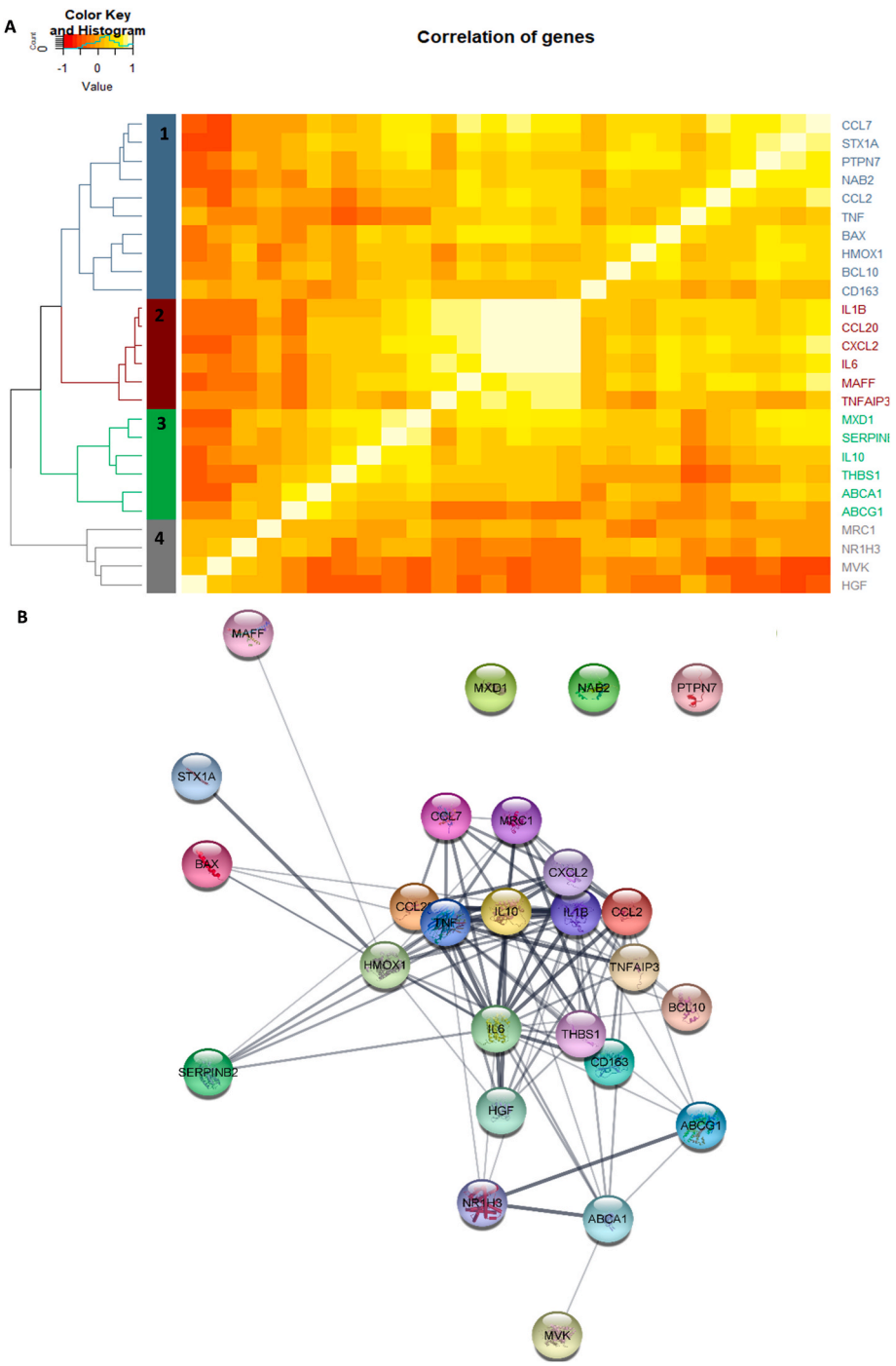

Fig. 1. A. *Heatmap showing the correlation of monocyte gene expression based on individual patients' ddCT values*. **B.** *Illustration of cytokine network created with the string analysis tool from* Cytoscape (version 3.9.1.) *showing the functional connections between the analysed genes based on the literature.*

12 months compared to baseline (t = 2.92, p.adj = 0.013), and 24 months (t = 3.87, p.adj=<0.001). Patients in the "downregulated" cluster who received statins (n = 21), also had a significantly improved outcome at 6 months (t = 3.35, p.adj = 0.020), and 24 months (t = 3.061, p.adj = 0.026) compared to baseline, but not at 12 months (t = 0.97, p.adj = 0.561) as seen in Fig. 3A and B and Supplementary Tables 6 and 7 Importantly, no change at all was observed in the placebo groups (up-and down-regulated), at any time point. Between group differences at 12 months and 24 months were not significant.

Linear Mixed Models revealed that depression scores decreased over time in the upregulated patients regardless of the condition (interaction of time*cluster: F = 2.94, p = 0.035) as seen in Supplementary Fig. 2. Post-hoc contrasts showed a significant decrease at 6 months (t = 2.79, df = 165.00, p = 0.012), 12 months (t = 4.02, df = 165.00, p < 0.001), and 24 months (t = 4.63, df = 165.00, p < 0.001) compared to baseline. In patients with "down-regulated" status, decreases were not observed (all p > 0.179).

3.5. *Validation of the inflammatory signature using secreted proteins and T-cells*

To assess whether the proposed clustering using inflammatory gene expression is also relevant for secreted proteins and the adaptive immune system we next analysed levels of hsIL-6, CRP, and T-cell

Table 2
Comparison of the monocytic gene expression by ddCT values between SSD patients and healthy controls by the Wilcoxon's rank sum test corrected for multiple comparisons with the Benjamini-Hochberg method. Genes that remained significant ($p < 0.05$) after adjustment are indicated by an asterisk.

Genes	Median	p value	p adj.
TNF_ddCT *	−0.963	<0.001	<0.001
HMOX1_ddCT *	−1.728	<0.001	<0.001
BCL10_ddCT *	−0.308	<0.001	<0.001
THBS1_ddCT *	0.923	<0.001	<0.001
MRC1_ddCT *	0.363	<0.001	<0.001
MVK_ddCT *	0.214	<0.001	<0.001
CD163_ddCT *	0.139	0.001	0.004
STX1A_ddCT *	−0.784	0.004	0.013
MXD1_ddCT *	−0.252	0.009	0.026
ABCG1_ddCT *	0.256	0.010	0.026
TNFAIP3_ddCT *	−0.004	0.013	0.031
ABCA1_ddCT *	0.170	0.015	0.032
PTPN7_ddCT *	−0.255	0.017	0.034
NR1H3_ddCT	0.085	0.028	0.052
CCL2_ddCT	−0.418	0.030	0.052
MAFF_ddCT	0.478	0.041	0.067
CCL20_ddCT	1.006	0.045	0.069
CCL7_ddCT	−0.925	0.061	0.088
NAB2_ddCT	−0.283	0.100	0.137
IL1B_ddCT	0.338	0.155	0.202
CXCL2_ddCT	0.443	0.177	0.208
IL6_ddCT	0.200	0.179	0.208
BAX_ddCT	−0.979	0.184	0.208
IL10_ddCT	−0.096	0.347	0.376
SERPINB2_ddCT	−0.310	0.736	0.765
HGF_ddCT	0.002	0.768	0.768

subpopulations between groups. Patients with and "upregulated" status had higher levels of T regulatory cells as compared to those of HC (Kruskal-Wallis, df = 2.00, $X^2 = 15.38$, $p < 0.001$, Dunn's test: z = 3.88, df = 2.00, $p < 0.001$, p.adj<0.001), and compared to "anti-inflammatory" patients (z = 2.40, p = 0.016, p.adj = 0.024) (Fig. 3C). The "upregulated" patient group also had numerically higher Th17 cell levels as compared to the "downregulated" patient (Fig. 3D). However, this difference was no longer significant after correction for multiple comparisons (trend in the Kruskal-Wallis test: $X^2 = 5.45$, df = 2.00, p = 0.065; Dunn's test compared to HC: z = 2.31, p = 0.02, p.adj = 0.06; compared to downregulated cluster: z = 1.90, p = 0.057, p.adj = 0.085). Other lymphocytic cell populations (cytotoxic T-cells, natural killer cells, monocytes, T_H1-cells, T_H2-cells, T memory cells, naïve T-cells, B-cells) did not differ between groups (all $p > 0.05$). No significant differences occurred for CRP between any cluster (Kruskal-Wallis chi-squared = 2.08, df = 2.00, p-value = 0.353). Levels of IL-6 were significantly higher in the patients with higher inflammatory gene expression compared to healthy controls, but this again was no longer significant after adjusting for multiple comparisons (z = 2.25, p = 0.024, p.adj = 0.073). The difference between "up- and downregulated" patients was not significant (z = 1.84, p = 0.065, p.adj = 0.098), see Fig. 3E.

4. Discussion

In our previous analysis of the randomized controlled trial with simvastatin in patients with SSD, the predefined primary outcome, i.e., symptom severity as measured with the PANSS total score at 12 months of treatment, was negative (Sommer et al., 2021). In the present analysis we stratified patients based on the expression of highly pro-inflammatory monocyte gene expression prior to therapy. A significant but limited statin-induced improvement at 12 (and 24 months) months was observed within the inflammatory patient group. Between-group effects of treatment (e.g., simvastatin vs placebo) were not significant. Importantly, placebo treatment in SSD patients for both the group with up- and downregulated inflammatory genes had no effect on PANSS scores.

The subset of patients with a monocyte pro-inflammatory state was also characterized by the highest levels of Th17 cells, Treg cells, and IL-6 serum levels (although significance reduced to a trend after correction for FDR) levels, confirming previous observations (Drexhage et al.,

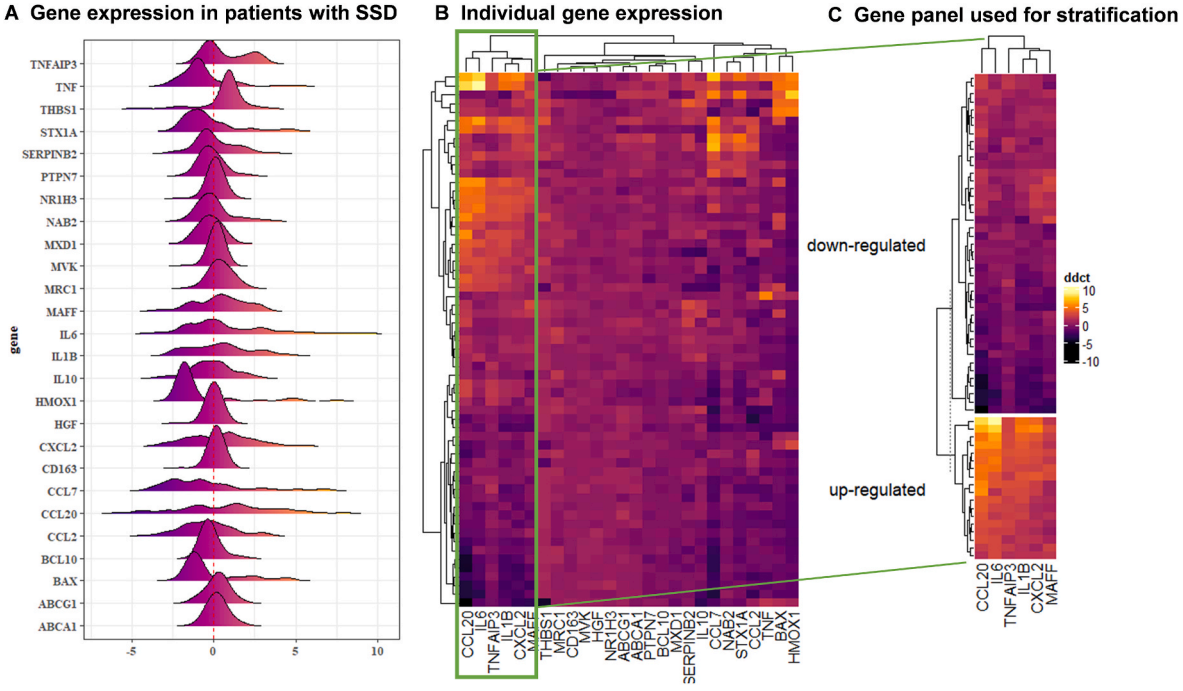

Fig. 2. A. *Representation of median ddCT gene expression in SSD patients* (e.g., dCT relative to healthy control expression). The significant differences were assessed using Wilcoxon's rank sum test. This figure shows that for some genes, such as TNFAIP3, the distribution shows two peaks, illustrating the heterogeneity within the patient group. **B.** *Complete heatmap of monocyte gene expression based on individual patient ddCT values*. Rows represent patients and columns represent ddCT expression of genes. **C.** *Selection of patient clusters based on "pro-inflammatory genes"* CCL20, IL6, TNFAIP3, IL1B, CXCL2, and MAFF.

Table 3
Demographic differences between clusters.

Cluster	"down-regulated"			"up-regulated"			Test statistic	p
	N	Mean	SD	N	Mean	SD		
BMI	43	23.60	3.70	18	24.91	4.23	F = 1.45	0.234
Age baseline	43	25.74	6.51	18	27.67	6.14	F = 1.14	0.290
Sex female (%)	43	26%		18	28%		$X^2 = 0.00$	>0.999
PANSS total	43	59.93	14.82	18	60.11	12.43	F = 0.00	0.964
CDSS	43	2.58	2.81	18	4.72	5.14	W = 302	0.174
Childhood Trauma	42	48.76	14.35	18	49.94	6.06	F = 0.11	0.738
Illness duration (years)	43	1.26	1.00	18	1.44	1.15	F = 0.41	0.523
Placebo/simvastatin	21/21	50%		9/9	50%			

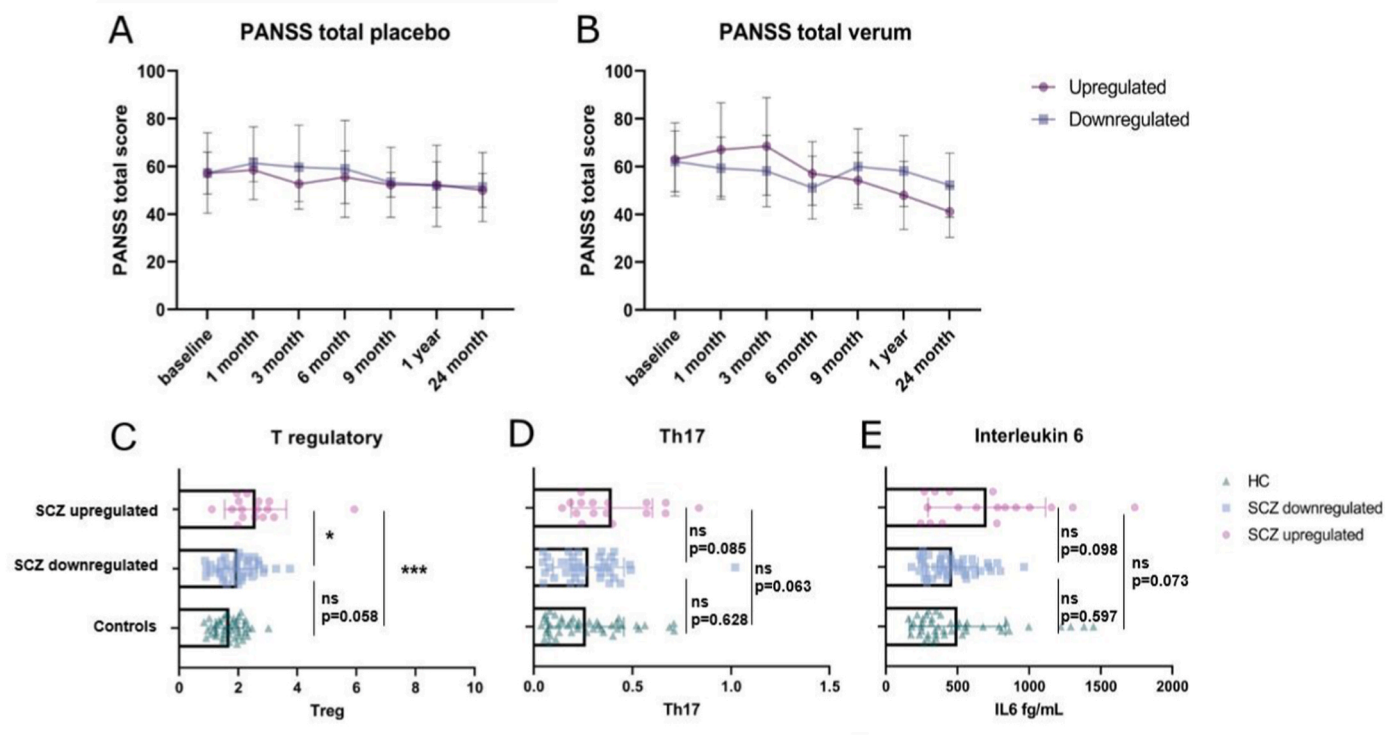

Fig. 3. PANSS total scores over time. In the placebo (**A**) and simvastatin (**B**) group at baseline, 1, 3, 6, 9, 12, and 24 months follow up in the two patient clusters: inflammatory vs. anti-inflammatory group. **C.** T regulatory cells in the SSD "upregulated", SSD "downregulated" group and HC. **D.** Th17 cells in the SSD "upregulated", SSD "downregulated" group and HC. **E.** High sensitive Interleukin-6 levels in the two patient clusters ("upregulated" and "downregulated") displayed versus HC. Displayed p values are adjusted with Benjamini-Hochberg correction. Values represent the mean and standard deviations. Stars represent significant differences as follows: *q < 0.05, **q < 0.01 and ***q < 0.001 level.

2010). Of note, in the present study only around 30% (18/61) had this pro-inflammatory profile, showing that an inflammatory pathway dysregulation is present in a minority of patients, in line with previously reported results (Fillman et al., 2013). Additionally, it should be noted that our inflammatory group was determined based on a specific profile of genes containing CCL20, TNFAIP3, IL1B, CXCL2, and MAFF, but in most of our patients the TNF monocyte gene was downregulated, suggesting that TNF is not involved in this inflammatory profile. However, others have observed elevated TNF serum levels (Goldsmith et al., 2016; Miller et al., 2011), and an upregulated TNF gene expression in monocytes (Drexhage et al., 2010) and leucocytes (Di Nicola et al., 2013), requiring further investigation into the role of TNF in SSD.

4.1. Anti-inflammatory therapies in schizophrenia-what is known so far?

Several previous clinical trials have attempted to treat patients with schizophrenia with anti-inflammatory agents, the best known of which are celecoxib, aspirin, and minocycline (Sommer et al., 2014; Çakici et al., 2019). The latter drug showed promising initial results in mouse models: minocycline was able to reverse ketamine-induced psychotic-like behaviour in mice (Monte et al., 2013), and some small clinical trials (Chaudhry et al., 2014; Kelly et al., 2015; Ghanizadeh et al., 2014) reported a beneficial effect. However, Deakin et al. (2018) reporting on the largest study using minocycline, did not find any improvement in patients with schizophrenia, and importantly, no effect on circulating inflammatory proteins. A key factor in this study is that while many studies report increased baseline levels of IL-6 and CRP in patients with SCZ, the patients in Deakin et al. (2018) did not show elevated inflammatory markers at baseline. Awareness of the necessity to stratify patients for such treatment approaches, especially in the presence of high inflammatory markers at baseline, has risen in the past years and is the way forward for precision medicine approaches.

4.2. Stratification of patients with SSD based on inflammation-is it worthwhile?

Precision medicine aims to identify target groups based on clinical and/or biological factors, who may benefit from specific treatments.

This approach has been successfully used in the past for other psychiatric conditions (Raison et al., 2013; Huet et al., 2021; Cussotto et al., 2022), but has only rarely been used for outcomes of RCTs with schizophrenia patients: Martinuzzi et al. (2019) successfully stratified a large number of first episode psychosis patients using the PANSS score in a clinical trial with amisulpride and Weiser pre-selected patients using a cut-off value of CRP in a trial with aspirin (Weiser et al., 2021). Interestingly, post-hoc associations in the first episode psychosis patients did reveal an association of one cluster with higher levels of inflammatory markers (Martinuzzi et al., 2019), but the study of Weiser and colleagues failed to show a significant effect of aspirin in the pre-selected patients. The latter finding is interesting, since in our analyses, CRP was also not significantly altered between groups. This may suggest that for precision medicine approaches in SSD, CRP may not useful. To the best of our knowledge, we are the first to apply clustering solely based on monocyte gene expression to predict outcome of an add-on treatment with anti-inflammatory properties. The differences we observed were subtle rather than striking and it should be re-iterated that at the group level, the outcome of people treated with statins versus placebo was not significantly different.

4.3. Demographic factors linked to inflammation

Demographic factors can have a large impact on inflammation. A recent meta-analysis showed a significant, and large effect size for IFN-γ, IL-6, and IL-12, and a moderate effect size for IL-17 in antipsychotic-naive patients with first episode psychosis compared to healthy controls, with a major impact for BMI and sex on IFN-γ as well as IL-6 (Dunleavy et al., 2022). However, in this paper we could not identify such associations. Our present analysis refutes childhood trauma, age, sex, BMI, and illness duration as determining factors. Interestingly, a large meta-analysis by Fraguas et al. also found no effect of sex, age and BMI. However, studies by Almulla et al. and Lee et al. demonstrated an association of depressive symptoms with immune-inflammatory pathways (Almulla et al., 2021) and levels of IL-6/TNF-alpha (Lee et al., 2017), making it worthwhile to take a closer look at the relationship between depressive symptoms and SSD in our study.

4.4. Impact of treatment on depression in SSD

An intriguing relationship we also uncovered in this study, is that regardless of add-on treatment (placebo/simvastatin), patients with an upregulated monocyte inflammatory gene expression experienced a significant improvement in depressive symptoms. This is interesting since a wealth of literature links monocyte and cytokine alterations to MDD (Simon et al., 2021; Lynall et al., 2020; Schiweck et al., 2020), and depressive symptoms are common in patients with SSD. Our data suggest that treatment as usual does improve depressive symptoms in SSD patients with pro-inflammatory monocytes. Indeed, also patients with depression who do not respond to treatment with solely SSRIs or SNRIs are often treated with second-generation antipsychotics such as aripiprazole, risperidone, and quetiapine, for augmentation and often with great success (Cantù et al., 2021; Nuñez et al., 2022). The anti-depressant effect of antipsychotics as an add-on treatment in patients with depression has been shown in several randomised controlled trials, e.g., Berman et al. (2009), Jon et al. (2013), and Marcus et al. (2008). Especially at doses of quetiapine between 150 and 300 mg per day have shown to ameliorate depressive symptoms when used as an augmentation therapy (McIntyre et al., 2014; Dorée et al., 2007). In line with the reported second-generation antipsychotics, risperidone (Keitner et al., 2009; Rapaport et al., 2006), and olanzapine (Shelton et al., 2001; Corya et al., 2006) have also been shown to decrease depressive symptoms when given as an add on to antidepressants. These studies support our finding: a reduction in depressive symptom burden might be due to the antipsychotics given their anti-depressive potential.

4.5. Statins in SSD are useful to combat atherosclerosis

Regardless of the impact on psychotic or depressive symptoms, as already noted (Sommer et al., 2021), statins should still be considered for the treatment of co-morbid cardiovascular disease, which is a real risk factor in patients with SCZ (Sommer et al., 2020; Kritharides et al., 2017; Nielsen et al., 2021). A large-scale randomised, placebo-controlled study on patients with coronary heart disease showed a significant reduction in all-cause mortality with low rates of side effects (Scandinavian Simvastatin Survival Study Group, 1994). Current guidelines strongly advise the use of statins for the primary prevention of cardiovascular disease (Robinson and Stone, 2015). While the death of cardiovascular disease is common in patients with SCZ (Sommer et al., 2020), the usage of simvastatin as an add-on therapy might positively influence the disease outcome in SSD patients.

4.6. Limitations

Firstly, only biomaterial at baseline was available for analysis of monocyte gene expression. It would have been particularly interesting to analyse monocytes after the simvastatin intervention, i.e., after 12 and/or 24 months, to see the effect of simvastatin on inflammation, but biomaterial at these time points was not available. Secondly, blood was not drawn from all participants of the initial simvastatin study, so fewer participants could be included in our secondary analysis. Even though 60 participants of this hard-to-recruit patient group were included, the sample size of each group mentioned above was relatively small. Thirdly, we did not correct for antidepressants or any other medication, which would have been interesting, especially regarding the antidepressant effects described above. Also, we analysed a set of predefined and validated monocyte genes, but this depicts only a relatively small part of the innate immune system and changes in other pathways might have been undetected by our analysis. The idea of precision medicine is to adjust the treatment options for individual patients/patient groups. For this, stratifying patients before treatment is initiated would be optimal. However, this analysis was done post-hoc, warranting future prospective studies with a larger sample size aiming for stratification before the intervention.

5. Conclusion

Our secondary analyses did not reveal significant differences between groups, even if stratified for inflammatory parameters. However, on an individual level, patients with a monocyte inflammatory profile did show significant improvement after statin treatment compared to baseline. In summary, statin treatment may well improve outcomes on an individual basis in patients with monocyte characteristics of inflammation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hemmo Drexhage reports financial support was provided by European Union. Iris Sommer, Lot de Witte and René Kahn report financial support was provided by Dutch Research Council. Shiral S Gangadin reports financial support was provided by Stichting De Cock - Hadders Foundation. Sabine Bahn reports a relationship with Psynova Neurotech Ltd, Psyomics Ltd that includes: board membership. M.A., S.S.G, I.E.C., A.W., and C.S. declare to have no competing interest related to this study. S.B. is director of Psynova Neurotech Ltd and Psyomics Ltd.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2022.100551.

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