Blood-based Biomarkers for Personalised Medicine Applications in Schizophrenia

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BLOOD-BASED BIOMARKERS FOR PERSONALISED MEDICINE APPLICATIONS IN SCHIZOPHRENIA

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BLOOD-BASED BIOMARKERS FOR PERSONALISED MEDICINE APPLICATIONS IN SCHIZOPHRENIA

Bloed gebaseerde biomarkers voor *personalised medicine* applicaties in schizofrenie

Thesis

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To my family

CONTENTS

CHAPTER 1: INTRODUCTION **3**
1.1. SCHIZOPHRENIA 4
1.2. BIOMARKER DISCOVERY IN SCHIZOPHRENIA 4
1.3. BLOOD-BASED BIOMARKER DISCOVERY 5
1.4. POTENTIAL OF BLOOD TESTING IN SCHIZOPHRENIA 9
1.5. SCOPE OF THE THESIS 13
CHAPTER 2: NEUROIMMUNE BIOMARKERS IN SCHIZOPHRENIA **15**
2.1. INTRODUCTION 16
2.2. NEUROIMMUNE ALTERATIONS AND SCHIZOPHRENIA FEATURES 18
2.3. TRIGGERS OF IMMUNE ACTIVATION IN SCHIZOPHRENIA 24
2.4. IMMUNOMODULATORY INTERVENTIONS IN SCHIZOPHRENIA 29
2.5. FUTURE DIRECTIONS 30
2.6. CONCLUSIONS 33
CHAPTER 3: CYTOKINE ALTERATIONS IN FIRST-EPISODE SCHIZOPHRENIA PATIENTS BEFORE AND AFTER ANTIPSYCHOTIC TREATMENT **35**
3.1. INTRODUCTION 36
3.2. MATERIAL AND METHODS 38
3.3. RESULTS 42
3.4. DISCUSSION 45
CHAPTER 4: IMMUNOMODULATORY EFFECTS OF PROBIOTIC SUPPLEMENTATION IN SCHIZOPHRENIA PATIENTS: A RANDOMIZED, PLACEBO-CONTROLLED TRIAL **49**
4.1. INTRODUCTION 50
4.2. MATERIALS AND METHODS 52
4.3. RESULTS 55
4.4. DISCUSSION 58
4.5. APPENDICES 62
CHAPTER 5: CHANGES IN THE FATTY ACID HANDLING PROTEINS H-FABP AND CD36 PREDICT RESPONSE TO OLANZAPINE TREATMENT IN RECENT-ONSET SCHIZOPHRENIA PATIENTS **65**
5.1. INTRODUCTION 66
5.2. MATERIALS AND METHODS 68
5.3. RESULTS 72
5.4. DISCUSSION 76
5.5. SUPPLEMENTARY INFORMATION 82
CHAPTER 6: DISCUSSION **87**
6.1. GENERAL DISCUSSION 88
6.2. STRENGTHS AND LIMITATIONS 94
6.3. FUTURE DIRECTIONS 95
6.4. CONCLUSION 96
SUMMARY **99**
SAMENVATTING **101**
CURRICULUM VITAE **105**
LIST OF PUBLICATIONS **107**
ACKNOWLEDGEMENTS **109**
REFERENCES **113**

CHAPTER 1
INTRODUCTION

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*"Blood test for schizophrenia". **Tomasik J**, Schwarz E, Guest PC, Bahn S. Eur Arch Psychiatry Clin Neurosci (2012)*

*"Proteomic profiling in schizophrenia: enabling stratification for more effective treatment". Guest PC, Martins-de-Souza D, Schwarz E, Rahmoune H, Alsaif M, **Tomasik J**, Turck CW, Bahn S. Genome Med (2013)*

*"Applications of blood-based protein biomarker strategies in the study of psychiatric disorders". Chan MK, Gottschalk MG, Haenisch F, **Tomasik J**, Ruland T, Rahmoune H, Guest PC, Bahn S. Prog Neurobiol (2014)*

1.1. SCHIZOPHRENIA

Schizophrenia is a mental disorder characterised by occurrence of positive and negative symptoms. Positive symptoms include delusions, hallucinations, disorganised speech and behaviour, and negative symptoms comprise reduced emotions, thinking processes and speech (1). Until recently, schizophrenia diagnosis was mostly based on the revised Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) (1) and classified schizophrenia into 5 subtypes: paranoid, disorganised, catatonic, undifferentiated and residual. Another diagnostic handbook used in psychiatry, the International Statistical Classification of Diseases and Related Health Problems - 10th Revision (ICD-10), also identifies several subtypes of schizophrenia (2). However, these classifications have given rise to criticism. In light of recent research, classification based on symptoms and not on underlying physiological processes is perceived as obsolete (3). DSM-5, published in May 2013, introduced the first substantial changes in schizophrenia diagnosis for 30 years and replaced the subtypes of schizophrenia with disease dimensions, originating from a combination of symptom domains (4). Similar to the Positive and Negative Syndrome Scale (PANSS) (5), this reflects better the complexity of schizophrenia and opens novel avenues for research. However, the DSM-5 schizophrenia diagnosis is still based on symptoms, which is not necessarily associated with aetiology. Significant efforts have been made to find biological signals associated with the disease and its symptoms. The results suggest that schizophrenia is a heterogeneous disease of various causes (genetic, environmental) and different physiological manifestations (altered metabolism, hormonal regulation, immune status). However, these investigations are at an early stage.

1.2. BIOMARKER DISCOVERY IN SCHIZOPHRENIA

Biomarkers (or biological markers) are defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (6). According to the Food and Drug Administration (FDA), biomarker research will be one of the main factors driving psychiatric drug development in the next 50 years (7). Biomarker

applications in clinical studies include differential diagnosis, prognosis, prediction of drug response and side effects, disease staging and monitoring treatment effects (8, 9). The translation of biomarker candidates into clinical tests has, however, been challenging. In the early 1980s, the niacin test found limited application to support the diagnosis of schizophrenia. This was based on an attenuated flush response of schizophrenia patients to cutaneous application of niacin (10). Current biomarker candidates in the schizophrenia field usually show small effect sizes, most likely due to the complexity of the disorder. Therefore, combinations of many biomarkers may be required to achieve good sensitivity and specificity for successful clinical translation. This requires measuring large numbers of biomarker candidates across hundreds of samples, a process which has been facilitated by recent technological advances. Such techniques include genomic, proteomic and immunoassay technologies.

1.3. BLOOD-BASED BIOMARKER DISCOVERY

Although schizophrenia is considered primarily a brain disease, it is now also regarded as a systemic disorder with effects on peripheral systems. Characteristic changes have been found for example in cerebrospinal fluid, liver and skin cells of patients. From a diagnostic perspective, the most promising material for investigation of such systemic alterations is blood, since this carries cells and molecules which regulate both central and peripheral functions. Although the information contained in blood is not fully comprehensive, the observed changes may still be useful for diagnostic, prognostic and monitoring purposes. Significant recent progress has been made with regards to investigation of blood alterations in schizophrenia. Disease-associated components found in the blood of schizophrenia patients include metabolic, immune, growth factor and hormonal alterations (11). Since individual molecular changes show low effect size, the changes may be specific for patient subgroups or related to distinct molecular phenotypes. The identification of such subgroups may aid in the development of novel therapeutics and targeted treatment approaches. In fact, researchers are now testing the targeting of specific alternate pathways such as inflammation in conjunction with treatment of schizophrenia symptoms (12). In this context, molecular stratification

of patients may be invaluable to increase efficacy and the success rate of clinical trials.

1.3.1. METABOLIC FINDINGS

Regarding peripheral metabolic disturbances, schizophrenia patients have a higher prevalence of metabolic syndrome compared to controls, independent of medication use (13). Metabolic conditions such as obesity, impaired glucose metabolism and hypertension occur frequently in schizophrenia patients. At the molecular level, high fasting glucose and insulin levels, and insulin resistance have been observed even in antipsychotic-naïve first-onset schizophrenia patients (14). Also, increased levels of circulating leptin in antipsychotic-naïve female schizophrenia patients have been found (15). Antipsychotics do not appear to reverse these changes, and may even augment them. Some atypical antipsychotics, for example clozapine and olanzapine, and to a lesser extent some typical antipsychotics, are known to induce metabolic side effects such as weight gain, hyperglycaemia, insulin resistance and diabetes, which limit the clinical use of these drugs (16). Hyper-prolactinaemia may also occur, particularly in women (17). Therefore, it is recommended that patients on antipsychotics are monitored regularly for changes in weight, waist circumference, blood pressure, fasting glucose and insulin levels. In line with these findings, impaired glycolytic response has been found in peripheral blood mononuclear cells of first-onset and mostly antipsychotic-naïve schizophrenia patients (18). Also, changes in levels of serum lipids have been correlated with response to atypical antipsychotics (19). Adiponectin has been proposed as a biomarker of metabolic syndrome (20) and triglyceride/HDL ratios have been used as biomarkers of insulin resistance in treated patients (21). Most recently, baseline insulin levels were found to predict reduction of negative symptoms and increased body weight (22). Also, baseline levels of leptin and insulin-related peptides have been identified as predictors of relapse time in patients treated with antipsychotics (22).

In line with these findings, analyses of *post mortem* brain tissues from schizophrenia patients have identified effects on proteins involved in glucose metabolism and insulin signalling pathways (23, 24). This suggests a link between the brain and periphery in the onset and

development of the disease. Likewise, there have been reports of hypothalamic-pituitary-adrenal (HPA) axis disturbance in schizophrenia, which has been linked to abnormal insulin signalling (**Figure 1**) (25, 26). These findings underline the importance of metabolic disturbances as part of schizophrenia aetiology and treatment response.

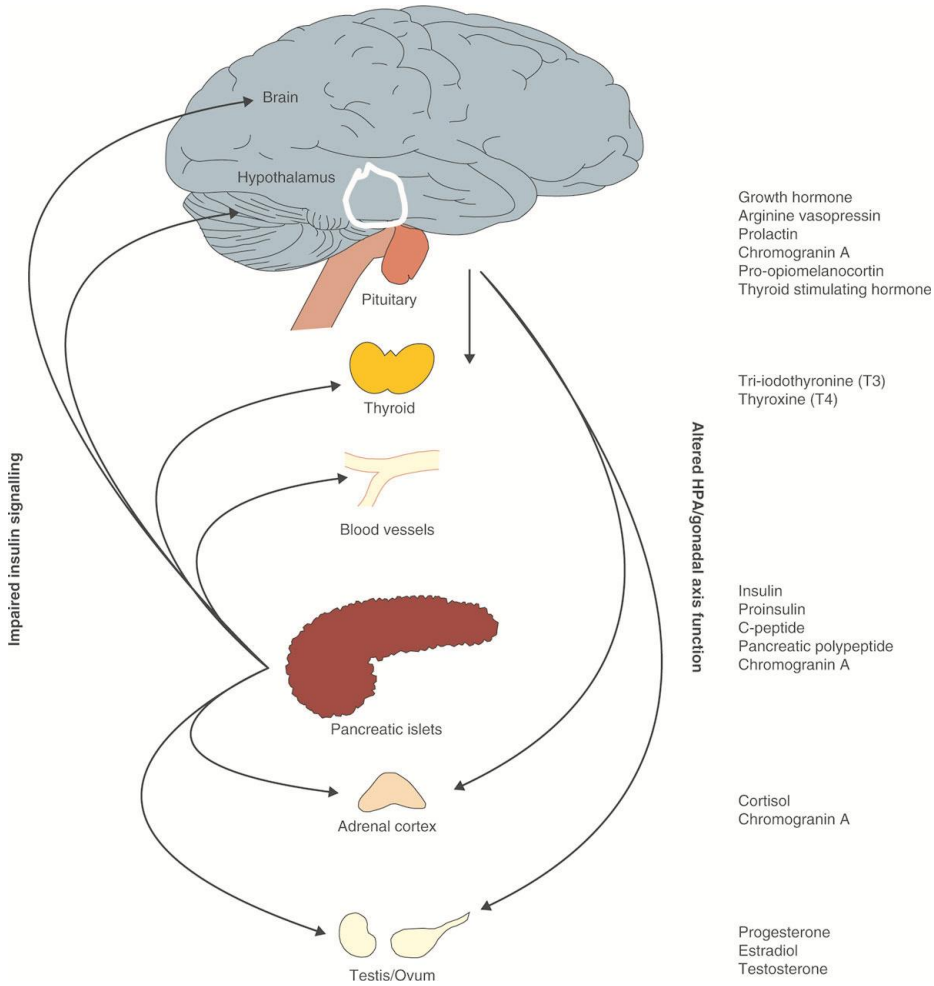

Figure 1. *Potential effects of insulin resistance on secretion of other hormones and bioactive molecules over the diffuse neuroendocrine system. HPA, hypothalamic-pituitary-adrenal. Figure from (27).*

1.3.2. IMMUNE SYSTEM FINDINGS

A range of factors, including genetic predisposition, autoimmune reactions and perinatal or adulthood exposure to infections, may induce immune imbalances seen in some schizophrenia patients (28). Inability to fight infections, resulting from attenuated cell-mediated immune response may result in chronic humoral responses (29). This may lead to central and peripheral immune activation, which is sometimes seen in schizophrenia. Characteristic molecular and cellular immune changes in schizophrenia have been reviewed recently (11, 30). Most consistently, the data suggest activation of monocytes, with increased levels of chemokine ligand 2 (CCL2), interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α. An imbalance between the cellular and humoral immune responses has been identified, but the direction of this imbalance is not clear. Increased levels of the cellular response cytokines IL-2, IL-12 and TNF- α were observed while changes in humoral cytokines showed increased (IL-6, IL-10) and decreased (IL-5) levels. A shift towards the humoral response was found in first-episode patients, suggesting its role in schizophrenia aetiology (11). Also, high levels of C-reactive protein have been associated with symptom severity in schizophrenia (31). These findings are consistent with *post mortem* and imaging studies of schizophrenia brain showing activation of microglial cells responsible for immune defence that may affect neuronal and synaptic function (32, 33).

Antipsychotics appear to help rebalance immune perturbations by modulating cytokine secretion, with clozapine and haloperidol showing distinct anti-inflammatory properties (34, 35). Also, various immunotherapeutics have been tested in combination with antipsychotic therapy (36). This includes the cyclooxygenase (COX)-1 inhibitor/COX-2 modulator aspirin, which works by blocking synthesis of proinflammatory prostaglandins (37).

1.3.3. HORMONAL REGULATION

Hormonal imbalances in schizophrenia appear to be related to metabolic syndrome and immune alterations (**Figure 1**). Release of hormones from the HPA axis controls functions such as food intake, energy turnover, growth, immune system and responses to stress. HPA axis dysfunction indicated by high cortisol, adrenocorticotropic hormone (ACTH) and

prolactin levels has been observed in schizophrenia regardless of medication status (26, 38). Following treatment with atypical antipsychotic medication, cortisol levels have been shown to decrease (39). In line with these findings, therapy discontinuation has been shown to lead to increased cortisol levels (40). Baseline cortisol levels also correlate with negative symptom severity, and altered cortisol levels are associated with a reduction of negative symptoms (40).

1.4. POTENTIAL OF BLOOD TESTING IN SCHIZOPHRENIA

1.4.1. DIAGNOSIS, PREVENTION AND EARLY INTERVENTION

Problems of diagnosing schizophrenia based on clinical interview have been shown by David Rosenhan in a famous experiment, in which healthy subjects simulating hallucinations were admitted to hospitals and diagnosed with psychiatric disorders (41). Although this experiment took place 40 years ago, misdiagnosis still remains a serious problem in modern psychiatry (42). Several reports have shown that molecular profiling of blood could aid in the diagnosis of schizophrenia (25, 43-47). Schwarz *et al.* (25) have described a 51-plex assay that was capable of discriminating schizophrenia patients from healthy controls with good performance. The most robust analytes included in this assay were related to immune or inflammatory functions although other pathways were also represented such as hormonal signalling, response to stress, growth factor signalling and metabolism. Diagnosis in the early stages of disease is perhaps the most critical time window (**Figure 2**). A study conducted by the same authors demonstrated changes in the levels of 20 peripheral biomarkers prior to the clinical manifestation of schizophrenia (48). Altogether, these findings suggest that with sufficient additional validation, future biomarker-based studies may enable the development of blood-based biomarker assays which can predict disease development, potentially in help-seeker individuals meeting the ultra-high risk criteria. In turn, early diagnosis and intervention will enable a better therapeutic effect and prevent devastating consequences of the disease.

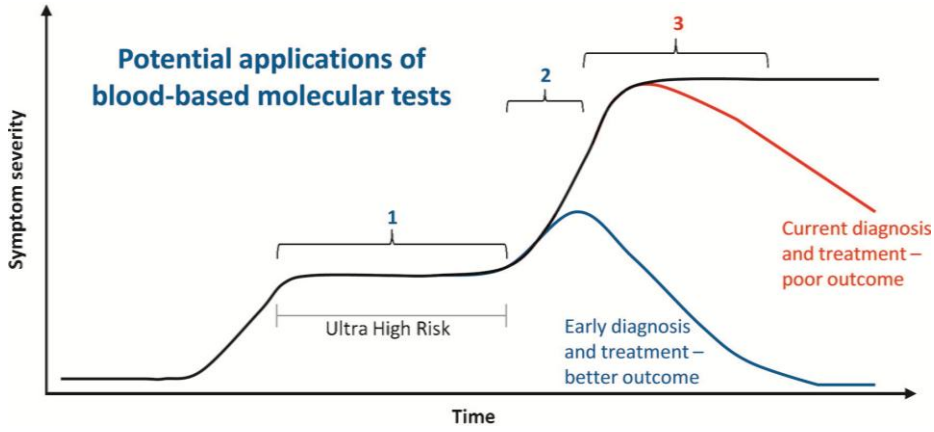

Figure 2. *Diagram illustrating disease progression in schizophrenia and potential applications of blood-based molecular tests. (1) Early diagnosis – estimating risk of developing schizophrenia. (2) Treatment response prediction – accurately identifying subjects who will benefit from antipsychotic treatment. (3) Patient monitoring/risk for side effects and relapse – monitoring biomarker concentration changes during and after treatment. Figure from (8, 49).*

1.4.2. PATIENT STRATIFICATION

A test confirming a diagnosis of schizophrenia could lead to an important progress in the psychiatric field. However, from the perspective of clinical practitioners, the identification of molecular subgroups requiring differing treatments or tests which permit differential diagnoses are more relevant applications than the confirmation of the current diagnostic concept of schizophrenia. One study has identified distinct subgroups of schizophrenia patients with changes in either immune molecules or growth factors and hormones (50). Nevertheless, considerably more research is needed in this area to assess the utility of more complex molecular tests.

1.4.3. DRUG RESPONSE AND DRUG DISCOVERY

Not all schizophrenia patients respond to initial treatment with antipsychotic medications (51). This is mainly due to the fact that there is not sufficient understanding of the underlying pathophysiology to aid diagnosis, patient stratification or treatment selection (52). Furthermore, the traditional treatment approach usually involves random selection and switching of drugs multiple times to achieve an adequate response. This has resulted in high drug attrition and lack of efficacy of blockbuster drugs in patient subpopulations. Therefore, reliable tests with a biological rationale to guide treatment selection are needed. Two studies have been performed which used serum biomarkers analyzed at baseline in schizophrenia patients for prediction of general response to mixed antipsychotic treatments (olanzapine, quetiapine, risperidone) (22, 27). These investigations showed that baseline levels of insulin correlated with the improvement in the negative subscale of the PANSS scores after a 6 week treatment period (22). This finding suggested that insulin signalling may play a role in the response to antipsychotic treatment and also suggests potential novel therapeutic strategies based on drugs which enhance insulin sensitivity. Also, weight gain and insulin resistance are well known side effects of antipsychotic treatment and a subsequent analysis of the same samples found that the baseline levels of 10 serum molecules were significantly associated with the change in body weight, while the insulin/glucose ratio was increased by approximately 40% between the baseline and 6 week follow-up periods (27). Consistent with these findings, the same study reported that the levels of insulin and 11 other serum molecules could predict time to relapse (22). The importance of this finding is underlined by the fact that a proportion of schizophrenia patients present with insulin resistance, which therefore cannot be attributed to antipsychotic medication alone (14, 26).

Besides the utility of blood for biomarker discovery, there is considerable scope in using cellular assays for identification of molecular alterations in schizophrenia. Lymphocytes have been proposed as an accessible alternative to nerve cells for biochemical investigations (53). These cells express a variety of receptors important for schizophrenia, including dopamine D_1-D_5, serotonin 5-HT_{1A}, 5-HT_2 and $GABA_A$ receptors.

Therefore, these cells could be targeted with antipsychotic drugs under different conditions and molecular response patterns identified to screen for the best drug candidates. As an example, it has been found that immune stimulation of blood cells reveals discrete differences in glycolysis between schizophrenia patients and controls, which would otherwise have remained unnoticed (18).

1.4.4. RELIEF FOR ANIMAL MODELS

The main advantage using blood and other samples from patients is that these originate from affected individuals and, therefore, should reflect the changes seen in the disease. Fibroblasts from schizophrenia patients have been used recently to create induced pluripotent stem cells (iPSCs) which can be differentiated into neurons (54). Neurons thus obtained from patients have shown diminished function compared to cells from healthy donors. Although iPSCs are potentially useful, it is not clear how accurately these can reflect the changes seen in neuronal cells. None of these cellular approaches will replace animal models in preclinical studies, although they may significantly reduce the number of compounds entering the stage of screening in animals. Therefore, the number of animals used in experiments would decrease.

1.5. SCOPE OF THE THESIS

Schizophrenia is now considered as an "umbrella" term for a group of conditions with similar manifestation of symptoms and various genetic and environmental aetiologies. Distinct molecular alterations in metabolic, immune and hormonal pathways are present in the blood of certain groups of schizophrenia patients. This suggests the possibility that different subtypes of patients occur with distinct biological aetiologies and that the molecular status of the patient may be informative from a clinical perspective. This thesis will address the study of schizophrenia based on molecular signatures in the blood related to deregulation in inflammatory, metabolic and hormonal pathways as well as their relevance to the disease pathophysiology and drug response in the context of personalised medicine.

Previous studies have suggested that neuroimmune processes may play a role in schizophrenia pathogenesis, at least in a subgroup of patients. **Chapter 2** will explore this evidence and elucidate which of the central and peripheral immune markers are most robustly changed in schizophrenia. It will also discuss the implications of the altered immune function in schizophrenia patients for personalised medicine approaches, including patient stratification by their immune status, more targeted drug interventions and the development of companion diagnostics.

Since studies on the role of immune system at the onset of schizophrenia have not yielded conclusive results due to limitations such as small sample size or dissimilarities in the clinical status of patients, **Chapter 3** will evaluate which of the immune system regulatory molecules known as cytokines are altered in the blood of first-onset schizophrenia patients after controlling for potential confounding factors such as age, gender, body mass index, smoking and medication status. This chapter will also investigate which of the cytokines altered in schizophrenia respond to antipsychotic treatment and whether this response correlates with the change in symptoms. Such analysis may yield potential novel targets for immunomodulatory interventions in schizophrenia.

As immune changes constitute a promising target for novel treatment approaches in schizophrenia, a range of adjunctive anti-inflammatory

agents has been tested for their antipsychotic efficacy. A similar study will be described in **Chapter 4**, which will investigate for the first time whether adjunctive probiotic treatment can modulate the immune function in schizophrenia and benefit to the patients. Probiotics are microorganisms known to restore immune homeostasis in a range of inflammatory conditions and affect brain function through the gut-brain axis. However, they have not been tested yet for their immunomodulatory effects in schizophrenia.

Another approach to improve the response rate in schizophrenia patients relies on the identification of those individuals who are more likely to respond to given antipsychotic treatment. **Chapter 5** will investigate whether there are any robust blood biomarkers associated with response prediction and molecular effects of specific antipsychotic medications in drug-free recent-onset schizophrenia patients. Such biomarkers could aid in increasing treatment response rate, reduce side effects of antipsychotic medication and be used as candidate drug targets for add-on or novel antipsychotic interventions.

Taken together, the research described in the present manuscript will investigate the association between distinct molecular endophenotypes in schizophrenia patients and the disease pathophysiology or response to treatment. **Chapter 6** will summarise how this research contributes to de-convoluting the complexity of schizophrenia and drug response mechanisms. It will also discuss potential applications of the presented findings in personalised medicine approaches and indicate future directions for biomarker research and drug discovery in schizophrenia.

CHAPTER 2
NEUROIMMUNE BIOMARKERS IN SCHIZOPHRENIA

Schizophrenia Research: Tomasik J*, Rahmoune H*, Guest PC and Bahn S (2014)

* These authors contributed equally.

ABSTRACT

Schizophrenia is a heterogeneous psychiatric disorder with a broad spectrum of clinical and biological manifestations. Due to the lack of objective tests, the accurate diagnosis and selection of effective treatments for schizophrenia remains challenging. Numerous technologies have been employed in search of schizophrenia biomarkers. These studies have suggested that neuroinflammatory processes may play a role in schizophrenia pathogenesis, at least in a subgroup of patients. The evidence indicates alterations in both pro- and anti-inflammatory molecules in the central nervous system, which have also been found in peripheral tissues and may correlate with schizophrenia symptoms. In line with these findings, certain immunomodulatory interventions have shown beneficial effects on psychotic symptoms in schizophrenia patients, in particular those with distinct immune signatures. In this review, we evaluate these findings and their potential for more targeted drug interventions and the development of companion diagnostics. Although currently no validated markers exist for schizophrenia patient stratification or the prediction of treatment efficacy, we propose that utilisation of inflammatory markers for diagnostic and theranostic purposes may lead to novel therapeutic approaches and deliver more effective care for schizophrenia patients.

2.1. INTRODUCTION

Schizophrenia affects about 1% of the population but the understanding of its aetiology remains incomplete. At present, schizophrenia is not considered a single disorder but a group of conditions with manifestations common to other psychiatric and non-psychiatric disorders. Those manifestations include clinical symptoms, such as hallucinations, delusions, disturbed emotions and social withdrawal, and involve biological mechanisms, in particular perturbations of the immune, metabolic and endocrine systems. In the absence of a biological marker, the current diagnosis of schizophrenia and its treatment are mainly based on clinical questionnaires and it is not surprising that the response rate is unsatisfactory, in particular after multiple treatment attempts, and relapse is common for those patients who discontinue medication (55). For decades, pathophysiological studies relating to

schizophrenia were focused on disturbances of dopaminergic and glutamatergic neurotransmission with limited clinical breakthroughs. Current antipsychotic drugs primarily alleviate the neurotransmitter imbalances, but most patients continue to experience residual symptoms on current treatment regimens (51, 56, 57). Furthermore, the rate of novel compounds coming to the market is far from satisfactory. However, recently there has been a greater focus on the identification of molecular changes in central and peripheral tissues obtained from schizophrenia patients to unravel the molecular signatures underpinning schizophrenia pathophysiology as a means of improving and accelerating this process (58).

A link between inflammatory diseases and schizophrenia has been proposed over decades. The evidence suggests that some clinical, epidemiological and genetic features may be shared between schizophrenia and certain autoimmune diseases (59-61). The co-prevalence between various autoimmune disorders and some cases of schizophrenia may contribute to the disease development (62). For example, Graves' disease (thyrotoxicosis) has been shown to share similar aetiological features with schizophrenia (63, 64). In addition, a relationship between perinatal and adulthood infections and schizophrenia is supported by various lines of evidence (65-70). More recently, genome-wide association studies (GWAS) have substantiated these findings by indicating a strong relationship between genes regulating immune response and schizophrenia (71).

In the past years, a significant proportion of clinical and molecular studies have attempted to unravel the role of immune dysregulation in schizophrenia and explore the possibility of targeting these pathways especially as add-on intervention to existing therapies (72-80). As the immune system is dynamic and sensitive to changes, the research into the relationship between schizophrenia and immune system abnormalities has yielded contradictory results. This is most likely due to a complex interplay between genetic predisposition, environmental risk factors, disease stage and side effects of antipsychotic medication. Recent findings from our group indicate that molecular changes in schizophrenia patients show an overlap with certain inflammatory as well as metabolic

disorders (11). The utility of the immunological markers for diagnosis and prognosis of schizophrenia is yet to be established.

In this review we will evaluate findings of neuroimmune changes in schizophrenia. We will discuss the evidence of central and peripheral immune findings in schizophrenia, their potential causes, effects of immunomodulatory therapies on symptoms and outline potential applications of these markers in managing the disease.

2.2. NEUROIMMUNE ALTERATIONS AND SCHIZOPHRENIA FEATURES

2.2.1. CENTRAL NERVOUS SYSTEM MARKERS

Imaging studies have shown that the brains of schizophrenia patients display characteristic structural changes at the onset of the disease, which cannot be attributed to drug effects or other confounding factors. Most often, decreased hippocampal and cortical volumes, accompanied by enlarged ventricular spaces, have been identified (81). Contrary to the findings in Alzheimer's disease, the changes in schizophrenia do not result from an ongoing neurodegenerative processes or neuronal death, but are related to changes in the organisation and size of neurons and other brain cells (81). Although central nervous system (CNS) changes show only low sensitivity and specificity for identification of patients compared to controls, they have improved our understanding of the mechanisms underlying schizophrenia symptoms (82). It has been postulated that psychotic symptoms, at least in part, are due to impaired dopaminergic and glutamatergic neurotransmission in the extended limbic system (hippocampus, dorsolateral prefrontal cortex and cingulate gyrus). However, the exact underpinning processes remain largely unknown.

Molecular profiling studies have suggested that molecules related to oxidative stress and immune regulation are implicated in the pathophysiology of certain brain regions in schizophrenia. However, their relation to the structural changes is not clear. These studies have repeatedly shown altered expression of immune-related markers in prefrontal (83-86) and temporal (87) cortices as well as in the

hippocampus (88) of schizophrenia patients. Since brain profiling studies can be performed only in *post mortem* brain tissue, and as most patients have been treated long-term before death, these results suggest that current treatment approaches are not effective in alleviating the immune manifestations of the disease. Some studies have found that only about 40% of schizophrenia patients display signs of immune activation, e.g. changes in IL1B, IL6, IL8 and alpha-1-antichymotrypsin (SERPINA3) transcript levels (85, 89). These findings are consistent with the proportion of schizophrenia patients displaying structural abnormalities (82), but further studies are required to assess precisely the association between immune activation and brain volume. Also, changes in other cytokines related mostly to the innate immune system have been observed in brains of schizophrenia patients, including tumour necrosis factor alpha (TNF-α) (90), and interferon-induced transmembrane protein 1 and 2 (IFITM2/IFITM3) (84); as well as the microglia marker CD11b (90) (**Table 1**). In line with these findings, immunohistochemical studies have shown that the density of microglial cells and their marker, HLA-DR, are higher in post-mortem schizophrenia brains, in particular in those patients who committed suicide (32, 85, 91, 92). Microglia are the equivalent of macrophages in the brain and one of their main roles is immune defence of the CNS. Therefore, activation of these cells indicates ongoing immunological processes in the CNS

Signs of immune dysregulation in schizophrenia have also been observed using *in vivo* brain imaging. Activated microglia express the 18 kDa translocator protein (TSPO) on the mitochondrial membrane. This protein has been targeted in positron emission tomography (PET) studies by measuring binding of the radiolabeled ligand, PK11195. Studies have shown increased binding of PK11195 in total grey matter of recent-onset patients with schizophrenia (33) and in hippocampus of recovering patients (93), suggesting activation of microglial cells in these regions at different stages of the disease. Also, astrocytes have been reported to show signs of activation in schizophrenia, as indicated by an increased release of S100B protein into the cerebrospinal fluid (CSF). S100B is a marker of nervous system damage and increased levels have been observed in the CSF of schizophrenia patients at disease onset and in

Marker	Description	Genetic association	CNS/CSF expression	Peripheral expression	References
IL-1β	Macrophage origin; pro-inflammatory; induces COX2 in CNS	rs16944, rs1143634	↑/↔	↑state	(85, 90, 94-97)
IL-1RA	Macrophage origin; anti-inflammatory	(86 bp)n repeats	↓	↑state	(96, 98, 99)
IL-6	Th2/macrophage origin; pro- and anti-inflammatory; role in autoimmune processes	rs1800795	↑/↔	↑state	(85, 95, 97, 100)
IL-10	Th2/macrophage origin; anti-inflammatory	rs1800896, rs1800872	-	↑state	(98, 100-104)
IL-12B	Macrophage origin; Th1 polarisation; linked to autoimmune diseases	rs2853694	-	↑trait/state	(97, 105, 106)
MHC	Expressed by antigen presenting cells and other cells; antigen recognition	rs6904071, rs6913660, rs13219354, rs6932590, rs13211507, rs3131296, rs114002140, other	↑HLA-DR	-	(32, 71, 85, 91, 92, 107-109)
S100B	Glial origin; neurotrophic factor	rs9722, rs1051169, rs2839357	↑	↑state	(110-114)
TNF-α	Th1/NK/macrophage origin; pro-inflammatory; viral inhibitor	rs1800629	↑	↑trait	(90, 97, 115, 116)

Table 1. *Overlap between genetic risk factors and most robust central (CNS/CSF) and peripheral immune markers in schizophrenia. The evidence indicates that mixed pro- and anti-inflammatory processes contribute to schizophrenia pathophysiology. CNS–central nervous system, CSF–cerebrospinal fluid, HLA-DR – human leukocyte antigen DR, NK–natural killer, state–state marker, Th–T helper, trait–trait marker.*

drug-naïve patients (110, 113). This protein induces the production of several other immune markers by microglia cells, including cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) (72), which are considered to be potential novel drug targets for the treatment of schizophrenia (37, 117).

Interestingly, many other cytokines showing changes in schizophrenia brains and CSF can be linked to microglia activation (IL-1β, IL-12, TNF-α) or are secreted by activated astrocytes [IL-6, IL-10, transforming growth factor beta (TGF-β)]. It is hypothesized that the interaction between these two glial cell types increases the production of quinolinic acid by microglia and kynurenic acid (KYNA) by astrocytes (75). These metabolites activate N-methyl D-aspartate (NMDA) receptors (29), which provides a direct link between immune activation and hypoglutamatergic neurotransmission in schizophrenia. KYNA has been found to be elevated in the CSF of drug-naïve first-episode schizophrenia patients (118), as well as in chronically ill patients (119), consistent with findings from *post mortem* studies (120). Drugs targeting the kynurenine pathway have shown positive effects on cognitive function in animal models (121), but have not yet been tested in schizophrenia patients.

2.2.2. PERIPHERAL MARKERS

Several studies have suggested that immune alterations in the CNS may originate from peripheral immune activation, crossing the blood-brain barrier in a subgroup of patients (122, 123). Peripheral cytokines can cross the blood-brain barrier and are known to perturb brain function through the hypothalamic-pituitary-adrenal (HPA) axis, precipitating changes in mood, behaviour and cognition (124). Although this causality is not well-established, characteristic immune imbalances are observed in the blood of schizophrenia patients (**Table 1**). The majority of studies have focused on cytokine changes in serum of schizophrenia patients and have been extensively reviewed in several meta-analyses (97, 106, 125). Importantly, a review from 2008 (125) challenged the previous hypothesis of blunted Th1 and enhanced Th2 responses in schizophrenia, reporting increased IL-1RA levels in both unmedicated and treated patients, elevated IL-6 only in the untreated group and high sIL-2R solely in drug-treated patients. A subsequent review (97) focused on evaluating

differences between first-episode and relapsed patients and found that the plasma concentrations of IL-1β, IL-6 and TGF-β were elevated in both patient groups and normalised with antipsychotic treatment. This suggested that these represent disease state markers. In contrast, the levels of IL-12, IFN-γ, TNF-α and sIL-2R were increased in patients but did not normalise with treatment, suggesting that these changes may represent trait markers. The most recent meta-analysis evaluated antipsychotic drug effects from follow-up studies (106), suggesting that treatment with antipsychotics decreases levels of IL-1β and IFN-γ, and increases IL-12 and sIL-2R levels. It should be noted that these studies did not control for potential confounding factors such as body mass index or smoking, which can significantly affect cytokine levels (126). The most robust analysis assessing cytokine levels in first-onset and drug-naïve schizophrenia patients to date has revealed a mixed pro- and anti-inflammatory profile, with limited response of cytokines to treatment, although some important cytokines were not investigated in this study (98).

It has been shown that blood-based molecular biomarker signatures can be utilised to distinguish schizophrenia patients from healthy controls and bipolar disorder patients (25, 45, 127). Interestingly, many of the differentially regulated molecules are involved in immune system regulation. We have shown that certain immune markers (macrophage migration inhibitory factor, IL-8, IL-1RA, IL-18, and IL-16) can be utilised to identify a subgroup of schizophrenia patients with prominent immune changes, in contrast to another distinct subgroup of patients with changes in growth factor and hormonal pathways (50). In another study, we found that certain immune markers (IL-6R, CD5L, IL-17) are specifically changed prior to the manifestation and diagnosis of schizophrenia, but not in pre-onset bipolar disorder patients (48). In addition, the levels of TGF-α, CD5L, CD40, macrophage-derived chemokine and tumor necrosis factor receptor like 2 protein have been associated with the prediction of relapse in schizophrenia (22). These results suggest that schizophrenia may be linked to a systemic change in inflammatory activity that also affects the brain.

Altered expression of plasma cytokines may originate from aberrant immune cell function. Profiling studies of lymphocytes from schizophrenia patients have provided evidence that immune processes are involved also at the cellular level (128, 129). Differences in the subtypes of immune cell populations are observed in schizophrenia patients. In drug-naïve patients, increased numbers of total lymphocytes, T lymphocytes (CD3-positive), T helper cells (CD4-positive) and a higher ratio between T helper and T cytotoxic cells (CD4/CD8) have been observed, while the proportion of T lymphocytes was reduced (130). In acutely relapsed patients, a higher proportion of CD4-positive and CD56-positive cells (T helper and natural killer cells, respectively) have been observed (130). Following treatment, the CD4/CD8 ratio decreased and the concentration of CD56-positive cells increased (130). It is important to mention that very few studies have investigated the distribution of rare but functionally important blood cell populations in schizophrenia patients (131), therefore these results require further critical validation. Several studies have suggested a role of the mononuclear phagocyte system in the pathophysiology of psychiatric disorders (131, 132). For example, changes in inflammatory gene expression patterns were observed in monocytes of 60% of recent-onset patients with schizophrenia (132).

Blood cells from schizophrenia patients show abnormalities not only in numbers, but also in function, such as altered responses to mitogenic stimulation (133) and in association with smoking (134). Processes that underlie abnormal blood cell function in schizophrenia are similar to those in the brain and involve mostly cell cycle, intracellular signalling, oxidative stress and metabolism pathways (18, 133, 135, 136). This similarity is not surprising, since it is known that blood and CNS gene expression patterns are correlated (137). We have also identified a reproducible cellular molecular signature associated with altered immune function of blood cells isolated from first-onset and drug-naïve schizophrenia patients (138). Further studies on lymphocytes as a functional model of the disease may help to unravel the complex molecular mechanisms underlying schizophrenia.

Recent studies suggest that the perturbations in immune system function seen in psychiatric disorders may result from failure to mount an appropriate inflammatory response. Such an event could be related to impaired metabolism (133, 139), as inflammatory responses consume large amounts of energy (140), largely due to rapid immune cell proliferation, migration and cytokine production. However, it is still not clear why the inflammatory response appears to be altered in some schizophrenia patients. Initially, it was thought that this was secondary to the frequently occurring weight gain, metabolic syndrome and type II diabetes, attributed as side effect of antipsychotic medications such as clozapine and olanzapine (141). This comes from an observation that metabolic disorders are associated with low grade systemic inflammatory conditions. However, a few studies have suggested that drug-naïve schizophrenia patients, as well as first-degree relatives of schizophrenia patients, also have impaired insulin signalling (14, 26, 142). In support of this, we have recently reported changes in glycolytic metabolism in stimulated peripheral blood mononuclear cells isolated from schizophrenia patients, suggesting a direct link between immune function and glucose metabolism (18). More recently, it has been reported that increased glucose transport via glucose transporter GLUT1 increases the pro-inflammatory response in macrophages (143), which may be relevant for the microglial hypothesis of schizophrenia. This occurs as GLUT1 is the rate limiting glucose transporter on proinflammatory macrophages and other immune cells (140, 143). These results indicate an association between energy metabolism pathways and inflammatory response, two processes that are often perturbed in schizophrenia.

2.3. TRIGGERS OF IMMUNE ACTIVATION IN SCHIZOPHRENIA

2.3.1. GENETIC FACTORS

Studies in monozygotic twins have shown that the genetic contribution to schizophrenia accounts for approximately 50% (144). Variants of several genes, including disrupted in schizophrenia-1 (DISC-1), neuregulin-1 (NRG-1) and catechol-O-methyltransferase (COMT), have been identified as potential risk factors for schizophrenia, but none of these showed statistical significance in subsequent genome-wide association studies.

Contradictory results have been reported for the role of cytokine-encoding genes, including polymorphism of the interleukin (*IL*) 1 gene complex (94, 99, 145-148), *IL2* (149), *IL3RA* (150), *IL6* (100, 149, 151), *IL10* (100-104), *IL12B* (105, 152), *IL18* (105, 153) and interferon (*IFN*) gamma (154). Although most of these genetic findings are not significant in genome-wide comparisons, they are consistent with studies that found altered levels of these cytokines in brain and plasma of schizophrenia patients (**Table 1**). For example, two studies have found that the high IL-10-producing haplotype is more frequent in schizophrenia patients (101, 103) and similar findings have been reported for the *TNFA* gene (115, 116). The above genes may also represent a link between genetic and environmental risk factors (**Figure 1**), such as the association reported between polymorphisms in IL-18 pathway genes and susceptibility to herpes simplex virus (HSV) 1 (105).

Recent progress in microarray and sequencing technologies has allowed the investigation of genetic associations at the genomic level. The first GWAS in schizophrenia examined about 500,000 single-nucleotide polymorphisms (SNPs) and revealed a strong effect only for the locus near the colony stimulator factor receptor 2 alpha (*CSF2RA*) gene (150). Subsequent sequencing revealed an association with the IL-3 receptor alpha (*IL3RA*) gene, which has been confirmed by other studies (150, 155, 156). The following extensive genome-wide scans excluded the single gene effects in schizophrenia and suggested multiple loci at distant genomic regions. One of the most reproducible findings involves the major histocompatibility complex (*MHC*) region (6p21.3-6p22.1) (108, 109, 157-160) [reviewed in (71)]. The *MHC* region spans more than 200 genes, many of which encode key regulators of immune system function, such as the human leukocyte antigen (*HLA*) genes, *TNF* superfamily genes and complement cascade genes (161). Two recent GWAS have examined the genetic background of five major psychiatric disorders (162, 163). Although overlap has been observed for the different conditions, in particular between schizophrenia and bipolar disorder (162), polymorphisms within the *MHC* region were specific for schizophrenia (163). MHC is a group of proteins involved in antigen recognition. Therefore, it is thought that *MHC* gene polymorphisms may render

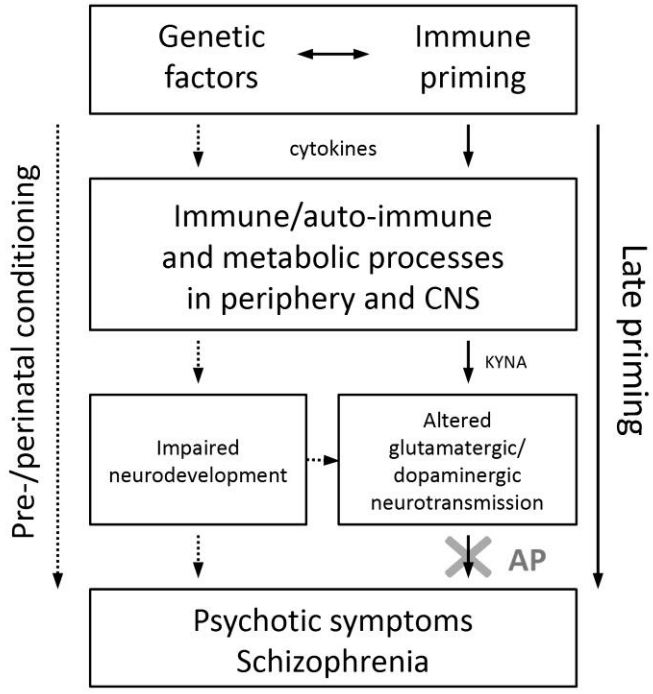

Figure 1. *Hypothetical immune mechanisms involved in schizophrenia pathogenesis as a target for novel treatments. Both genetic and environmental factors contribute to dysfunction of the immune system. Pre- and perinatal infections may result in chronic inflammatory processes leading to neurodevelopmental changes and psychotic symptoms. Immunological alterations in adult life may perturbed glutamatergic and dopaminergic neurotransmitter systems, e.g. via kynurenic acid (KYNA) pathway. Current antipsychotic treatments (AP) target only the neurotransmitter imbalance. Alternative treatments could not only alleviate symptoms, but also restore normal function of the processes underpinning schizophrenia.*

individuals more susceptible to disease by altering immune system function. A few studies have specifically addressed the expression of schizophrenia susceptibility genes in *post mortem* brains from schizophrenia patients and showed concordance with the genetic findings regarding the *MHC* region (164, 165). A recent study has suggested that epigenetic regulation of immune-related genes by methylation may also play a role in schizophrenia pathogenesis (166).

2.3.2. INFECTIOUS AGENTS

Genetic factors most likely contribute to schizophrenia pathogenesis only in a subgroup of patients and the immune activation in schizophrenia often cannot be attributed to genetic underpinning. This suggests a significant role of environmental factors such as infections in activating the immuno-phenotype. There is evidence that viral infections during pregnancy may increase the risk to develop schizophrenia in the offspring (70). An association between schizophrenia and prenatal exposure to influenza has been reported for decades (67) but only a study from 2004 has substantiated the direct link between maternal anti-influenza antibody levels and the risk of schizophrenia, in particular during early pregnancy (167). The prevalence of schizophrenia is also higher in offspring of mothers seropositive for HSV-2 and *Toxoplasma gondii* (68, 69), although contradicting results have also been reported (168). In addition, high maternal IL-8 and TNF-α levels during pregnancy have been associated with an increased risk of schizophrenia in the offspring (169, 170).

Perinatal infections with other viruses such as cytomegalovirus, mumps virus, CBV-5, but not with bacteria, increase the risk of schizophrenia (66). In addition, infections in adulthood have been linked with the onset of schizophrenia. High levels of IgG antibodies against cytomegalovirus and *Toxoplasma gondii* were measured in serum and CSF of individuals with recent-onset schizophrenia and normalised with antipsychotic treatment (65). Interestingly, markers related to infectious agents show correlations with functional deficits in schizophrenia. IgG antibodies against *Toxoplasma gondii* have shown positive correlations with psychotic symptoms in ultra-high risk patients (171). In another study, levels of antibodies to HSV-1 have been associated with cognitive symptoms in schizophrenia (172), consistent with the known impact of this virus on brain areas involved in cognition (173, 174). In addition, the effect of HSV-1 exposure on cognitive symptoms was additive to the effect of serum levels of C-reactive protein (CRP) (175).

2.3.3. AUTOIMMUNE REACTIONS

The altered function of central and peripheral immune system in schizophrenia patients and the existence of psychotic features in patients

suffering from autoimmune diseases has led to studies of the link between autoimmune disorders and schizophrenia. For example, some studies have shown that schizophrenia is associated with type 1 diabetes mellitus (62), although this is thought to be rare (176). Also, increased levels of certain auto-antibodies have been observed in schizophrenia patients. In first-episode patients, the prevalence of anti-cardiolipin and NMDA receptor antibodies is increased, while in the general patient population a high prevalence of antibodies against molecules such as DNA, the dopamine receptor, lupus anticoagulant and rheumatoid factor have been reported [reviewed in (177)]. Antibodies against the NMDA receptor have been found in approximately 5-10% of schizophrenia patients, but not in bipolar disorder patients (177-180). These findings suggest an explicit link to impaired glutamatergic transmission in schizophrenia and related cognitive perturbations.

Psychosocial stress is known to contribute to the onset of autoimmune disease or affect disease progression (59). Clinical features such as cognitive deficit and acute psychosis known to be associated with schizophrenia can also be present in patients suffering from systemic lupus erythematosus (SLE) (60). At the molecular level, similar pro-inflammatory molecules have been found to be elevated in first-onset schizophrenia patients and in SLE patients (127, 181). In a comprehensive Danish study on 7,704 subjects carried out between 1981 and 1998, patients with a history of autoimmune disease had a 45% increased risk of developing schizophrenia (62). Conversely, nine autoimmune conditions had higher lifetime prevalence in schizophrenia than in control groups (thyrotoxicosis, intestinal malabsorption, acquired hemolytic anemia, chronic active hepatitis, interstitial cystitis, alopecia areata, myositis, polymyalgia rheumatic, Sjögren's syndrome) (62). In a similar study, prior autoimmune disease was associated with 29% higher risk of schizophrenia and increased synergistically with previous infections (61). Interestingly, an opposite relationship has been observed for schizophrenia and rheumatoid arthritis (RA). Schizophrenia patients have a 2-3 fold decreased risk of RA in comparison to healthy subjects, and patients with RA appear to have reduced risk of schizophrenia (182-184). There is also an association of schizophrenia with atopic disorders and allergies. A nation-wide study has shown altered risk of atopic

conditions such as asthma, allergic rhinitis and urticaria among schizophrenia patients (185).

2.4. IMMUNOMODULATORY INTERVENTIONS IN SCHIZOPHRENIA

Inflammatory pathways constitute a potential target for the development of future schizophrenia treatments (**Figure 1**). It is interesting in this regard that the most effective antipsychotic drug to date, clozapine, has been shown to mediate long-term immune suppression (34, 35, 186) and attenuate microglial activation (187). The anti-inflammatory properties of clozapine might also be related to its side effects – agranulocytosis and neutropenia (decreased levels of mononuclear leukocytes), hence the pre-requisite for monitoring the immune parameters during clozapine treatment (188). Since other antipsychotic drugs show only limited anti-inflammatory effects on the immune system, several adjunctive treatments have been tested for alleviating psychotic symptoms. Aspirin is an inhibitor of cyclooxigenase (COX)-1 and modulator of COX-2 activity. Both of these enzymes produce prostaglandins, which mediate inflammation. In a study from 2010 (37), aspirin administration was found to reduce total and positive PANSS (Positive and Negative Syndrome Scale) scores. Importantly, higher efficacy was observed with lower Th1/Th2 activity measured by evaluating the IFN-γ/IL-4 ratio. In another study, aspirin add-on therapy significantly reduced general psychopathology PANSS scores and also showed a trend for reduced total and positive PANSS scores (117).

Similarly, treatment with a selective COX-2 inhibitor celecoxib decreased symptoms in recent-onset and chronic schizophrenia patients (12, 189, 190), in particular those with lower soluble TNF-α receptor-1 concentrations (12). However, an extensive meta-analysis revealed that the overall effect of celecoxib supplementation in schizophrenia was not significant (36). Also adjunctive minocycline, an antibiotic with anti-inflammatory and neuroprotective properties targeting microglia, yielded inconclusive results (36). Only one study has been carried out utilising N-acetylcysteine (NAC) supplementation in schizophrenia (191) and this resulted in reduced total, negative and general psychopathology PANSS scores. NAC is a glutathione precursor with anti-inflammatory and anti-oxidant properties, and it also has modulatory glutamatergic and

neurotropic effects that may benefit schizophrenia patients (192-194). Further studies are required to assess the utility of immunomodulatory therapies at different stages of the disease and their relationship to inflammatory markers.

2.5. FUTURE DIRECTIONS

2.5.1. CLINICAL NEED

To enable personalised medicine strategies, there is an urgency to further investigate the biological abnormalities associated with severe mental illnesses such as schizophrenia (195). Thus, early and accurate empirical biomarkers are needed to aid the current interview-based clinical diagnosis (**Figure 2**). Moreover, establishing the molecular signatures underpinning the prodromal stages, early onset of the disease and managing the chronicity of the disease, will not only aid in increasing our understanding of the pathophysiology of schizophrenia, but will also improve the disease diagnosis or guide clinicians towards a better pharmacotherapeutic selection. Furthermore, current antipsychotic treatments targeting patients suffering from schizophrenia are not effective for all patients and many suffer from side effects. This can lead to problems with compliance and switching of drugs by clinicians multiple times in order to achieve efficacious responses (196).

As immune dysregulation is an intrinsic part of schizophrenia at the early onset as well as the late stages of the disease, using molecular biomarkers to identify subgroups of patients with prominent immune changes could help to inhibit disease progression and improve outcomes (50). Molecular profiles aiding patient stratification may also be used for identifying those patients who are most likely to respond to a particular drug intervention (197). A biomarker-guided treatment in schizophrenia would lead to improvements in clinical practice, as it would enable development of new and personalised treatment strategies and decision rules for continuation or termination of a selected treatment. This would reduce unnecessary drug exposure and side effects for the non-responders. The healthcare system would benefit by cutting costs associated with the large number of non-responders.

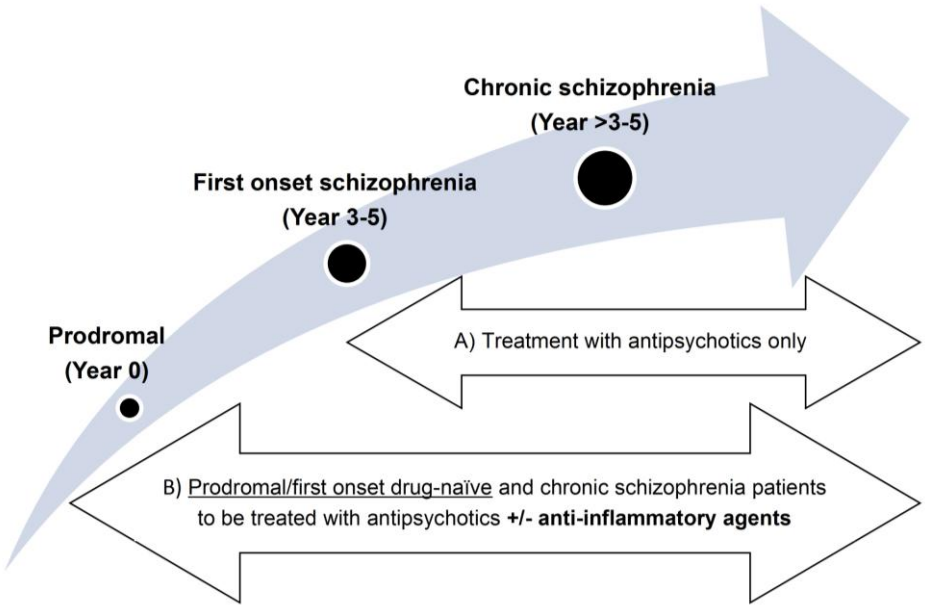

Figure 2. *Schematic diagram depicting opportunities for anti-inflammatory drug interventions in schizophrenia. A) Current monotherapy with antipsychotics after disease onset. B) Future treatment based on patient stratification and targeting the inflammatory status at early onset and throughout the disease progression. This is based on the premise that treatment of schizophrenia patients with anti-inflammatory drugs in combination with antipsychotics will lead to symptom improvement. This evidence has come from adjunctive treatment studies using aspirin (37, 117) , the selective COX-2 inhibitor celecoxib (12, 189, 190), N-acetylcysteine (191) and minocycline, an antibiotic with anti-inflammatory and neuroprotective properties targeting microglia (36).*

2.5.2. LIMITATIONS AND IMPLICATIONS

In regards to the adjuvant therapies for schizophrenia, it has been observed that anti-inflammatory intervention is more effective at the early stages of the disease, when the pathological changes associated with chronic inflammatory processes are not severe (189). This assumes that alterations at the later stages of the disease might be irreversible or require prolonged anti-inflammatory treatment. Despite evidence that

there are subgroups of patients with distinct molecular profiles in serum or plasma related to response to treatment (12, 37, 50), no clinical trials to date have applied patient enrichment strategies in their design. We suggest that stratifying patients according to their inflammatory profile would improve outcomes of the clinical trials and help to minimise adverse effects (**Table 2**). This stratification approach could be based on genetic, imaging and molecular biomarker data regarding immune dysfunction in schizophrenia, as described in this review. This would help to facilitate a personalised medicine approach in schizophrenia, which could ultimately lead to improved treatment outcomes for patients. In addition, it has been suggested that indiscriminate suppression of the immune system may not be the most optimal way to treat immune imbalances in schizophrenia (75). Instead, more targeted approaches should be tested, such as specific suppression of excessive cytokine secretion via treatment with humanised monoclonal antibody approaches. Also, the relationship between particular immune markers and symptom severity should be further evaluated for identification of additional relevant drug targets.

It should be noted that a significant proportion of the reports related to neuroimmune schizophrenia biomarkers are underpowered or have ill-defined or non-stringent inclusion and exclusion criteria (**Table 2**). The outcome of such studies is generally only suggestive and the authors recommend follow up investigations on a larger scale, ideally in independent cohorts. Moreover, few longitudinal follow-up studies have been performed to validate potential candidate biomarkers, which might only represent a snapshot of the disease progression and differ between prodromal, recent-onset and chronic stages of the disease, or be related to gender (198). Surrogate markers should be used in conjunction with clinical endpoints, with careful assessments of reproducibility and validity. A combination of clinical, genetic, imaging, "omics" and multivariate analysis methods (systems biology) would help to achieve a comprehensive correlative assessment throughout disease progression. Finally, the issue of specificity of the neuroinflammatory biomarkers between schizophrenia and other neuropsychiatric disorders should be evaluated in more detail, in particular in relation to bipolar disorder and other relevant differential diagnostic disease groups.

Conf. factor	Recommendation
Patient heterogeneity	➢ Stringent inclusion/exclusion criteria ➢ Patient stratification by immune status ➢ Systems biology approaches ➢ Clinical and molecular target identification ➢ Diagnostic and prognostic biomarkers ➢ Gender associations
Heterogeneity of disease stages	➢ Evaluation of the immune biomarkers in prodromal, recent-onset and chronic patients ➢ Longitudinal studies ➢ Impact of antipsychotic treatment ➢ Prevention/early intervention trials
Low response to add-on treatment	➢ Patient enrichment strategies in clinical trial design ➢ Application of prognostic biomarkers ➢ Prior power calculations to enhance effect sizes ➢ Biomarker-targeted treatments ➢ Use of drugs crossing blood-brain barrier ➢ Replication studies
Side effects	➢ Prognostic and monitoring biomarkers ➢ Adjunctive pharmacological intervention (e.g. anti-diabetic)
Specificity	➢ Differential expression of neuroimmune biomarkers between schizophrenia, bipolar disorder and other neuropsychiatric diseases ➢ Molecular similarities between schizophrenia and immune conditions for improved therapeutic strategies

Table 2. *Confounding factors hampering the interpretation of outcomes from immune biomarker studies in schizophrenia and recommendations for overcoming these limitations. Despite many reports on immune alterations in schizophrenia, only a limited number of studies have investigated immune changes as a means of patient stratification (50), at different disease stages (189), in relation to therapeutic response and side effects (12, 37, 199) or in comparison to other neuropsychiatric diseases (162, 163) (left column). For successful implementation of biomarker studies in personalised medicine approaches, the above listed confounding factors need to be controlled in the clinical trial design. In the right column, we list recommendations for the design of future biomarker studies investigating immune alterations in schizophrenia to address the above mentioned limitations.*

2.6. Conclusions

Central nervous system disorders, especially those of psychiatric nature, may present unique challenges to the acceptance of peripheral markers (200). Although there are currently no established validated biomarkers

for treatment efficacy or patient stratification in schizophrenia, it is has been shown that central and peripheral inflammatory status is a significant component of the early and late stages of the disease. As ongoing studies aim to investigate the relationship between the cause and effect of the inflammatory component of schizophrenia, advances in molecular profiling platforms, imaging and genetic studies have opened the possibility to understand the disease at a more fundamental level. This should pave the way for designing biomarker-based tests for stratification of patients based on molecular profiles at different stages of the disease.

Stratification of schizophrenia patients based on inflammation profiles to assign the right treatments to the right patients is consistent with the personalised medicine approach that is emerging in other areas of medicine such as oncology. In breast cancer, for example, over-expression of the human epidermal growth factor receptor 2 (*HER2*) has been used to identify those female patients who are most likely to benefit from treatment with the monoclonal antibody-based treatment, Trastuzumab (201). A related approach in schizophrenia research could lead to novel therapeutic targets and to the personalisation of treatment approaches, increasing the chances of a positive therapeutic outcome for each patient. Testing of blood samples could be used for stratification of patients based on whether they show distinct changes in key biological pathways such as the effects on immune dysfunction described here. In addition, new adjunctive drug treatment strategies could be developed which target the inflammatory pathway for combined treatments with either existing or newly developed antipsychotics. Targeting the inflammatory component of multi-factorial diseases such as schizophrenia requires well-designed clinical studies to correlate molecular data with clinical ratings. This comprehensive strategy should enable not only the use of anti-inflammatory agents at late stages to manage disease symptoms, but also at the prodromal and in the early phases of psychosis or schizophrenia. Moreover, the use of such approaches at different stages of the disease might lead to alleviation of some symptoms, preventing disease onset or slowing its progression.

CHAPTER 3

CYTOKINE ALTERATIONS IN FIRST-EPISODE SCHIZOPHRENIA PATIENTS BEFORE AND AFTER ANTIPSYCHOTIC TREATMENT

Schizophrenia Research (2014) de Witte L*, Tomasik J*, Schwarz E, Guest PC, Rahmoune H, Steiner J, Rothermundt M, Leweke FM, van Beveren NJM, Kahn RS and Bahn S

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ABSTRACT

Schizophrenia has been associated with central nervous system and peripheral immune system imbalances. However, most studies have not yielded conclusive results due to limitations such as small sample size, dissimilarities in the clinical status of patients and the high variability of cytokine levels within the normal human population. Here, we have attempted to account for these limitations by carrying out standardised multiplex immunoassay analyses of 9 cytokines in serum from 180 antipsychotic-naïve first-episode schizophrenia patients and 350 matched controls across 5 clinical cohorts. All subjects were matched for potential confounding factors including age, gender, smoking and body mass index. We found that the levels of interleukin (IL)-1RA, IL-10 and IL-15 were increased significantly in patients across the cohorts. We also found that the levels of IL-1RA and IL-10 were decreased in 32 patients who had been followed up and treated for 6 weeks with atypical antipsychotics. Interestingly, we found that the changes in IL-10 levels were significantly correlated with the improvements in negative, general and total symptom scores. These results indicate that mixed pro- and anti-inflammatory responses may be altered in first-onset patients, suggesting a role in the aetiology of schizophrenia. The finding that only the anti-inflammatory cytokine IL-10 responded to treatment in parallel with symptom improvement suggests that this could be used as a potential treatment response biomarker in future studies of schizophrenia.

3.1. INTRODUCTION

Schizophrenia is a severe psychiatric disorder with a complex and heterogeneous aetiology. Previous studies have indicated that alterations in immune system function may be involved in the disease process in at least a subgroup of patients (202, 203). Well-established risk factors for schizophrenia include auto-immune and allergic diseases (62, 204), genetic variations in the human leukocyte antigen (HLA)-region on chromosome 6 (109), winter and spring birth seasons (205), and perinatal and childhood infections (206). Other evidence for immune system dysfunction in schizophrenia include the finding of activated microglia in the brain (33, 93) and altered levels of cytokines and other

inflammatory markers in the cerebrospinal fluid and blood (97, 125). These findings suggest that the immune system may constitute a potential target for novel treatment approaches in schizophrenia. Indeed, recent clinical trials have shown that augmentation of antipsychotic treatment with non-steroidal anti-inflammatory drugs (NSAIDs) improves symptoms in schizophrenia patients (207).

The finding that immune alterations in schizophrenia have been found within the brain and cerebrospinal fluid, as well as in peripheral systems such as blood serum and leukocytes, suggests that systemic inflammatory processes are involved in schizophrenia pathogenesis (11, 30, 97, 125, 127, 131, 208). A previously published meta-analysis of 14 studies on serum cytokine alterations in first-episode schizophrenia patients (N between 4 and 83) found increased levels of interleukin (IL)-1β, IL-6, IL-12, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, interferon (IFN)-γ and soluble IL-2-receptor, although significant heterogeneity was observed across the different studies (97). This may be due to the small sample size, differences in inclusion and exclusion criteria, the use of diverse and non-standardised assay platforms, the sporadic presence of inflammatory or metabolic co-morbidities, and the large variability of serum cytokine levels within the normal human population (209). Moreover, peripheral inflammatory markers can be affected by confounding factors such as age, gender, smoking, weight and anti-psychotic medication (97, 210, 211). Therefore, larger sample numbers, stringent inclusion and exclusion criteria, and control of confounding factors are needed to draw reliable conclusions.

With this in mind, we have used a multiplex immunoassay to measure the levels of 9 cytokines in serum of antipsychotic-naïve first-episode schizophrenia patients and controls matched for age and gender. We also attempted to control for body mass index (BMI) and smoking, although this information was not available for all subjects. The main aim was to assess the reproducibility of cytokine alterations across independent cohorts to gain further insight into the role of these molecules in the aetiology of schizophrenia. In addition, we also carried out a pilot study to investigate cytokine changes in a subgroup of these patients who were treated with antipsychotics for 6 weeks.

3.2. MATERIAL AND METHODS

3.2.1. CLINICAL SAMPLES

First-onset and antipsychotic-naïve schizophrenia patients are difficult to obtain since annual recruitment rates range from 10 to 15 subjects for most clinical centres. We recruited subjects in collaboration with three centres in Germany and one in the Netherlands over a period of 10 years (**Table 1**). Cohort 1 was from the Department of Psychiatry, University of Cologne. Cohort 2 was recruited at the Department of Psychiatry, University of Muenster. Cohorts 3 and 5 were from the Department of Psychiatry, University of Magdeburg and cohort 4 was from the Erasmus Medical Centre in the Netherlands. A subgroup of patients (n = 32; **Table 2**) from cohort 3 was used in follow-up studies to investigate antipsychotic treatment effects. The respective institutional ethical committees approved the study protocols and written informed consent was given by all participants. All studies were conducted according to the Declaration of Helsinki. Diagnoses were carried out by experienced psychiatrists using the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) criteria and patients were assessed for psychopathology using the Positive and Negative Syndrome Scale (PANSS) (5). All clinical tests were performed according to Good Clinical Practice Guidelines. Healthy controls were recruited from the same hospitals and associated universities and matched the respective patient populations for age, gender and body mass index (BMI). Patients and controls with a family history of mental disease or other serious medical conditions such as type II diabetes mellitus, hypertension, cardiovascular or autoimmune diseases were excluded from the study. The study protocol, clinical samples and test methods were carried out in compliance with the Standards for Reporting of Diagnostic Accuracy (STARD) initiative (212).

3.2.2. SAMPLE PREPARATION

Blood samples were collected in the morning into S-Monovette 7.5 mL serum tubes (Sarstedt, Numbrecht, Germany). The serum was prepared according to standard protocols by leaving the samples at room temperature for 2 h to allow clotting, followed by centrifugation at 4000 g

Cohort	1	2	3	4	5	Total
Centre	Cologne, Germany	Muenster, Germany	Magdeburg, Germany	Rotterdam, Netherlands	Magdeburg, Germany	-
N	130	92	183	54	71	530
Patients n	71	46	33	14	16	180
Controls n	59	46	150	40	55	350
Patients (M/F)	42/29	35/11	22/11	11/3	8/8	118/62
Controls (M/F)	31/28	35/11	79/71	33/7	28/27	206/144
Patients Age	31 ± 10	27± 9	31 ± 10*	24 ± 7	35 ± 11	30 ± 10
Controls Age	30 ± 8	27 ± 9	36 ± 10	27 ± 4	34 ± 10	32 ± 10
Patients BMI	24 ± 5	22 ± 2	26 ± 5	na	22 ± 3	na
Controls BMI	23 ± 4	na	24 ± 4	na	23 ± 3	na
Patients Tobacco (Y/N/na)	25/23/23	16/26/4	24/9/0*	10/4/0	6/9/1	81/71/28
Controls Tobacco (Y/N/na)	25/34/0	na	41/109/0	na	12/42/1	na
Patients Cannabis (Y/N/na)	33/22/16	15/27/4	9/24/0	8/6/0	0/15/1	65/94/21
Controls Cannabis (Y/N/na)	31/25/3	na	2/148/0	na	0/55/0	na
Antipsychotic-naïve patients	all	all	all	all	all	all
PANSS positive item score	23 ± 6	18 ± 7	21 ± 6	na	19 ± 8	na
PANSS negative item score	23 ± 8	18 ± 7	19 ± 9	na	16 ± 4	na
Sample storage time (years)	6.9 ± 2.6	na	na	2.5 ± 1.1	na	na

Table 1. *Demographic details of patients from the analysed cohorts. M/F = male/female, BMI = body mass index, Y/N = yes/no (based on interview), na = not available. Values are shown as mean ± sd. *p<0.05 (Mann-Whitney test).*

Number	32
Treatment (ola/quet/risp/mix)	7/7/9/9
Gender (M/F)	22/10
Age	31.2 ± 10.5
BMI T_0	24.0 ± 4.4
BMI T_6	24.7 ± 3.9**
Smoking (Y/N)	23/9
Cannabis (Y/N)	9/23
Antipsychotic-naïve at T_0	32
PANSS positive score T_0	21.5 ± 6.5
PANSS negative score T_0	18.3 ± 8.9
PANSS general psychopathology score T_0	42.4 ± 12.1
PANSS positive score T_6	11.7 ± 3.9***
PANSS negative score T_6	14.2 ± 6.5**
PANSS general psychopathology score T_6	28.1 ± 8.6***

Table 2. *Demographic details of patients used for investigating drug effects. Patients were assessed before (T_0) and after (T_6) 6 week treatment with different antipsychotics (ola, = olanzapine; quet = quetiapine; risp = risperidone; mix = mix of different antipsychotics). M/F = male/female, BMI = body mass index, Y/N = yes/no, na = not available. Values are shown as mean ± sd. ** p<0.01, *** p<0.001 (Wilcoxon signed-rank test).*

for 5 min to remove clotted cells and other debris. The resulting supernatants were stored at -80 °C in Lo-Bind Eppendorf tubes (Hamburg, Germany) prior to analysis.

3.2.3. *MULTIPLEXED IMMUNOASSAY ANALYSES*

The levels of 21 serum cytokines were measured using a HumanMAP immunoassay platform as described previously (25). The same platform has been successfully applied in a number of immunological (213, 214) and psychiatric (25, 45) studies for identification of biomarker candidates. The assays were performed in a Clinical Laboratory Improvement Amendments-certified laboratory at Myriad-RBM (Austin, TX, USA). In brief, assays were calibrated and absolute protein concentrations were determined using duplicate 8-point standard antigen curves. Assay performance was verified using quality control serum samples.

3.2.4. STATISTICAL ANALYSES

Assays for 9 cytokines [interferon (IFN)-γ, interleukin (IL)-1α, IL-1RA, IL-5, IL-10, IL-12p40, IL-15, IL-18 and tumour necrosis factor (TNF)-α] fulfilled the following strict inclusion criteria and were therefore included in the study: 1) measurement in all 5 cohorts, 2) only one cohort with more than 80% missing data allowed and 3) did not show a significant correlation with sample storage time. To account for missing values in the measurement of specific analytes, readings below the lower limit of quantitation were replaced with half the minimum value. Analysis of covariance (ANCOVA) indicated that the levels of most cytokines varied significantly across the cohorts (p value for the group ∗ cohort interaction < 0.05). Therefore, each cohort was analysed for cytokine alterations individually.

The application of Shapiro–Wilk tests indicated that none of the measured analyte values were normally distributed ($p < 0.05$). Therefore, Cliff's delta was chosen as a non-parametric estimate of effect size and calculated for each cohort (215). To obtain an overall effect size, the estimates determined for individual cohorts were combined without weighting. Unweighted Cliff's delta has been shown to outperform parametric effect size estimates such as Hedge's g and Cohen's d in terms of bias under non-normality and variance heterogeneity conditions (216). This approach minimised any strong influence of individual cohorts on the overall effect size, which could give rise to centre-specific findings. Confidence intervals for the overall Cliff's delta estimates were determined using the adjusted bootstrap percentile interval (bias corrected and accelerated, BCa) (217). This method finds approximate confidence intervals based on the percentiles of the bootstrap distribution derived from re-sampling the set of effect sizes 5000 times determined from all cohorts. For assessment of the changes in cytokine levels following antipsychotic treatment, non-parametric Wilcoxon signed-rank tests were applied. Spearman correlation testing was also performed to determine if any analyte showed a significant correlation with symptom improvement (ΔPANSS). All analyses were carried out using the free software package R (http://cran.r-project.org/) and GraphPad Prism.

3.3. RESULTS

3.3.1. PATIENT CHARACTERISTICS

The serum samples were obtained from 180 first-episode antipsychotic-naïve schizophrenia patients and 350 healthy controls matched for age and gender (**Table 1**). Patients and controls were also matched for BMI and smoking in cases where this information was available. As an exception, cohort 3 showed significant differences in the age of patients and the distribution of smokers in relation to controls. The patients in all cohorts had high average scores for PANSS positive and negative scores and were antipsychotic-naïve at the time of blood collection.

3.3.2. CYTOKINE ALTERATIONS IN SCHIZOPHRENIA

The serum concentrations of 9 cytokines were measured by multiplexed immunoassay analysis. This showed that the levels of these cytokines were not distributed uniformly across the cohorts. Since the distributions of age, gender, BMI and smoking were not significantly different, it is likely that other confounding factors which had not been controlled might have affected the results. To account for this possibility, we performed a non-parametric meta-analysis to determine the reproducibility of cytokine alterations across the 5 cohorts. This showed that 3 analytes (IL-1RA, IL-10 and IL-15) were reproducibly increased in first-onset schizophrenia patients compared to controls (**Figure 1c**, **e** and **g**). The most robust change was observed for IL-10 with average Cliff's delta reading of 0.36 and only 2% missing values.

Secondary analyses revealed that levels of IL-18 were significantly higher in smoking patients from cohort 3 (1.33 fold change, $p = 0.017$) and cohort 5 (3.91 fold change, $p = 0.008$) when compared to non-smoking patients. However, these effects were not reproducible in the remaining cohorts. In addition, no reproducible effects were observed with cannabis use or BMI, although this analysis was limited to only those patients for whom data on smoking, cannabis use and BMI was available.

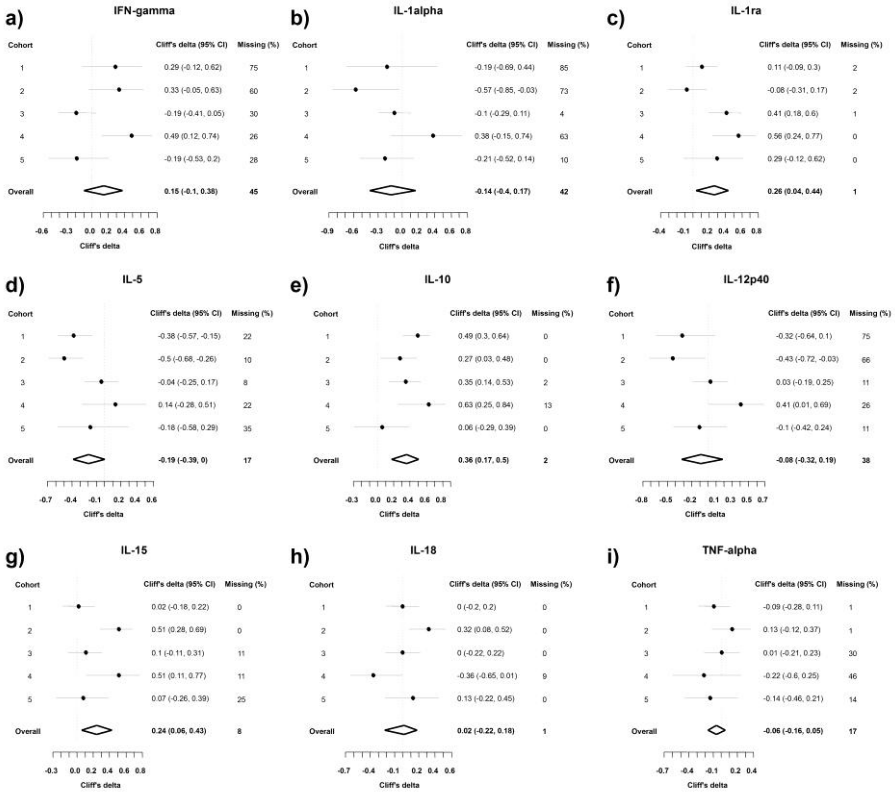


Figure 1. *Non-parametric meta-analysis of nine cytokines across five clinical cohorts. Horizontal lines indicate 95% confidence intervals (CI) and black circles the average effect sizes for individual cohorts. Diamonds represent the overall effect size estimates and their CIs. The figure also indicates the percentage of values outside the linear range of the assay system [Missing (%)].*

3.3.3. ANTIPSYCHOTIC TREATMENT EFFECTS

We also carried out multiplex immunoassay analysis of serum from 32 patients in cohort 3 after 6 weeks of treatment with atypical antipsychotics. The treatment resulted in significant decreases in PANSS positive, negative and general scores and an average increase in BMI (**Table 2**). The immunoassay profiling analysis showed that IL-1RA and IL-10, which were both increased in the first-onset patients, were now

Cytokine	All patients (n=32)		Quetiapine (n=7)		Mix of antipsychotics (n=9)		Risperidone (n=9)		Olanzapine (n=7)	
	RC	P	RC	P	RC	P	RC	P	RC	P
IFN -γ	0.99	1.000	1.10	0.553	1.40	0.527	0.95	0.752	0.50	0.219
IL-1α	0.91	0.299	1.00	1.000	1.05	0.820	0.82	0.203	0.85	0.578
IL-1-RA	0.76	0.005	0.82	0.141	1.23	0.820	0.53	0.035	0.64	0.047
IL-5	0.95	0.510	1.09	0.578	0.86	0.353	0.74	0.050	1.26	0.352
IL-10	0.86	0.059	0.91	0.834	1.02	0.933	0.79	0.042	0.73	0.036
IL-12p40	0.87	0.223	1.11	0.834	1.02	0.933	0.53	0.021	1.10	0.578
IL-15	0.94	0.495	0.76	0.469	1.15	0.575	0.93	0.800	0.88	0.675
IL-18	1.01	0.666	1.17	0.297	1.14	0.374	0.84	0.014	0.94	0.469
TNF-α	1.28	0.070	1.68	0.059	0.83	0.529	1.08	0.800	2.16	0.100

Table 3. *Changes in cytokine levels following 6 week treatment with atypical antipsychotics. Reproducible significant findings are shaded. P = p value, RC = ratio change; p values were calculated with Wilcoxon signed-rank tests.*

decreased in at least two of the antipsychotic treatment groups (**Table 3**). Both molecules showed significant decrease following treatment with risperidone and olanzapine (**Figure 2**). Furthermore, Spearman correlation testing showed that the changes in IL-10 levels were significantly correlated with symptom improvement (ΔPANSS) for negative, general and total scores (**Table 4**). Neither IL-1RA nor IL-10 showed a correlation with BMI which increased moderately (24.0 to 24.7) after the 6 week treatment period.

	ΔPANSS positive	ΔPANSS negative	ΔPANSS general	ΔPANSS total	ΔBMI
IL-1RA T_0	0.12 (0.519)	0.04 (0.812)	0.10 (0.572)	0.10 (0.575)	0.17 (0.365)
ΔIL-1RA	0.22 (0.227)	0.15 (0.410)	0.29 (0.110)	0.25 (0.164)	-0.07 (0.711)
IL-10 T_0	0.07 (0.711)	0.11 (0.558)	0.04 (0.807)	0.04 (0.821)	-0.06 (0.756)
ΔIL-10	0.30 (0.089)	0.41 (0.018)	0.37 (0.038)	0.45 (0.009)	-0.26 (0.142)

Table 4. *Univariate correlation analysis showing relationship between IL-1RA and IL-10 levels (initial, T_0, and change from baseline to week 6, Δ) and clinical outcomes. The table shows Spearman's correlation coefficients rho and corresponding p values (in brackets). Significant correlations are shaded.*

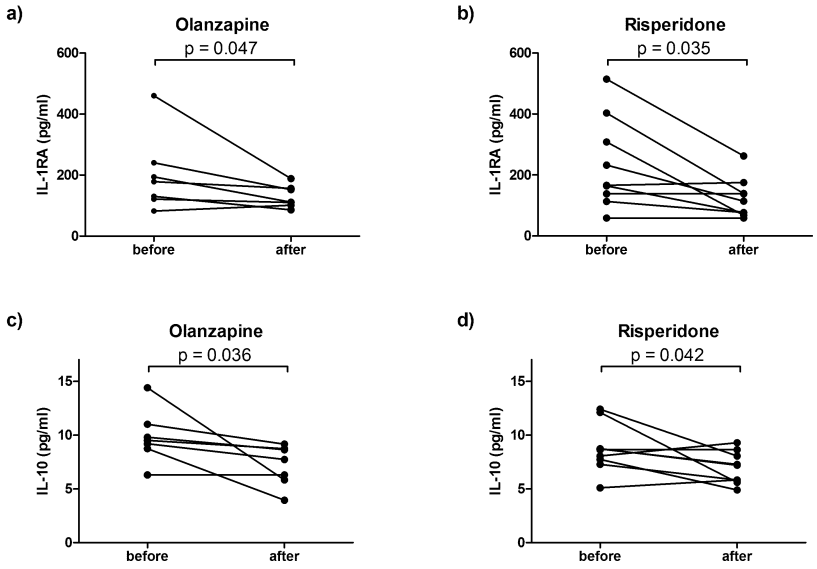


Figure 2. *Significant changes following 6 week treatment with atypical antipsychotics. Scattergrams show changes in IL-1RA (a-b) and IL-10 (c-d) levels following treatment with olanzapine (a, c) and risperidone (b, d). Quetiapine and mixture of antipsychotics did not cause significant effects on IL-1RA and IL-10 levels. P values were calculated with Wilcoxon signed-rank tests.*

3.4. Discussion

This is the first meta-analysis of cytokine changes in first-onset drug-naïve schizophrenia patients compared to well-matched healthy controls across multiple clinical cohorts. All participants underwent extensive clinical assessment, and the samples were processed according to strict standard operating procedures. To achieve maximum coherence, sera from different cohorts were analysed with a robust HumanMAP immunoassay platform in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory at Myriad-RBM Inc. (Austin, Texas). This showed that IL-1RA, IL-10 and IL-15 levels were consistently increased in first-onset patients compared to controls throughout the cohorts. Furthermore, we demonstrated that the levels of IL-1RA and IL-10 were decreased after 6 weeks treatment of 32 patients with atypical antipsychotics and the change in IL-10 levels was correlated with

symptom improvement. The strength of the present study was the high number of antipsychotic-naïve patients (n = 180) and controls (n = 350) that were matched for potential confounding factors such as age, gender, BMI and smoking, and the use of a standardised assay platform.

A major consideration of this study was the heterogeneity of reported serum cytokine changes in schizophrenia. In line with this, we found that even after excluding subjects with co-morbidities and matching for potential confounding factors, cytokine variation was still apparent across the cohorts. Therefore, this variation may be dependent on other factors which have not been considered in our and other studies. In a meta-analysis carried out by Miller et al., increased levels of the pro-inflammatory cytokines IL-12, TNF-α and IFN-γ were found in antipsychotic-naïve first-episode patients (97), although we found no changes in the same molecules. However, these analytes had high percentages of missing values in our study (17% to 45%) which could have biased our effect size estimates. Another possible explanation for this discrepancy might be the inclusion of data from non-standardised assays or the potential presence of confounding factors in meta-analysis described above. Based on our secondary analyses, it is unlikely that the differences were caused by smoking status, cannabis use or BMI. However, other factors, such as differences in the levels of physical exercise, socioeconomic status, psychosocial stress, race, or treatment regimes in different countries, have not been controlled for in the current investigation or virtually all previous studies.

The cytokines IL-1RA and IL-10 have anti-inflammatory functions, whereas IL-15 is categorised as pro-inflammatory cytokines. Therefore the finding that both sets of molecules were increased in first-onset schizophrenia patients suggests a combination of increases in pro- and anti-inflammatory pathways. This contrasts with some studies which have identified mainly pro-inflammatory changes in first-onset schizophrenia patients (97), although it is consistent with others which have shown mixed responses as we have found here (127, 218, 219).

Although we selected first-episode antipsychotic-naïve patients for this study, it still remains to be determined whether the altered cytokine

levels are a cause or consequence of the disease process. Interestingly, a polymorphism in the IL-10 gene (IL10) promoter region that leads to higher production of IL-10 as well as polymorphisms in the IL-1RA gene (IL1RN) has been associated with genetic susceptibility to schizophrenia (99, 103, 220). These genetic studies suggest that IL-10 and IL-1RA may have a causal role in schizophrenia pathogenesis. Moreover, it is now widely accepted that peripheral immune alterations can modulate brain function. For example, peripheral infections, inflammatory responses, or injection with pro-inflammatory cytokines such as IFN-α can lead to altered brain and behavioural responses (221). Furthermore, animal studies have shown that peripheral injection with specific cytokines can lead to either neuroprotection or neurotoxicity, depending on the class of molecule administered (222). One study suggested that peripheral IL-15 may induce depression-like behaviour and others reported an increase of IL-15 in schizophrenia (45, 223).

The finding that IL-1RA and IL-10 levels were decreased after treatment with atypical antipsychotics suggests that these molecules might be involved in the therapeutic response to antipsychotic treatment. Indeed, reduction in IL-10 was significantly correlated with symptom improvement. As these molecules are anti-inflammatory, a reduction could signify that antipsychotics promote certain inflammatory responses. This is consistent with the findings that side-effects by atypical antipsychotics are associated with increased inflammatory markers (224, 225). It has previously been shown that antipsychotics can directly modulate response of immune cells such as macrophages and microglia (226, 227). Moreover, different classes of neurotransmitter receptors are expressed by immune cells and the appropriate neurotransmitters can alter cytokine responses of these cells (228, 229). However, more complex mechanisms may be involved in the observed cytokine responses, such as the potential effect of decreased psychological stress on serum cytokine levels after antipsychotic treatment (230).

One limitation of the current study was the small number of cytokines investigated. This can lead to a potential bias. This procedure was based on the commercial availability of a multiplexed immunoassay platform

and did not specifically target all key regulators of immune- and inflammation-related pathways. Future studies should investigate a broader range of molecules and include those from other molecular classes such as growth factors or hormones. Another potential limitation comes from the fact that samples were not necessarily collected from participants under fasting conditions. This could be important as previous studies have shown that cytokine levels can fluctuate according to dietary intake (231). Further clinical and pre-clinical studies are required to unravel the role of cytokine alterations in schizophrenia and find strategies for better treatment approaches. Indeed, the first trials investigating therapies for schizophrenia targeting the immune system have shown promising results (207). Evaluation of the cytokine alterations before and during treatment in these trials may help to target the relevant patient population by stratification based on these signatures, predict treatment responses to current medications and identify potential novel immunomodulatory approaches for adjunctive treatments with traditional antipsychotics.

CHAPTER 4

IMMUNOMODULATORY EFFECTS OF PROBIOTIC SUPPLEMENTATION IN SCHIZOPHRENIA PATIENTS: A RANDOMIZED, PLACEBO-CONTROLLED TRIAL

Submitted. Tomasik J, Yolken RH, Bahn S and Dickerson FB

ABSTRACT

The prevalence of gastrointestinal infections in schizophrenia patients is higher than in the non-psychiatric population and may be linked to schizophrenia pathophysiology. In a recent report from a randomized, double-blind, placebo-controlled trial, we showed that supplementation of probiotics to schizophrenia patients undergoing long-term antipsychotic treatment improved gastrointestinal functioning, but did not reduce psychotic symptoms. In this follow-up study we examined the effects of probiotic supplementation on circulatory immune markers in the same patient population. Blood samples were collected from the patients before and after 14 weeks of treatment with probiotics (Lactobacillus rhamnosus strain GG and Bifidobacterium animalis subsp. lactis strain Bb12) or placebo as an adjuvant to standard antipsychotic treatment. In total, 58 out of 65 participants completed the trial, 31 in the probiotic arm and 27 in the placebo arm. The concentrations of 47 immune-related serum proteins were measured using multiplexed immunoassays (Human InflammationMAP panel; Myriad RBM, Austin, TX, USA). Probiotic add-on treatment significantly reduced levels of von Willebrand factor (ratio change = 0.84, p-value = 0.047) and increased levels of MCP-1, BDNF, RANTES and MIP-1 beta with borderline significance ($p \leq 0.08$). In silico pathway analysis revealed that these probiotic-specific alterations are related to regulation of macrophage cell function, T helper cell function and intestinal epithelial cells by the IL-17 family of cytokines. We hypothesize that supplementation of probiotics to schizophrenia patients may restore intestinal homeostasis by improved control of gastrointestinal leakage, resulting in improved bowel functioning, but does not reduce psychotic symptoms.

4.1. INTRODUCTION

A range of gastrointestinal problems such as irritable bowel syndrome and constipation have been reported in schizophrenia patients (232, 233). Defects in the gastrointestinal barrier in schizophrenia patients can lead to penetration of gut microbiota and food antigens into the host periphery (234). This was accompanied by increased levels of antibodies against microbial and food antigens in both first-onset and chronic schizophrenia patients. Resulting inflammatory responses have been

found to be abnormally activated in many individuals with schizophrenia, regardless of disease stage or medication status. It has been hypothesized that schizophrenia can originate from early exposure to microbial infections, which may contribute to the etiology through chronic neuroinflammatory and autoimmune processes (29, 235). Although the exact mechanism of gut-brain interaction has not been completely elucidated, it is known that bacteria inhabiting the human intestine can communicate with the central nervous system in multiple ways (236, 237). For example, these microorganisms interact with the innate immune system, affecting secretion of pro- and anti-inflammatory cytokines which, in turn, can regulate brain development and function (238). In addition, bacteria of the species *Lactobacillus* and *Bifidobacterium* are capable of producing neurotransmitters such as gamma-aminobutyric acid (GABA) and acetylcholine, which directly target receptors in the central nervous system (239).

Current antipsychotic medications show limited immunomodulatory effects (240). Only recently, clinical trials have attempted to target schizophrenia-related inflammation using anti-inflammatory agents. Supplementation with celecoxib, acetylsalicylic acid and minocycline in addition to standard antipsychotic medication has resulted in overall patient improvement, in particular in a reduction of positive psychotic symptoms (37, 189, 241). Probiotics can also modulate the immune response of the host by affecting the composition of gut microbiota (242). Several probiotic species have been tested for health benefits, including the gram-positive anaerobic genra *Lactobacillus* and *Bifidobacterium*. These have shown beneficial effects on intestinal permeability (243), systemic inflammatory cytokine levels (244), neurotransmitter and neurotrophic factor production (245) and oxidative stress in animal models (246).

In humans, oral administration of probiotics has resulted in increased systemic antioxidant capacity (247), restored normal inflammatory status (248), changed the activity of brain regions responsible for processing of emotion and sensation (249) and reduced anxiety (250). Therefore, probiotics have been suggested as a potential novel therapeutic approach for a range of neurodevelopmental disorders (251).

In addition, probiotics have been found to lower the levels of cholesterol and prevent eczema and other forms of allergies (252, 253). They have also been proposed as a means of treating the symptoms of celiac disease, which has an incidence in schizophrenia patients that is more than 3-fold higher than in the general population (62, 254, 255).

We showed recently that supplementation of probiotic strains *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 improved bowel function in schizophrenia patients remaining on long-term antipsychotic treatment (256). The present study was undertaken to assess the systemic immunomodulatory effects of probiotic supplementation in the same patient population. Using multiplexed immunoassays, we measured the levels of 47 immune molecules in patient sera collected before and after treatment with adjunctive probiotics or placebo. Group comparisons revealed probiotic-specific changes in levels of molecules involved in innate and adaptive immune responses and intestinal epithelial cell function. These alterations may be related to improved function of the intestinal tract in the probiotic arm of the trial.

4.2. MATERIALS AND METHODS

4.2.1. PARTICIPANTS AND STUDY PROCEDURES

The patient population and probiotic compound investigated in this study have been described in detail previously (256). Briefly, 65 outpatients from psychiatric rehabilitation programs in the Baltimore area (Maryland, USA) diagnosed with schizophrenia or schizoaffective disorder according to DSM-IV criteria, with at least moderately severe psychotic symptoms [Positive and Negative Syndrome Scale (PANSS) positive score ≥ 1 and/or PANSS negative symptom score ≥ 4 or total PANSS score ≥ 50, containing at least 3 positive or negative items with scores ≥ 3 at screening] were enrolled in the study between December 2010 and August 2012. Participants were randomized into a double-blind 14 week treatment protocol with adjunctive probiotic (n=33) or placebo (n=32), with initial 2 week placebo run-in (**Figure 1**). All patients received antipsychotic treatment for at least 8 weeks prior to starting the trial and did not change the medication within the previous 21 days.

Patients suffering from any clinically significant or unstable medical condition, including congestive heart failure, celiac disease or immunodeficiency syndromes, as well as those receiving antibiotics within the previous 14 days, were excluded from the study.

The active study compound consisted of one tablet containing approximately 10^9 colony forming units of the probiotic organism *Lactobacillus rhamnosus* GG and 10^9 colony forming units of the probiotic organism *Bifidobacterium animalis* subsp. *lactis* BB12 (Ferrosan; Denmark) or placebo. The probiotic microorganisms were grown in media which do not contain casein, lactose, other milk products or gluten, to reduce the risk of allergic reactions to these ingredients.

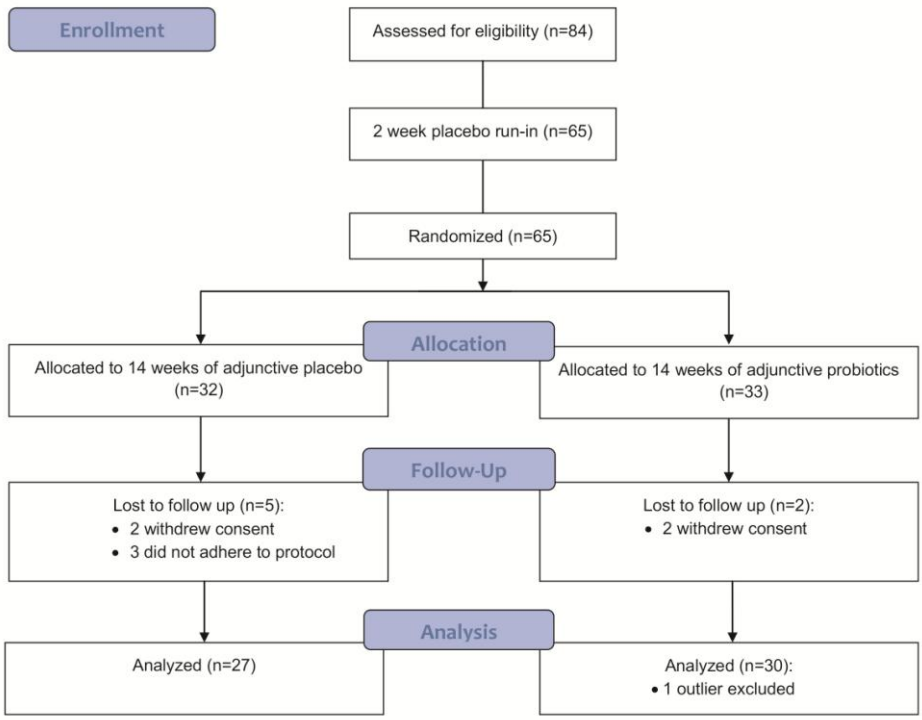


Figure 1. *CONSORT (Consolidated Standards of Reporting Trials) flow diagram of the trial.*

In total, 58 participants completed the trial, comprising 31 in the probiotic arm and 27 in the placebo arm. Blood samples were collected from all subjects at the beginning and at the end of the trial. Serum was prepared by allowing clot formation for 2 h at room temperature and subsequent centrifugation at 4000 g for 5 min. The resulting serum supernatants were stored at -80 degrees until analysis.

4.2.2. MULTIPLEXED IMMUNOASSAYS

Serum samples were analyzed using the Human InflammationMAP panel in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory (Myriad RBM, Austin, TX, USA). The panel consisted of 47 multiplexed immunoassays targeting selected inflammatory markers, including cytokines, chemokines and acute-phase reactants (**Appendix A**). Analyte levels were estimated in each sample from the 8-point standard curves, and assay performance was validated using 3 control samples. The same multiplex immunoassay platform has been applied successfully for serum biomarker profiling in a range of high-impact studies (257-259).

4.2.3. DATA ANALYSIS

For those participants who completed the double-blind phase, immune marker data acquired from multiplex immunoassay analyses were filtered separately for each treatment group and time point. Principal Component Analysis was applied to identify artifactual effects on the overall variance. This resulted in detection and removal of one outlier from the probiotic-supplemented group. Molecules with more than 60% low values were excluded from further analysis. This equated to 20 analytes (**Appendix A**). For the 27 analytes remaining in the dataset, values below the lower detection limit were replaced with half the minimum value for that specific assay. Shapiro-Wilk tests showed that the majority of analyte levels were non-normally distributed, therefore Wilcoxon signed-rank test was applied to compare analyte levels before and after treatment. Resulting p-values were controlled for false discovery rate with a conservative Benjamini-Hochberg approach (260).

Pathway analysis was performed with the Ingenuity Pathways Knowledge Database (IPKB; Ingenuity® Systems; Mountain View, CA,

USA). Only molecules from the datasets that met the p-value cut-off of 0.10 and were associated with the biological functions and/or canonical pathways in the IPKB were considered for the analysis. A right-tailed Fisher's exact test was used to calculate p-values associated with the identified pathways. The significance of the association between the dataset and canonical pathways was measured by the ratio between the number of molecules from the dataset divided by the total number of known molecules in that pathway and by the p-value (Fisher's exact test).

4.2.4. ETHICAL CONSIDERATIONS

The study was approved by Institutional Review Boards of the Sheppard Pratt Health System and the Johns Hopkins School of Medicine. The trial was registered at ClinicalTrials.gov corresponding to NCT01242371 and monitored by a Data Safety Monitoring Board. Written informed consent was obtained from all study participants.

4.3. RESULTS

A total of 65 patients were enrolled in the study and randomized, 33 to the adjunctive probiotics arm and 32 to the adjunctive placebo arm. A total of 58 participants (89%) completed the study (**Figure 1**). The clinical characteristics of the completers are shown in **Table 1.** There were no significant differences in age, gender, race, duration of education,

	Probiotic supplement	**Placebo supplement**	**p-value**
N	31	27	-
Age	44.8 ± 11.2	48.1 ± 9.4	0.236 [a)]
Gender (male/female)	22/9	16/11	0.413 [b)]
Race (white/other)	16/15	20/7	0.106 [b)]
PANSS [c)] total start	67.3 ± 11.9	70.2 ± 11.6	0.258 [a)]
PANSS [c)] total end	66.8 ± 11.6	67.3 ± 11.9	0.773 [a)]

Table 1. *Demographical and clinical data of study completers. The table shows mean values ± standard deviation. Detailed patient characteristics has been described in (256).* [a)] *Mann-Whitney U test.* [b)] *Fisher's exact test.* [c)] *Positive and Negative Syndrome Scale.*

PANSS scores and proportion of patients receiving clozapine between the groups at the beginning of the study (256). PANSS psychiatric symptom scores did not change over the course of the trial, but patients receiving probiotic supplement were less likely to report severe bowel difficulties (p = 0.003) (256).

In terms of the mechanism of action, treatment with the probiotics significantly decreased the levels of the acute phase reactant von Willebrand factor (**Table 2**). Uploading the accession numbers of all analytes which showed a change at a significance level of <0.10 [von Willebrand factor, monocyte chemotactic protein 1 (MCP-1), brain derived neurotrophic factor (BDNF), T cell specific protein RANTES and macrophage inflammatory protein 1 beta (MIP-1 beta)] into the Ingenuity Pathways Knowledge Base (www.ingenuity.com) indicated that supplementation with probiotics most significantly affected the regulation of cytokine production in macrophages, T helper cells and intestinal epithelial cells by IL-17A and IL-17F pathways (top canonical pathways, p = 9.34E-06 and 1.54E-05, ratio 0.111 and 0.087; **Table 3**).

Analyte	UniProtKD ID	Probiotic supplement				Placebo supplement			
			FC [a]	p	q [b]		FC [a]	p	q [b]
von Willebrand Factor	P04275	↓	0.84	0.047	0.431				
Monocyte Chemotactic Protein 1	P13500	↑	1.22	0.054	0.431				
Brain Derived Neurotrophic Factor	P23560	↑	1.28	0.063	0.431				
T Cell Specific Protein RANTES	P13501	↑	1.28	0.069	0.431				
Macrophage Inflammatory Protein 1 beta	P13236	↑	1.1	0.080	0.431				
Vascular Cell Adhesion Molecule 1	P19320					↓	0.88	0.016	0.313
Intercellular Adhesion Molecule 1	P05362					↓	0.83	0.023	0.313
Tumor necrosis factor receptor 2	P20333					↓	0.87	0.072	0.450
Ferritin	P02794 P02792					↓	0.85	0.073	0.450
Matrix Metalloproteinase 3	P08254					↓	0.88	0.099	0.450

Table 2. *Changes in immune marker levels following probiotic and placebo supplementation with p-value < 0.10. The full list of changes is available in* ***Appendix B****.* [a] *Average fold change.* [b] *q-value – p-value controlled for false discovery rate with Benjamini-Hochberg procedure.*

Probiotic supplement	Placebo supplement
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F (p = 9.34E-06, ratio = 1.11E-01)	Atherosclerosis Signaling (p = 2.54E-08, ratio = 3.1E-02)
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F (p = 1.54E-05, ratio = 8.7E-02)	Leukocyte Extravasation Signaling (p = 2.37E-05, ratio = 1.6E-02)
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza (p = 4.99E-05, ratio =4.9E-02)	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis (p = 8.33E-05, ratio = 1.0E-02)
Role of IL-17A in Arthritis (p = 8.7E-05, ratio = 3.3E-02)	HMGB1 Signaling (p = 4.3E-04, ratio = 2.1E-02)
Role of MAPK Signaling in the Pathogenesis of Influenza (p = 1.3E-04, ratio = 3.0E-02)	Hepatic Fibrosis/Hepatic Stellate Cell Activation (p = 9.31E-04, ratio = 1.4E-02)

Table 3. *List of canonical pathways most significant to the probiotic and placebo groups revealed by Ingenuity Pathway Analysis. Significance of the association was measured by the p-value and by the ratio of the involved molecules. Probiotic supplementation showed enrichment in IL-17A and IL-17F-related pathways.*

Changes identified in the placebo group [vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1); **Table 2**] may have resulted from abnormally high levels of these proteins at baseline and therefore cannot be assigned as true placebo effects. Initial levels of VCAM-1 in the placebo group were 21% higher than in the group supplemented with probiotics (p = 0.021, Mann-Whitney test). After treatment, VCAM-1 returned to similar levels (3% difference, p = 0.581), with the greatest change observed for patients with the highest initial VCAM-1 levels (Spearman's rho = -0.61, p = 0.0007). This suggests that changes in the placebo group may be due to the regression to the mean effect (261, 262).

4.4. DISCUSSION

This is the first study to investigate the immunomodulatory effects of a probiotic supplementation in schizophrenia. In this investigation, 65 patients undergoing long-term antipsychotic treatment were randomized to 14 weeks of either probiotic or placebo add-on supplementation; 58 participants completed the trial (**Figure 1**). Using multiplexed immunoassays, we measured levels of 47 immune markers before and after add-on treatment, of which 27 satisfied the strict criteria for analysis. We found that probiotic supplementation significantly reduced levels of von Willebrand factor, and levels of MCP-1, BDNF, T cell specific protein RANTES and MIP-1 beta were found to be increased with borderline significance ($p \leq 0.08$). These changes were related mostly to the regulation of cytokine production in macrophages, T helper cells and intestinal epithelial cells as shown by the effects on IL-17A and IL-17F pathways. Although IL-17 levels were not detectable in our study, we identified increased levels of other cytokines related to IL-17, namely MCP-1 and RANTES. The decreased levels of VCAM-1 and ICAM-1 in the placebo group were interpreted as a regression to the mean effect (261, 262), i.e. they resulted from abnormally high initial levels in the placebo group and did not differ between the groups at the end of the study. This is consistent with the fact that the placebo was provided in the context of long-term stable antipsychotic treatment and, therefore, no changes were expected in this group.

The only molecule that changed when applying conventional significance criteria ($p < 0.05$) after probiotic supplementation was von Willebrand factor (FC = 0.84). Von Willebrand factor is a positive acute phase reactant produced by endothelial cells in response to injury. This protein has been found to be positively correlated with cardiovascular risk factors in schizophrenia and bipolar disorder, and does not change with second-generation antipsychotic treatment (224). In our study, probiotic supplementation decreased the levels of von Willebrand factor. Probiotics are known to decrease levels of certain cardiovascular risk parameters (263). However, their effect on antipsychotic-related cardiovascular risk should be assessed in future longitudinal studies by measuring cardiovascular markers such as triglycerides, HDL and LDL.

Pathway analysis suggested that probiotic add-on treatment, but not standard antipsychotic therapy, modulates immune function via type 17 immune responses. This pathway involves the IL-17 family of cytokines, which are regulatory proteins produced by T helper 17 (Th17) cells involved in cellular responses to extracellular bacteria such as those colonizing the intestinal lumen (264). Therefore, we hypothesize that the observed cytokine changes are related to improved bowel functioning, which we reported in this group of patients previously (256). These cytokines are also critical mediators of autoimmune reactions. Deregulation of the type 17 response has been observed in schizophrenia. Studies have shown that this pathway was blunted in psychotic episodes (265). Also, autoimmune processes against central nervous tissue components, which are regulated by type 17 cytokines, are a known phenomenon that may contribute to schizophrenia etiology (266). Here we showed that molecules associated with the type 17 response, in particular MCP-1 and RANTES, increased with borderline significance in the group of patients treated with probiotic supplement. Pathway analysis suggested that these changes may be associated with improved control of gastrointestinal leakage by the innate and adaptive immune systems (**Figure 2**). This is consistent with the observed decrease in von Willebrand factor levels after probiotic supplementation, which might be a secondary effect of improved intestinal epithelium integrity. IL-17 levels were not detectable in any of the samples (<5 pg/ml). Therefore, further studies using assays with improved sensitivity are necessary to identify any direct effects on T helper type 17 pathways.

The finding that BDNF may have been increased by probiotic add-on treatment is interesting as it is a neurotrophin involved in neuronal survival and plasticity and has been associated previously with schizophrenia pathophysiology (267). Reduced levels of BDNF have been associated with increased positive and negative symptom severity in unmedicated first-onset schizophrenia patients (127, 268, 269). Also, other studies have shown that treatment with antipsychotics alone has no effects on circulating BDNF levels [summarized in (270)]. To our knowledge, these are the first results which show that probiotics may lead to an increase in BDNF levels, as suggested elsewhere (271). However, this finding did not translate into improved symptoms in the

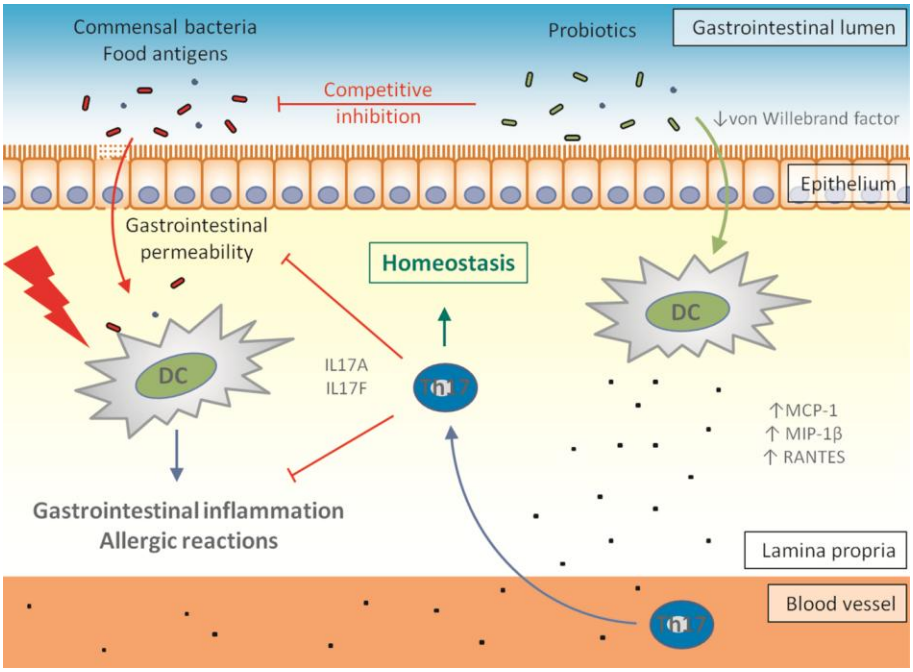


Figure 2. *Hypothetical mechanism of restoring intestinal homeostasis by probiotic bacteria indicated by pathway analysis. Probiotics competitively inhibit growth of commensal bacteria and induce release of chemokines from intestinal epithelial and dendritic cells that attract T helper 17 cells. Resulting type 17 immune response restores integrity of the epithelium (indicated by decreased levels of von Willebrand factor), attenuating gastrointestinal inflammation and allergic reactions to bacterial and food antigens. DC – dendritic cell, Th17 – T helper 17 cell.*

probiotic arm and requires further validation to show statistical significance.

Several limitations of the molecular profiling results should be considered when interpreting the results of this study. Firstly, we were not able to detect all targeted cytokines in our clinical samples. This included molecules which have been previously shown to be modulated by probiotics, such as IL-1 beta, IL-6, TNF alpha and IFN gamma. The reduced number of analyzed proteins resulted in relatively high q-values ($q = 0.431$ for $p < 0.1$ in the probiotic arm). Therefore, the identified changes require further validation studies. Repeating these experiments

using more sensitive and diverse multiplex immunoassays is essential to provide a more complete picture of the effects.

Secondly, although we investigated only patients who remained on stable, long-term antipsychotic treatment during the trial period, it is still possible that the antipsychotic compound exhibited certain immunomodulatory effects. This relates in particular to the changes identified in VCAM-1 and ICAM-1 levels in the placebo group. Furthermore, due to the lack of a control group, it was not possible to determine whether baseline levels of the analytes were altered. Lastly, the small number of analytes identified as significantly different between the two groups was a limiting factor for the pathway analysis. For example, although MCP-1 has been linked to IL-17 pathways, this chemokine can be produced by various cell types, including endothelial and epithelial cells, fibroblasts, smooth muscle cells, astrocytes and microglia, and is capable of regulating migration of not only Th17 lymphocytes, but also other Th cell types as well as monocytes, natural killer cells, basophils and eosinophils (272). Similarly, RANTES is known to have chemotactic properties for different T cell subtypes, monocytes, basophils and eosinophils (273). This suggests that more complex immune networks may be affected by probiotic supplementation.

We conclude that probiotics decrease serum levels of von Willebrand factor and may have immunomodulatory effects in schizophrenia patients, affecting molecules that do not respond to standard antipsychotic therapy. These changes may be associated with the improvement in bowel functioning reported previously in the same group of patients through IL-17-related immune responses, which control the intestinal microbiome-host interaction. However, supplementation of probiotics does not reduce psychotic symptoms. We suggest that future studies should be carried out which test the exact biological and neurobiological mechanisms of probiotic supplementation.

4.5. APPENDICES

Protein	UniProtKB ID	Protein	UniProtKB ID
Alpha-1-Antitrypsin (AAT)	P01009	Interleukin-10 (IL-10)	P22301
Alpha-2-Macroglobulin (A2Macro)	P01023	Interleukin-12 Subunit p40 (IL-12p40)	P29460
Beta-2-Microglobulin (B2M)	P61769	Interleukin-12 Subunit p70 (IL-12p70)	P29459
Brain-Derived Neurotrophic Factor (BDNF)	P23560	Interleukin-15 (IL-15)	P40933
C-Reactive Protein (CRP)	P02741	Interleukin-17 (IL-17)	Q16552
Complement C3 (C3)	P01024	Interleukin-18 (IL-18)	Q14116
Eotaxin-1	P51671	Interleukin-23 (IL-23)	Q9NPF7
Factor VII	P08709	Macrophage Inflammatory Protein-1 alpha (MIP-1 alpha)	P10147
Ferritin (FRTN)	P02794, P02792	Macrophage Inflammatory Protein-1 beta (MIP-1 beta)	P13236
Fibrinogen	P02671, P02675, P02679	Matrix Metalloproteinase-2 (MMP-2)	P08253
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	P04141	Matrix Metalloproteinase-3 (MMP-3)	P08254
Haptoglobin	P00738	Matrix Metalloproteinase-9 (MMP-9)	P14780
Intercellular Adhesion Molecule 1 (ICAM-1)	P05362	Monocyte Chemotactic Protein 1 (MCP-1)	P13500
Interferon gamma (IFN-gamma)	P01579	Stem Cell Factor (SCF)	P21583
Interleukin-1 alpha (IL-1 alpha)	P01583	T-Cell-Specific Protein RANTES (RANTES)	P13501
Interleukin-1 beta (IL-1 beta)	P01584	Tissue Inhibitor of Metalloproteinases 1 (TIMP-1)	P01033
Interleukin-1 receptor antagonist (IL-1ra)	P18510	Tumor Necrosis Factor alpha (TNF-alpha)	P01375
Interleukin-2 (IL-2)	P60568	Tumor Necrosis Factor beta (TNF-beta)	P01374
Interleukin-3 (IL-3)	P08700	Tumor necrosis factor receptor 2 (TNFR2)	P20333
Interleukin-4 (IL-4)	P05112	Vascular Cell Adhesion Molecule-1 (VCAM-1)	P19320
Interleukin-5 (IL-5)	P05113	Vascular Endothelial Growth Factor (VEGF)	P15692
Interleukin-6 (IL-6)	P05231	Vitamin D-Binding Protein (VDBP)	P02774
Interleukin-7 (IL-7)	P13232	von Willebrand Factor (vWF)	P04275
Interleukin-8 (IL-8)	P10145		

Appendix A. *Inflammatory markers measured using Human InflammationMAP multiplexed immunoassay platform. Analytes excluded from the analysis due to high proportion of missing values are shown in grey.*

Analyte	Function[a)]	Probiotic supplement				Placebo supplement			
			FC	*P* value	*q* value		FC	*P* value	*q* value
von Willebrand Factor	AP	↓	0.84	0.047	0.431	↑	1.15	0.751	0.881
Monocyte Chemotactic Protein 1	C	↑	1.22	0.054	0.431	↓	0.93	0.525	0.782
Brain Derived Neurotrophic Factor	GF	↑	1.28	0.063	0.431	↑	1.06	0.895	0.966
T Cell Specific Protein RANTES	C	↑	1.28	0.069	0.431	↓	0.96	0.551	0.782
Macrophage Inflammatory Protein 1 beta	C	↑	1.1	0.080	0.431	↓	0.97	0.62	0.821
Factor VII	O	↑	1.05	0.221	0.759	↓	0.92	0.117	0.450
Ferritin	AP	↓	0.91	0.225	0.759	↓	0.85	0.073	0.450
Vascular Endothelial Growth Factor	GF	↑	1.08	0.225	0.759	↑	1.01	0.957	0.994
Stem Cell Factor	GF	↑	1.06	0.285	0.855	↓	0.91	0.243	0.513
Haptoglobin	AP	↓	0.93	0.365	0.876	↑	1.04	0.4	0.720
Fibrinogen	AP	↓	0.95	0.375	0.876	↑	1.02	0.511	0.782
Complement C3	AP	↓	0.96	0.421	0.876	↓	0.93	0.247	0.513
Interleukin 8	C	↓	0.95	0.449	0.876	↑	1.03	0.713	0.875
Interleukin 1 receptor antagonist	C	↑	1.04	0.559	0.876	↓	0.83	0.104	0.450
Alpha 2 Macroglobulin	AP	↑	1.02	0.581	0.876	↑	1.02	1.000	1.000
Vascular Cell Adhesion Molecule 1	O	↑	1.02	0.584	0.876	↓	0.88	0.016	0.313
Beta 2 Microglobulin	TR	↓	0.99	0.589	0.876	↓	0.93	0.139	0.461
Alpha 1 Antitrypsin	AP	↑	1.03	0.613	0.876	↓	0.98	0.638	0.821
Interleukin 18	C	↑	1.03	0.65	0.876	↓	0.9	0.343	0.661
Vitamin D Binding Protein	O	↑	1.02	0.713	0.876	↓	0.95	0.234	0.513
Interleukin 23	C	↑	1.02	0.727	0.876	↓	0.88	0.154	0.461
Eotaxin 1	C	↑	1.02	0.757	0.876	↓	0.96	0.431	0.727
Tumor necrosis factor receptor 2	TR	↑	1.01	0.758	0.876	↓	0.87	0.072	0.450
Intercellular Adhesion Molecule 1	TR	↑	1.03	0.805	0.876	↓	0.83	0.023	0.313
Matrix Metalloproteinase 3	O	↓	0.98	0.811	0.876	↓	0.88	0.099	0.450
Tissue Inhibitor of Metalloproteinases 1	O	↑	1.02	0.914	0.949	↓	0.93	0.242	0.513
C Reactive Protein	AP	↓	0.44	0.992	0.992	↑	1.54	0.882	0.966

Appendix B. *Changes in immune marker levels following probiotic and placebo supplementation. FC – average fold change; p-value < 0.05 was considered significant (shaded); q-value – p-value controlled for false discovery rate with Benjamini-Hochberg procedure.* [a)] *Abbreviations: AP - acute phase protein, C – cytokine, GF - growth factor, TR - transmembrane receptor, O – other.*

CHAPTER 5

CHANGES IN THE FATTY ACID HANDLING PROTEINS H-FABP AND CD36 PREDICT RESPONSE TO OLANZAPINE TREATMENT IN RECENT-ONSET SCHIZOPHRENIA PATIENTS

Ready for submission, patent application pending. Tomasik J, Schwarz E, Lago SG, Rothermundt M, Leweke FM, Steiner J, van Beveren NJM, Guest PC, Rahmoune H and Bahn S

ABSTRACT

Traditional schizophrenia pharmacotherapy remains a subjective trial and error process involving administration, titration and switching of drugs multiple times until an adequate response is achieved. Despite this time-consuming and costly process, not all patients show an adequate response to treatment. As a consequence, relapse is a common occurrence and early intervention is hampered. Here, we have attempted to identify candidate blood biomarkers associated with drug response in 124 initially antipsychotic-free recent-onset schizophrenia patients treated with widely-used antipsychotics, namely olanzapine (n=41), quetiapine (n=23), risperidone (n=31) and a mixture of these drugs (n=29). Patients were recruited and investigated as two separate cohorts to allow biomarker validation. Data analysis showed that patients with higher levels of heart-type fatty acid binding protein (H-FABP) at baseline responded better to olanzapine ($p=0.034$, $\beta=67.8$ in the discovery cohort and $p=0.042$, $\beta=16.2$ in the validation cohort, adjusted for baseline symptoms, age, gender and body mass index). In a functional validation analysis, we also tested an independent cohort of 10 patients treated with olanzapine and found that baseline expression of the binding partner for H-FABP, fatty acid translocase (CD36), on monocytes correlated with reduction of psychotic symptoms ($p=0.047$, $\beta=-0.0074$). We also identified a set of molecules changed after treatment with antipsychotic medication, in particular olanzapine. These molecules are predominantly involved in cellular development and metabolism. Taken together, our findings suggest an association between biomarkers involved in fatty acid metabolism and response to olanzapine, while other proteins may serve as surrogate markers associated with drug efficacy and side effects.

5.1. INTRODUCTION

Schizophrenia is a severe mental illness affecting approximately 24 million people worldwide (274). The disorder is treated primarily with antipsychotic medication but the exact mechanism of action of these drugs remains largely unknown. As a consequence, traditional schizophrenia pharmacotherapy can be a subjective trial and error process involving administration, titration and switching of drugs

multiple times until an adequate response is achieved. This results in the problem that not all patients respond well to initial treatment and many experience relapse (275).

Treatment with antipsychotic drugs has also been associated with adverse side effects which can lead to health problems and non-compliance (276-278). The effects of second-generation (atypical) antipsychotics, for example olanzapine, quetiapine and risperidone, include metabolic-related side effects such as weight gain, hyperglycaemia, insulin resistance and type II diabetes mellitus (16). Side effects of atypical antipsychotics have been associated with the high affinity of these compounds for histamine H_1, α_1 adrenergic, 5-hydroxytryptamine 2C (5-HT_{2C}) and 5-HT_6 receptors, which are known to be involved in appetite regulation (279, 280). Considering the potential negative health impact of these side effects, clinicians are now required to monitor patients receiving atypical antipsychotics closely for their glucose, insulin and fatty acids blood levels.

In addition, these deficits have led academic and pharmaceutical industry researchers to launch biomarker discovery initiatives to facilitate treatment decisions and identify novel therapeutic approaches. It is anticipated that the application of biomarker strategies into the clinical decision making process could lead to benefits such as better stratification of patients, monitoring of therapeutic effects, or early detection of side effects (personalised medicine). There have already been some molecular studies which have investigated the association between antipsychotic treatment effects and biomarker readouts (22, 281, 282). For example, changes in immune and metabolic markers have been associated with psychotic relapse in schizophrenia patients after treatment with atypical antipsychotics (22).

With this in mind, we have attempted to characterise serum molecular profiles in early stage schizophrenia patients, before and after treatment with some of the most widely-used atypical antipsychotics. We performed a comprehensive molecular analysis on serum collected from recent-onset, drug-free schizophrenia patients before and after treatment with olanzapine, risperidone, quetiapine or a mixture of these

antipsychotics. Sera were analyzed using the Human MAP® multiplex immunoassay panel which targets a selection of hormones, growth factors, transport proteins and immune-related molecules, which may be relevant to the antipsychotic mechanism of action. Proteins which showed significant predictive value for treatment response or alterations after treatment were evaluated in an independent clinical cohort treated with the same medications. In addition, we carried out functional validation of the findings using blood cells isolated from an independent cohort of recent-onset drug-free patients.

5.2. Materials and Methods

5.2.1. Clinical samples

Subjects were recruited from the Departments of Psychiatry at the Universities of Cologne (discovery cohort), Muenster (discovery cohort), Magdeburg (validation cohort) and the Erasmus Medical Centre in Rotterdam (follow-up cohort; **Table 1**). Schizophrenia was diagnosed based on the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV). The medical faculty ethical committees of the respective universities approved the protocols of the study. Informed consent was given in writing by all participants and clinical investigations were conducted according to the Declaration of Helsinki.

All patients were antipsychotic-naïve (n=55) or had been free of antipsychotic medication at the start of the study (n=69 for at least 6 weeks, n=12 for less than 6 weeks). Patients with co-morbidities, such as type II diabetes mellitus, hypertension, cardiovascular, autoimmune, inflammatory or infectious diseases, were excluded from the study. Patients were assessed for psychopathology by experienced clinicians using the Positive and Negative Syndrome Scale (PANSS) before and after 4-6 weeks treatment with antipsychotics.

	Discovery	Validation	Follow-up
Number	47	77	12
Treatment (ola/quet/risp/mix)	23/9/7/8	18/14/24/21	12/0/0/0
Gender (male/female)	35/12	51/26	12/0
Age (years)	29.0 ± 10.8	35.6 ± 11.1	26.4 ± 6.2
Body mass index (kg/m^2)	22.6 ± 3.1[1]	25.4 ± 4.6	24.0 ± 4.8
Smoking (yes/no/nk)	6/16/25	51/26/0	8/4/0
Cannabis (yes/no/nk)	0/1/22	17/54/6	2/10/0
Drug status (naïve/free)	23/24	32/45	0/12
PANSS positive score T_0	19.9 ± 6.8	23.0 ± 7.2	20.5 ± 5.3[2]
PANSS negative score T_0	19.0 ± 8.3	19.3 ± 8.7	22.1 ± 5.4[2]
PANSS general psychopathology score T_0	39.6 ± 10.3	43.0 ± 10.3	39.6 ± 6.0[2]
PANSS positive score T_1	13.4 ± 3.6	12.7 ± 5.3	13.7 ± 4.8[2]
PANSS negative score T_1	16.1 ± 5.7	14.4 ± 6.8	19.8 ± 5.1[2]
PANSS general psychopathology score T_1	31.3 ± 7.6	28.7 ± 9.1	33.7 ± 8.6[2]

Table 1. *Demographic details of schizophrenia patients from discovery, validation and follow-up cohorts. BMI – body mass index, T_0 – baseline measurement, T_1 – follow-up measurement (4-6 weeks). Values are shown as mean ± standard deviation. nk = not known. [1] Data available for 23 patients. [2] Data available for 10 patients.*

5.2.2. *MULTIPLEX IMMUNOASSAY PROFILING*

Blood samples from the discovery and validation cohorts were collected between 8:00 and 12:00 am into S-Monovette 7.5 mL serum tubes (Sarstedt). The blood was left at room temperature for 2 hours according to standard protocols to allow clotting and centrifuged at 4000 g for 5 minutes to remove particulate material. Resulting supernatants were stored at -80°C in Low Binding microcentrifuge tubes (Eppendorf). Levels of 147 analytes were measured in 250 µL serum using the Human MAP® multiplexed immunoassay platform (**Supplementary Table S1**) in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory (Rules Based Medicine; Austin, TX, USA) as described previously (25).

5.2.3. UNIPLEX IMMUNOASSAY

Whole blood was collected from the follow-up cohort into 7.5 mL heparin tubes (Sarstedt), centrifuged at 500 g for 10 min and the supernatants (plasma) were stored at -80°C. Plasma levels of H-FABP were determined using human H-FABP enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotech) according to manufacturer's protocol.

5.2.4. FLOW CYTOMETRY

Peripheral blood mononuclear cells (PBMCs) were prepared from blood collected into 7.5 mL heparin tubes from the follow-up cohort, as above. The pellets containing blood cells after the centrifugation step were diluted 1:1 with phosphate-buffered saline (PBS; Sigma-Aldrich) and centrifuged over Ficoll (GE Healthcare) at 750 g for 20 min. PBMCs were extracted from the interphase, washed three times with PBS at 300 g for 10 min and cryopreserved in foetal bovine serum (FBS) containing 10% dimethyl sulfoxide (Sigma-Aldrich) at $5x10^6$ cells/mL. For analysis, PBMCs were thawed at 37°C and suspended in pre-warmed sterile RPMI-1640 medium (Sigma-Aldrich) containing 10% heat-inactivated FBS, 50 U/mL penicillin and 50 µg/mL streptomycin, 2 mM L-alanyl-L-glutamine dipeptide (Life Technologies) and 20 µg/mL DNAse (Sigma-Aldrich). The cells were centrifuged at 500 g for 5 min and suspended at $1x10^6$ cell/mL in 200 µL of FACS buffer (PBS with 0.5% bovine serum albumin; Sigma Aldrich) containing 20% human Fc receptor binding inhibitor (eBioscience) to block non-specific antibody binding sites. Following 20 min incubation at room temperature, $1x10^5$ cells were stained with 0.5 µL anti-human CD3-PeCy7, 0.5 µL anti-human CD4-PerCP-eFluor710, 0.5 µL anti-human CD8-APC-eFluor780 (eBioscience) and 0.3 µL anti-human CD14-V500 (BD Biosciences) in a total volume of 135 µL. Another $1x10^5$cells were stained for CD36 expression using the above staining cocktail plus 2.5 µL anti-human CD36-eFluor660 (eBioscience) in a total volume of 135 µL. The cells were stained for 30 min in the dark at room temperature, washed twice at 500 g for 5 min with 3 mL FACS buffer and resuspended in 0.5 mL FACS buffer with 1 µM DAPI (Sigma-Aldrich). A minimum of 40,000 cells were acquired from each sample using an 8-colour BD FACSVerse flow cytometer (BD Biosciences).

5.2.5. *DATA ANALYSIS*

R version 2.15.2 (283) was used for statistical analyses and FlowJo version 10 was used for analysis of flow cytometry data. Treatment efficacy was determined by comparing PANSS scores before and after treatment using Wilcoxon signed-rank tests. Multiplex immunoassays with more than 30% missing values (41 out of 147; **Supplementary Table S1**) were excluded from further analysis. To account for values below the lower and upper limits of detection, these were imputed with half the minimum and 1.5 the maximum values, respectively. Association of baseline molecular levels with response to treatment (ΔPANSS) was determined using analysis of covariance (ANCOVA) after controlling for age, gender, body mass index (BMI) and baseline PANSS scores to account for any regression to the mean effects (262). ΔPANSS scores were calculated as percentages of the baseline score (T_0) after subtraction of the minimal possible PANSS score. For ANCOVA, the values from multiplex immunoassays were $\log_{10}$-transformed to approximate normality. Distribution of analyte levels was assessed using Shapiro-Wilk tests. Receiver operating characteristic (ROC) analysis was used to calculate the area under the curve (AUC), sensitivity and specificity for the identified predictor of response. Due to known association, one-tailed Spearman's rank correlation test was used to calculate the correlation between plasma H-FABP levels and monocyte CD36 expression. Wilcoxon signed-rank tests were used to identify changes in protein levels following antipsychotic treatment. Fold changes were calculated as the ratio of the mean levels after/before treatment. P values for biomarkers identified in the discovery cohort were controlled for false discovery rate (Q value) with the Benjamini-Hochberg procedure (284). $P<0.05$ was considered significant. *In silico* functional analysis was performed using the Ingenuity Pathway Analysis software (IPA; Ingenuity® Systems).

5.3. RESULTS

5.3.1. PATIENT CHARACTERISTICS AND TREATMENT EFFICACY

Patient samples in the discovery and validation cohorts were matched for age, gender and BMI (**Table 1**; BMI was not available for all samples). On average, total PANSS scores decreased in the discovery cohort from 78.5 to 60.8 and in the validation cohort from 85.3 to 55.8 after 4 to 6 weeks of treatment. There was a significant decrease in PANSS general and total scores in both cohorts for all treatment groups (**Table 2**). However, the decrease in PANSS positive scores was significant in both cohorts for patients treated with olanzapine, risperidone and the antipsychotic mixture, but not after quetiapine treatment. Furthermore, the decrease in PANSS negative scores was significant in both cohorts only after olanzapine treatment.

5.3.2. PREDICTION OF RESPONSE

Multiplexed immunoassay profiling of baseline molecular levels for the four treatment groups allowed reproducible identification of one molecule [heart-type fatty acid binding protein (H-FABP); UniProtKB ID P05413], which could predict response to olanzapine treatment. After correcting for age, gender, BMI and baseline PANSS total score, higher baseline levels of H-FABP were associated with a greater reduction in total PANSS scores in the discovery (P=0.034, β=67.8; **Figure 1a**) and validation (P=0.042, β=16.2; **Figure 1b**) cohorts. This association was

	Olanzapine		**Quetiapine**		**Risperidone**		**Mixture**	
	Disc n=23	*Val* n=18	*Disc* n=9	*Val* n=14	*Disc* n=7	*Val* n=24	*Disc* n=8	*Val* n=21
PANSS positive	0.03	<0.001	0.011	0.091	0.027	<0.001	0.017	<0.001
PANSS negative	0.024	0.003	0.035	0.345	0.225	<0.001	0.091	0.026
PANSS general	0.009	<0.001	0.035	0.001	0.045	<0.001	0.018	<0.001
PANSS total	0.005	<0.001	0.008	0.002	0.028	<0.001	0.012	<0.001

Table 2. *Effects of olanzapine, quetiapine, risperidone and mixture of atypical antipsychotics on PANSS positive, negative, general psychopathology and total scores in the discovery (Disc) and validation (Val) cohorts. The table shows P values from Wilcoxon signed-rank tests. Reduction in scores was considered significant for P<0.05. Findings which were not reproducible are shown in grey.*

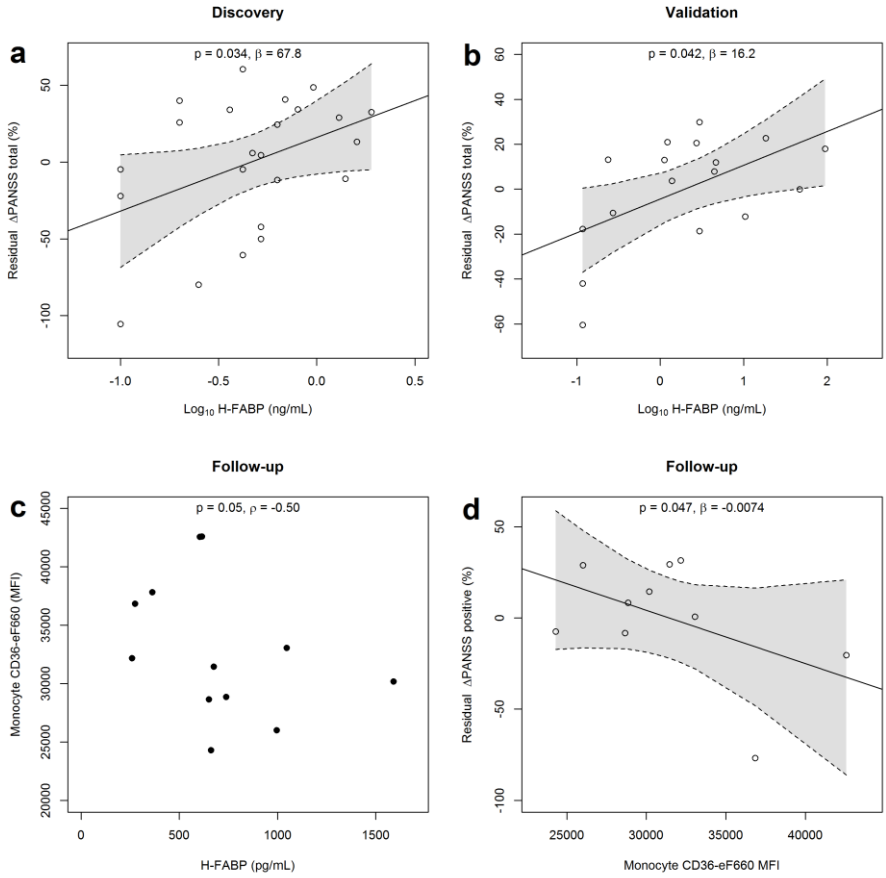


Figure 1. *Molecular predictors of response to olanzapine. Baseline H-FABP levels were associated with the reduction of total PANSS scores in the discovery (a) and in the validation (b) cohorts of patients. In a follow-up study, monocyte CD36 expression inversely correlated with plasma levels of H-FABP (c) and was predictive of improvement in psychotic symptoms (ΔPANSS positive; d). P, β and ΔPANSS values were adjusted for age, gender, BMI and baseline PANSS scores using ANCOVA models. Black line and grey area represent the fitted model and its 95% confidence intervals, respectively. β – estimate from linear regression modelling, ρ – Spearman's rank correlation coefficient.*

also significant without correcting for covariates (P=0.008, β=70.4, discovery cohort; P=0.020, β=16.3, validation cohort). The ROC-AUC for this prediction was 0.71 for the discovery cohort (sensitivity=0.69, specificity=0.60) and 0.66 for the validation cohort (sensitivity=0.90,

specificity=0.57). No other molecules showed predictive power of response using samples from any treatment group.

5.3.3. *FUNCTIONAL VALIDATION*

In an independent pilot study performed in 12 recent-onset drug-free patients treated with olanzapine (ΔPANSS data was available for 10 of them), plasma levels of H-FABP were negatively correlated with CD36 expression in monocytes, with borderline significance (P=0.05, ρ=-0.50; **Figure 1c**). CD36 (UniProtKB ID P16671) is a fatty acid translocase known to interact with H-FABP. Among the tested T helper cells, T cytotoxic cells, B cells and monocytes, CD36 molecules were predominantly expressed in the latter (**Supplementary Figure S1**). After controlling for age, gender, BMI and baseline PANSS scores, monocyte CD36 expression did not correlate with the reduction in PANSS total scores (P=0.75, β=-0.0008). However, CD36 levels did correlate with PANSS positive score reduction (P=0.047, β=-0.0074; **Figure 1d**).

5.3.4. *TREATMENT EFFECTS*

In the discovery cohort, multiplexed immunoassay profiling led to identification of 50 molecules with altered levels after treatment with olanzapine (**Supplementary Table S2**), whereas only 5, 10 and 15 analytes were changed after the quetiapine, risperidone and mixed antipsychotic treatments, respectively (**Supplementary Table S3**). The highest number of consistent changes (P<0.05, same directional change) between the discovery and validation cohorts was observed for the olanzapine treatment group. In both cohorts, the levels of 14 proteins were increased and 3 were decreased (**Table 3**). In addition, one protein [angiotensin-converting enzyme (ACE)] which met the same criteria, was identified for the quetiapine treatment and one (prolactin) for the risperidone treatment groups. The mixed antipsychotic therapy resulted in significant changes in two proteins, insulin-like growth factor binding protein 2 (IGFBP2) and prolactin. Finally, two proteins were reproducibly increased after treatment with the different medications: prolactin (after treatment with olanzapine, risperidone and antipsychotic mixture) and ACE (after treatment with olanzapine and quetiapine).

Analyte	UniProtKB ID	Discovery			Validation	
		RC	P value	Q value	RC	P value
Olanzapine						
Angiotensin-converting enzyme	P12821	1.32	0.001	0.005	1.42	<0.001
Alpha-fetoprotein	P02771	1.37	<0.001	0.002	1.66	0.008
Beta-2 microglobulin	P61769	1.28	<0.001	0.002	1.15	0.006
EN-RAGE	P80511	0.67	0.033	0.069	0.59	0.005
Factor VII	P08709	1.09	0.029	0.064	1.27	0.004
Fas (FASLG receptor)	P25445	1.24	0.001	0.004	1.25	0.001
ICAM-1	P05362	1.28	0.001	0.004	1.28	0.005
MDC	O00626	1.20	0.002	0.009	1.31	0.001
MMP-3	P08254	0.75	0.028	0.063	0.38	<0.001
Prolactin	P01236	1.52	0.015	0.037	4.04	<0.001
Prostatic acid phosphatase	P15309	1.30	0.001	0.007	1.43	0.002
Resistin	Q9HD89	0.78	0.006	0.019	0.83	0.001
Stem cell factor	P21583	1.16	0.013	0.034	1.24	0.006
Thyroid-stimulating hormone	P01215	1.28	0.031	0.067	1.83	<0.001
TNF-alpha	P01375	1.50	<0.001	0.002	2.03	0.013
TNF RII	Q92956	1.58	<0.001	<0.001	1.22	0.001
VCAM-1	P19320	1.23	<0.001	0.002	1.13	0.016
Mixture						
IGFBP2	P18065	0.74	0.035	0.338	0.79	0.035
Prolactin	P01236	4.64	0.016	0.315	2.04	0.011
Quetiapine						
Angiotensin-converting enzyme	P12821	1.28	0.024	0.694	1.28	0.002
Risperidone						
Prolactin	P01236	3.84	0.016	0.394	3.96	<0.001

Table 3. *Consistency of changes revealed by multiplexed immunoassays between discovery and validation cohorts for four different treatments. Only significant changes (P<0.05, Wilcoxon signed-rank test) with the same direction of change are shown. Q value – P value adjusted for false discovery rate; RC – ratio change.*

Pathway analysis of combined multiplexed immunoassay data from olanzapine-treated patients revealed that the most significantly altered molecular and cellular functions were cell-to-cell signalling, cellular development, cellular growth, proliferation and movement (P values 5.35E-09 – 3.64E-03; with 9 or more molecules involved in each function; **Supplementary Table S4**). The top canonical pathway affected by the olanzapine treatment was atherosclerosis signalling (P = 2.41E-07; 5 proteins involved) and the disorder that was most significant to the dataset was cardiovascular disease (P values 3.98E-08 – 3.28E-03; 9 out of 17 molecules involved).

5.4. DISCUSSION

This is the first molecular profiling study to identify and validate serum biomarkers in recent-onset schizophrenia patients associated with response prediction and molecular effects of different antipsychotic treatments. Serum was collected from 124 drug-naïve and drug-free patients following four different treatment regimes. On average, the patients showed reduction in positive, negative and cognitive symptoms, with the most significant changes observed following olanzapine treatment. Multiplexed immunoassay analysis of 147 serum analytes before and after therapy resulted in identification of H-FABP as a baseline predictor of response to olanzapine. This was validated at the functional level using samples from 10 drug-free patients with the finding that monocyte expression of the H-FABP molecular partner, CD36, predicted change in psychotic symptoms. Since H-FABP levels are age and gender-dependent (285), we adjusted the ANCOVA models for these covariates together with BMI and baseline PANSS scores to account for potential regression to the mean effects. Importantly, the levels of H-FABP were associated with response in both the discovery and validation cohorts with and without adjustment for these covariates. In line with this finding, expression of CD36 on monocytes was significantly associated with changes in positive (psychotic) symptoms by olanzapine using samples from a separate cohort. This association was significant both with and without adjusting for covariates.

H-FABP is a lipid carrier protein expressed primarily in cardiac and skeletal muscles, brain, and mammary gland (286). At concentrations >17.7 ng/ml, it has been used as a biomarker for acute coronary syndrome (287). However, across the 41 patients treated with olanzapine in the current study, we found that samples from only 3 individuals had H-FABP concentrations exceeding this threshold. Furthermore, patients with cardiovascular disease had been excluded from analysis in this study, making it unlikely that these effects were associated with such conditions. There have also been links from *post mortem* studies of other brain disorders, which show changes in H-FABP levels. Decreased levels of H-FABP have been found in brain tissues from patients with Down's syndrome and Alzheimer's disease (288). The same researchers

suggested that H-FABP may affect brain function by altering membrane fluidity and, therefore, signal transduction in neuronal cells (288). More recently, H-FABP has been shown to be highly expressed in dopaminergic neurons (289) and regulate dopamine D_2 receptor function (290) as well as synaptogenesis and myelination (291) in animal models. This is interesting considering the proposed role of elevated dopamine levels in schizophrenia (292).

CD36 (or fatty acid translocase) is a membrane protein expressed by many cell types, which binds to a variety of ligands including thrombospondin, long-chain fatty acids, oxidised low density lipoproteins and pathogen associated molecular patterns (293). It is involved in fatty acid and glucose metabolism, and has been linked to insulin resistance (294). CD36 and H-FABP are known to physically interact *in vivo* (295) and contain similar fatty acid-binding domain (296). We showed that levels of H-FABP and monocyte CD36 were inversely correlated and patients with low concentrations of H-FABP and high expression of CD36 on monocytes were less likely to respond to olanzapine. We suggest that low treatment response may be due to a deregulation of signal transduction in these patients involving fatty acid transport or metabolism (**Figure 2**). This is consistent with studies which have shown that cell membrane fatty acids can be depleted in some schizophrenia patients and higher intake of unsaturated fatty acids has been associated with less severe symptoms (297). Pre-treatment lipid levels have been also used to distinguish responders from non-responders to atypical antipsychotic treatment (298). Taken together, these findings suggest that fatty acid metabolism affects signal transduction in the brain and peripheral H-FABP and monocyte CD36 levels provide a good indicator of response to treatment and constitute potential therapeutic targets (286, 293).

Multiplexed immunoassay profiling of patients' serum revealed also molecular changes associated with different atypical antipsychotic treatments. Common effects of therapy with different atypical antipsychotics included increased prolactin and ACE levels. The highest number of consistent changes was observed after treatment with olanzapine, which resulted in changes in the levels of 17 proteins.

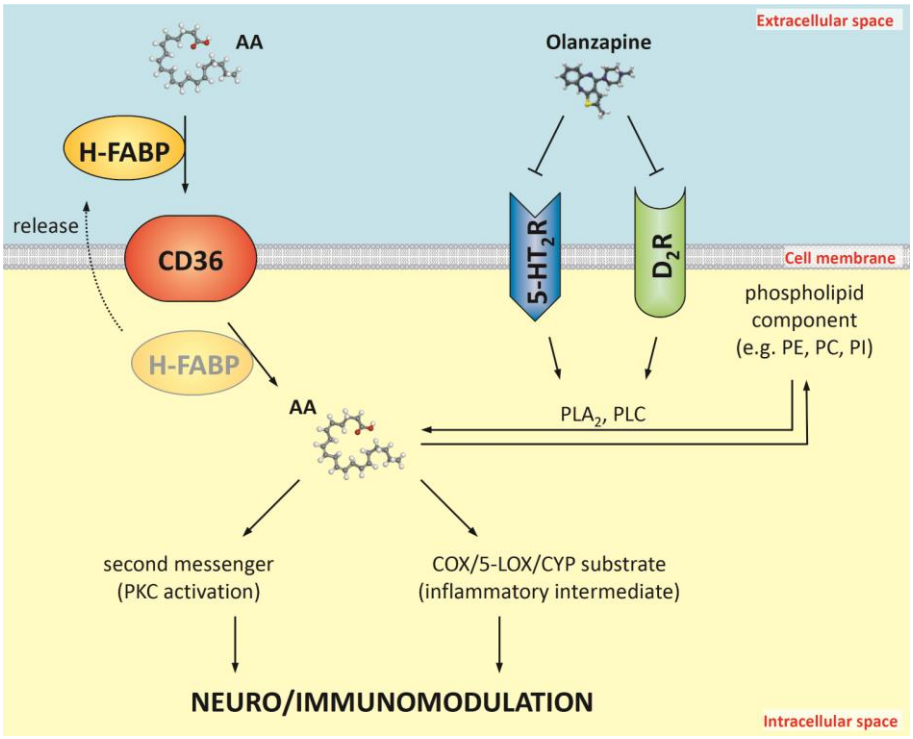


Figure 2. *Hypothetical mechanism of the interaction between fatty acid handling proteins H-FABP and CD36 and response to olanzapine. H-FABP is a critical factor for arachidonic acid (AA) transport and metabolism (299), especially in the brain (289). Also CD36 is known to mediate AA uptake inside the cell (300). Alterations in AA transport may result in altered membrane fluidity, which affects receptor function, or impaired signal transduction from dopamine D2 (D_2R) and 5-hydroxytryptamine 2 ($5\text{-}HT_2R$) receptors, for which AA acts as a second messenger (301, 302). This can diminish response to antipsychotic treatment and other processes. Abbreviations: 5-LOX – 5-lipoxygenase, COX – cyclooxygenase, CYP – cytochrome P450, PC – phosphatidylcholine, PI – phosphatidylinositol, PE – phosphatidylethanolamine, PKC – protein kinase C, PLA_2 – phospholipase A2, PLC – phospholipase C.*

Pathway analysis indicated that these proteins were most significantly involved in cell signalling, development, growth and cardiovascular system regulation. We propose that these effects may be associated with response to treatment in terms of therapeutic efficacy and side effects, and that the identified biomarkers could be used for critical decision making purposes during antipsychotic treatment of first- and early-onset schizophrenia patients.

Prolactin and ACE were altered after treatment with different atypical antipsychotics, which could be related to the clinical effects of these compounds. Prolactin levels were increased significantly following treatment with olanzapine, risperidone and mixed antipsychotic drugs. Prolactin is a peptide hormone produced by the pituitary gland and lymphocytes (303). It is involved in many functions including lactation, immune modulation and behavioural modification. It is also known to be involved in stimulation of insulin release from pancreatic β cells, which may explain why many patients treated with these compounds develop insulin resistance (304). An increase in prolactin levels following typical antipsychotic treatment is well known and has also been ascribed to the antagonistic activity of some atypical antipsychotics such as olanzapine on dopamine D_2 receptors in the anterior pituitary (305). These receptors regulate the prolactin production and release and, therefore, high levels of this hormone could be related to therapeutic outcome or metabolic side effects of antipsychotic treatment (306, 307). Our study also confirmed previous reports that treatment with quetiapine does not affect prolactin levels (57, 308).

The finding that ACE was increased after treatment with olanzapine and quetiapine is interesting as this protein normally facilitates vasoconstriction by catalysing conversion of angiotensin I to angiotensin II. Therefore, the increased levels of this protein may be involved in adverse cardiovascular side effects which are sometimes associated with antipsychotic treatments, in particular olanzapine (309, 310). In addition, the *in silico* pathway analysis identified cardiovascular disease as the top disorder associated with the molecular changes observed after treatment with olanzapine, and atherosclerosis signalling as the top canonical pathway related to those changes. Further studies should be undertaken to determine the potential use of ACE measurement as an early indicator of cardiovascular side effects associated with antipsychotic treatment.

Although the affected proteins were measured in the blood, some may also regulate function of cells within the central nervous system. For example, MMP-3 is produced by microglia and macrophages and is known to induce white matter injury in patients with vascular dementia (311) and also to cause de-myelination (312). Therefore, the decreased

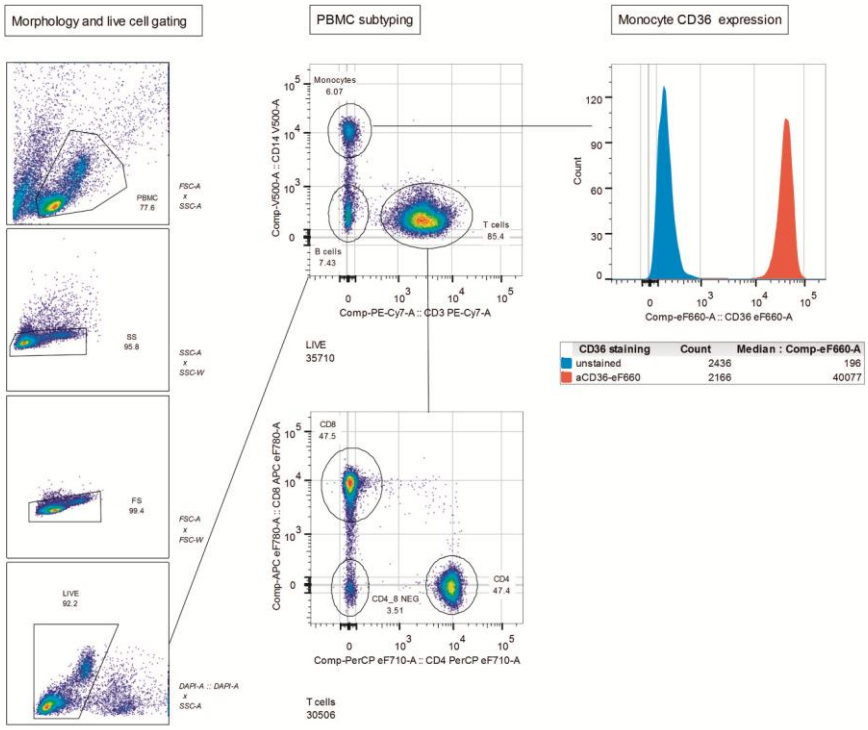
levels of MMP-3 observed following olanzapine treatment may be associated with increased myelination. This is consistent with the pro-myelination effects of some atypical antipsychotics, which have been described previously (313).

We also identified biomarkers that may be related to the metabolic side effect profile of olanzapine. This included increased levels of thyroid-stimulating hormone, which stimulates appetite and may be at least partly responsible for drug-induced weight gain (314). The olanzapine treatment also showed changes in the levels of tumour necrosis factor (TNF)-alpha and TNF RII, which have been implicated in weight gain during antipsychotic treatment (315). Additionally, treatment with a mixture of antipsychotics resulted in decreased levels of IGFBP2, which is known to have anti-diabetic effects in animal models (316). Taken together, these findings indicate that there is considerable scope for further studies in the identification of serum biomarkers which are associated with metabolic side effects of atypical antipsychotics.

There are potential limitations which should be considered in this study. The fact that olanzapine showed superior therapeutic efficacy compared to the other antipsychotics may have been affected by the limited number of patients in the other treatment groups. Patients with more severe symptoms were more likely to receive olanzapine due to its well known effectiveness in treatment of psychotic symptoms, even though this drug has been associated with substantial side effects. Thus, less severely ill patients were more likely to receive other antipsychotics, leading to fewer side effects. Therefore, the absolute change of PANSS scores for patients treated with olanzapine was likely to be higher than for the patients treated with other antipsychotics. In addition, it is conceivable that this would also lead to a greater number of changes in serum biomarkers. To reduce the impact of these effects on our analyses, we calculated change in PANSS scores in percentage and applied statistical tests to these transformed values. Also our follow-up study included only 12 patients, therefore it is important that these studies are validated in larger cohorts.

The present results constitute the first molecular profiling study to identify and validate the effects of treatment with different atypical antipsychotics in recent-onset schizophrenia patients. This revealed that fatty acid handling proteins, serum H-FABP and its binding partner, monocyte CD36, could be used as potential pre-treatment predictors of response to olanzapine. The results also showed that prolactin and ACE levels were increased in common by different treatment protocols and the highest number of consistent changes was observed after treatment with olanzapine. We suggest that these effects may be associated with response to treatment and that the identified biomarkers could be useful to guide critical decision making during selection of antipsychotic treatments of schizophrenia patients. Further molecular profiling studies, such as the one described here, can help to increase our understanding of the molecular basis of drug action as well as disease processes. Our hope is that these findings can enhance the effectiveness of schizophrenia treatment approaches by helping to identify those patients who are most likely to respond to a given drug using surrogate markers of treatment response. In this way, future interventions can be tailored to specific patient populations, thereby facilitating early intervention and reducing the prevalence of medication non-response, adverse side effects and non-compliance. The identified biomarkers could also serve as candidate drug targets to improve efficacy of current antipsychotic medications by add-on or novel treatments.

5.5. SUPPLEMENTARY INFORMATION



Supplementary Figure S1. *Workflow for the flow cytometry data analysis of CD36 expression in monocytes.*

Analyte	UniProtKB ID	Analyte	UniProtKB ID	Analyte	UniProtKB ID
1. ACE	P12821	50. G-CSF	P09919	99. MIF	P14174
2. ACTH	P01189	51. GLP-1 Total	P01275	100. MIP-1alpha	P10147
3. Adiponectin	Q15848	52. Glucagon	P01275	101. MIP-1beta	Q8NHW4
4. AgRP	O00253	53. GM-CSF	P04141	102. MMP-2	P08253
5. Alpha-1 Antitrypsin	P01009	54. GRO-alpha	P09341	103. MMP-3	P08254
6. Alpha-2 Macroglobulin	P01023	55. Growth Hormone	P01241	104. MMP-9	P14780
7. Alpha-Fetoprotein	P02771	56. GST	P08263	105. Myeloperoxidase	P05164
8. Amphiregulin	P15514	57. Haptoglobin	P00738	106. Myoglobin	P02144
9. ANG-2	O15123	58. HB-EGF	Q99075	107. NGF-beta	P01138
10. Angiotensinogen	P01019	59. HCC-4	O15467	108. NrCAM	Q92823
11. Apolipoprotein A1	P02647	60. H-FABP	P05413	109. PAI-1	P05121
12. Apolipoprotein CIII	P02656	61. HGF	P14210	110. Pancreatic Polypeptide	P01298
13. Apolipoprotein H	P02749	62. I-309	P22362	111. PAPP-A	Q13219
14. AXL	P30530	63. ICAM-1	P05362	112. PARC	P55774
15. BDNF	P23560	64. IFN-gamma	P01579	113. PDGF	P01127
16. Beta-2 Microglobulin	P61769	65. IgA	na	114. Peptide YY	P10082
17. Betacellulin	P35070	66. IgE	na	115. Progesterone	na
18. BLC	O43927	67. IGF BP-2	P18065	116. Prolactin	P01236
19. BMP-6	P22004	68. IGF-1	P05019	117. Prostate Specific Antigen, Free	P07288
20. C Reactive Protein	P02741	69. IgM	na	118. Prostatic Acid Phosphatase	P15309
21. Calcitonin	P01258	70. IL-10	P22301	119. RANTES	P13501
22. Cancer Antigen 125	Q8WXI7	71. IL-11	P20809	120. Resistin	Q9HD89
23. Cancer Antigen 19-9	Q9BXJ9	72. IL-12p40	P29460	121. S100b	P04271
24. Carcinoembryonic Antigen	P06731	73. IL-12p70	P29459	122. Secretin	P09683
25. CD40	Q6P2H9	74. IL-13	P35225	123. Serum Amyloid P	P02743
26. CD40 Ligand	P29965	75. IL-15	P40933	124. SGOT	P17174
27. CgA	P10645	76. IL-16	Q14005	125. SHBG	P04278
28. CNTF	P26441	77. IL-17	Q16552	126. SOD	P00441
29. Complement 3	P01024	78. IL-18	Q14116	127. Sortilin	Q99523
30. Cortisol	na	79. IL-1alpha	P01583	128. sRAGE	Q15109
31. Creatine Kinase-MB	P12277	80. IL-1beta	P01584	129. Stem Cell Factor	P21583
32. CTGF	P29279	81. IL-1ra	P18510	130. TECK	O15444
33. EGF	P01133	82. IL-2	P60568	131. Tenascin C	P24821
34. EGF-R	P00533	83. IL-23	Q9NPF7	132. Testosterone	na
35. ENA-78	P42830	84. IL-3	P08700	133. TGF-alpha	P01135
36. Endothelin-1	P05305	85. IL-4	P05112	134. TGF-b3	P10600
37. EN-RAGE	P80511	86. IL-5	P05113	135. Thrombopoietin	P40225
38. Eotaxin	P51671	87. IL-6	P08887	136. Thyroid Stimulating Hormone	P01215
39. Eotaxin-3	Q9Y258	88. IL-7	P13232	137. Thyroxine Binding Globulin	P05543
40. Epiregulin	O14944	89. IL-8	P10145	138. TIMP-1	P01033
41. Erythropoietin	P01588	90. Insulin	P01308	139. Tissue Factor	P13726
42. Factor VII	P08709	91. Leptin	P41159	140. TNF RII	Q92956
43. Fas	P25445	92. LH	P01215	141. TNF-alpha	P01375
44. Fas-Ligand	P48023	93. Lipoprotein (a)	P08519	142. TNF-beta	P01374
45. Ferritin	P02794	94. Lymphotactin	P47992	143. TRAIL-R3	O14798
46. FGF basic	P09038	95. MCP-1	P13500	144. TSP-1	P07996
47. FGF-4	P08620	96. MCP-3	P80098	145. VCAM-1	P19320
48. Fibrinogen	P02671	97. M-CSF	P09603	146. VEGF	P15692
49. FSH	P01225; P01215	98. MDC	O00626	147. von Willebrand Factor	P04275

Supplementary Table S1. *Analytes included in Human MAP® multiplexed immunoassay platform. Analytes excluded from the analysis due to missing values are shown in grey.*

Analyte	RC	P value	Q value	Analyte	RC	P value	Q value
ACE (Angiotensin Converting Enzyme)	1.32	0.001	0.005	IL -17	1.86	<0.001	0.002
Alpha-1 Antitrypsin	1.21	0.001	0.005	IL -18	1.26	0.006	0.019
Alpha-2 Macroglobulin	1.11	0.048	0.098	IL -7	0.81	0.003	0.010
Alpha-Fetoprotein	1.37	<0.001	0.002	MCP-1	1.17	0.022	0.051
ANG-2 Angiopoietin 2	1.84	<0.001	0.002	MDC	1.20	0.002	0.009
Angiotensinogen	1.33	0.048	0.098	MIP-1beta	1.16	0.009	0.024
Apolipoprotein H	1.22	<0.001	0.002	MMP-3	0.75	0.028	0.063
Beta-2 Microglobulin	1.28	<0.001	0.002	Myoglobin	1.42	0.001	0.004
BLC (B Lymphocyte Chemoattractant)	2.06	0.001	0.005	NrCAM	1.34	0.005	0.019
Carcinoembryonic Antigen	1.46	<0.001	0.003	PAI-1	1.11	0.010	0.027
CD40	1.20	0.007	0.021	Prolactin	1.52	0.015	0.037
Complement 3	1.10	0.023	0.054	Prostatic Acid Phosphatase	1.30	0.001	0.007
Creatine Kinase MB	1.54	0.004	0.016	Resistin	0.78	0.006	0.019
CTGF (Connective Tissue Growth Factor)	1.19	0.009	0.025	SGOT	1.18	0.008	0.024
EGF-R	1.17	<0.001	0.002	SOD	0.81	0.048	0.098
EN-RAGE	0.67	0.033	0.069	Sortilin	0.69	<0.001	<0.001
H-FABP	1.61	0.007	0.021	Stem Cell Factor	1.16	0.013	0.034
Factor VII	1.09	0.029	0.064	Thyroid Stimulating Hormone	1.28	0.031	0.067
Fas	1.24	0.001	0.004	Thyroxine Binding Globulin	1.15	0.001	0.006
G-CSF	1.47	0.0498	0.099	TIMP-1	1.11	0.007	0.021
HB-EGF	1.42	<0.001	0.002	Tissue Factor	1.62	<0.001	0.002
HCC-4	1.22	0.003	0.012	TNF-alpha	1.50	<0.001	0.002
ICAM-1	1.28	0.001	0.004	TNF RII	1.58	<0.001	<0.001
IL -10	1.17	0.009	0.025	VCAM-1	1.23	<0.001	0.002
IL -15	0.88	0.002	0.007	VEGF	1.31	<0.001	0.002

Supplementary Table S2. *Significant changes revealed by multiplexed immunoassay profiling in the discovery cohort after treatment with olanzapine (P<0.05, Wilcoxon signed-rank test). Q value – P value adjusted for false discovery rate; RC – ratio change.*

Quetiapine (n=9)				Risperidone (n=7)				Mixture (n=8)			
Analyte	RC	P value	Q value	Analyte	RC	P value	Q value	Analyte	RC	P value	Q value
ACE	1.28	0.024	0.694	AXL	1.21	0.036	0.394	Adiponectin	0.78	0.008	0.315
BDNF	1.19	0.035	0.694	EGF	0.48	0.016	0.394	Alpha-1 Antitrypsin	0.83	0.034	0.338
Carcinoembryonic Antigen	1.46	0.035	0.694	Factor VII	1.29	0.047	0.394	CTGF	0.83	0.022	0.315
Creatine Kinase MB	1.33	0.013	0.694	ICAM-1	1.13	0.034	0.394	H-FABP	0.32	0.039	0.338
von Willebrand Factor	0.75	0.044	0.694	MIF	0.74	0.031	0.394	HB-EGF	0.60	0.023	0.315
				Myeloperoxidase	0.43	0.031	0.394	HCC-4	0.83	0.042	0.339
				Prolactin	3.84	0.016	0.394	IGFBP2	0.74	0.035	0.338
				RANTES	1.52	0.022	0.394	IL-17	0.78	0.022	0.315
				Thyroid Stimulating Hormone	1.62	0.016	0.394	IL-5	1.91	0.035	0.338
				TNF-alpha	0.66	0.035	0.394	MMP-3	0.52	0.016	0.315
								Myoglobin	0.43	0.023	0.315
								Prolactin	4.64	0.016	0.315
								sRAGE	1.45	0.008	0.315
								Tenascin C	0.78	0.023	0.315
								TIMP-1	0.77	0.039	0.338

Supplementary Table S3. *Significant changes revealed by multiplexed immunoassays in the discovery cohort after treatment with quetiapine, risperidone and a mixture of antipsychotics (P<0.05, Wilcoxon signed-rank test). Q value – P value adjusted for false discovery rate; RC – ratio change.*

Top Networks	Top Bio Functions			Top Canonical Pathways
	Diseases and Disorders	Molecular and Cellular Functions	Physiological System Development and Function	
Cell Death and Survival, Tumour Morphology, Liver Necrosis/Cell Death (Score 13)	Cardiovascular Disease (P value 3.98E-08 – 3.28E-03, 9 molecules)	Cell-To-Cell Signalling and Interaction (P value 5.35E-09 – 3.64E-03, 9 molecules)	Haematological System Development and Function (P value 1.01E-08 – 3.64E-03, 11 molecules)	Atherosclerosis Signalling (P value 2.41E-07, ratio 5/131 (0.038))
Cellular Compromise, Cell-To-Cell Signalling and Interaction, Inflammatory Response (Score 2)	Hypersensitivity Response (P value 5.18E-08 – 3.64E-03, 4 molecules)	Cellular Development (P value 3.18E-08 – 3.64E-03, 9 molecules)	Tissue Morphology (P value 1.01E-08 – 3.64E-03, 7 molecules)	Hepatic Fibrosis / Hepatic Stellate Cell Activation (P value 1.89E-05, ratio 4/140 (0.029))
Cancer, Cell Death and Survival, Cellular Development (Score 2)	Inflammatory Response (P value 5.18E-08 – 3.64E-03, 9 molecules)	Cellular Growth and Proliferation (P value 3.18E-08 – 3.64E-03, 9 molecules)	Immune Cell Trafficking (P value 2.52E-08 – 3.64E-03, 7 molecules)	Allograft Rejection Signalling (P value 2.48E-05, ratio 3/59 (0.051))
Gene Expression, Cell Morphology, Haematological System Development and Function (Score 2)	Organismal Injury and Abnormalities (P value 5.18E-08 – 3.64E-03, 5 molecules)	Cellular Movement (P value 4.95E-08 - 3.64E-03, 10 molecules)	Tissue Development (P value 2.52E-08 – 3.64E-03, 9 molecules)	HMGB1 Signalling (P value 1.8E-04, ratio 3/97 (0.031))
Cellular Development, Cellular Growth and Proliferation, Haematological System Development and Function (Score 2)	Nutritional Disease (P value 1.81E-06 – 3.64E-03, 5 molecules)	Cell Death and Survival (P value 1.81E-06 – 3.64E-03, 9 molecules)	Cardiovascular System Development and Function (P value 7.59E-08 - 3.64E-03, 6 molecules)	Glucocorticoid Receptor Signalling (P value 2.21E-04, ratio 4/277 (0.014))

Supplementary Table S4. *Top networks, biological functions and canonical pathways affected by treatment with olanzapine as revealed by Ingenuity Pathway Analysis.*

CHAPTER 6
DISCUSSION

6.1. General discussion

In this thesis, we investigated molecular biomarker alterations present in the blood of schizophrenia patients and their relation to the disease pathophysiology and response to treatment. We also discussed potential utility of these biomarkers for future personalised medicine applications. We found that:

1. Serum levels of IL-1RA, IL-10 and IL-15 were increased in recent-onset antipsychotic-naïve schizophrenia patients. Treatment with antipsychotic medication decreased levels of IL-1RA and IL-10 and the decrease in IL-10 levels correlated with the improvement in symptoms.
2. Probiotic supplementation to antipsychotic medication of long-term schizophrenia patients decreased serum levels of von Willebrand factor although it was not superior to placebo in reducing symptoms.
3. Pre-treatment levels of serum H-FABP and monocyte CD36 predicted response to olanzapine treatment in recent-onset drug-free schizophrenia patients.
4. Serum ACE and prolactin levels increased after treatment with different atypical antipsychotics. The blood biomarker changes after treatment with olanzapine are related predominantly to cardiovascular disease.

Individual results summarised here were discussed in respective chapters. Here, we will evaluate the link between these results, their contribution to the current knowledge about schizophrenia and their potential implications for personalised medicine approaches and drug discovery in schizophrenia.

Mixed pro- and anti-inflammatory responses in schizophrenia

The findings presented in **Chapter 3** were the biggest study to date of cytokine alterations in recent-onset drug-naïve schizophrenia patients in terms of sample size. We analysed levels of 9 cytokines in sera from 180 patients and 350 healthy donors recruited from four different clinical centres. Among the previous studies, the biggest was carried out in 88 patients and 36 controls, and compared levels of 8 markers assigned by

their origin and function as type-1, type-2, type-17 and regulatory cytokines (265). Changes in type-1/type-2 cytokine ratios in schizophrenia have been reported before (317-319) and it has been suggested that the altered immune function may affect neuronal cell function in the brain (203). However, it is still not clear whether these effects are related solely to the disease or if they are a response to the effects of antipsychotic medication or other confounding factors such as body weight or smoking. A study by Borovcanin *et al.* (265) did not yield conclusive results since this showed an increase only in the levels of TGF-β (regulatory cytokine) in never-medicated patients. A meta-analysis by Upthegrove's *et al.* (320) published shortly after our paper, showed that levels of the pro-inflammatory cytokines IL-1β, sIL-2r, IL-6, and TNF-α were significantly elevated in neuroleptic-naïve first-episode psychosis patients, while levels of IL-2, IL-4 and IFN-γ were unaffected. However, only two cytokines, IFN-γ and TNF-α, were analysed in both studies. The finding that IFN-γ (type-1 cytokine) levels were not changed was consistent across the studies. However, Upthegrove *et al.* showed an increase in levels of TNF-α in 141 patients from 3 cohorts and we did not observe changes in the levels of this same cytokine across 180 patients from 5 cohorts. This difference may be explained by the fact that Upthegrove *et al.* analysed a heterogeneous population of drug-naïve patients and did not control for certain confounding factors which may affect cytokine levels such as age, gender and body mass index, while patients and controls in our analysis were matched for these covariates. However, most discrepancies could be explained by the fact that these studies analysed similar number of patients, but focused on different cytokines. Upthegrove *et al.* concluded that pro-inflammatory cytokine levels are increased in the serum of neuroleptic-naïve first-episode schizophrenia patients and that this "adds the evidence of a clear indication of pro-inflammatory immune deregulation in schizophrenia" (320). However, the only cytokine with direct anti-inflammatory properties analysed in this study was IL-4. Also IL-6 may have anti-inflammatory effects (321), and this was increased in patients. In turn, our study found that mostly anti-inflammatory cytokines, IL-1RA and IL-10, were increased in antipsychotic-naïve first-episode schizophrenia patients. Taken together, these findings show that both pro- (IL-1β,

sIL-2r, IL-6, IL-15) and anti-inflammatory (IL-1RA, IL-10) cytokines are increased at the onset of schizophrenia and cannot be attributed to the effects of neuroleptic drugs. These results suggest that both pro- and anti-inflammatory pathways may underpin schizophrenia pathophysiology and constitute one or more potential targets for the development of future treatments. However, indiscriminate suppression of the immune system may not be the most optimal way to treat immune imbalances in schizophrenia and approaches with broader mechanisms of action should be tested.

IL-10, H-FABP and CD36 as response biomarkers – personalised treatment implications

Treatment with atypical antipsychotics has limited effects on immune markers. In **Chapter 3**, we showed that only olanzapine and risperidone treatments decreased levels of IL-1RA and IL-10, while quetiapine and combination of antipsychotics did not alter any of the cytokine levels. It is interesting in this regard that unlike other neuroleptics, the most effective antipsychotic drug to date, clozapine, has been shown to mediate long-term immune suppression (34, 35, 186). These results suggest a link between therapeutic efficacy of antipsychotic drugs and immune function. In line with these findings, we observed that changes in IL-10 levels during treatment correlated with changes in negative, general psychopathology and total symptoms. Also, certain *IL-10* gene polymorphisms are known risk factors for schizophrenia (322) and high serum IL-10 levels have been reported in schizophrenia previously (218). This combined evidence suggests that IL-10 has a significant role in the disease process and response to treatment. IL-10 is a potent anti-inflammatory cytokine. It inhibits synthesis of pro-inflammatory cytokines, in particular those of type-1, such as IFN-γ and IL-2, and stimulates T helper 2- and B cell-mediated humoral responses. It also regulates production of pro-inflammatory prostaglandins by inhibition of cyclooxygenase-2 (COX-2) (323, 324).

Also the other treatment response markers identified in this thesis converge on COX pathways. In our study presented in **Chapter 5**, pre-treatment levels of proteins involved in fatty acid transport, serum

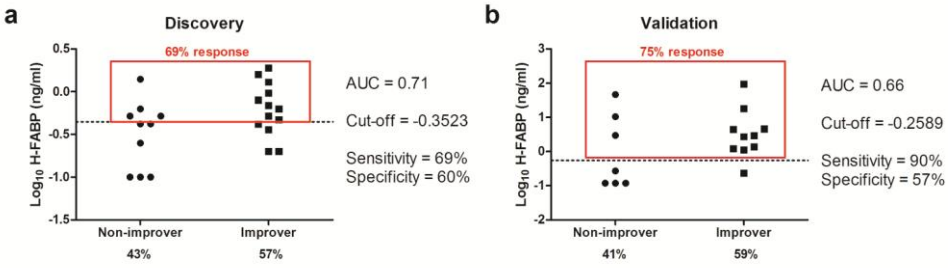


Figure 1. *H-FABP for personalised treatment approaches in schizophrenia. Receiver operating characteristic analysis of (a) the discovery and (b) validation cohorts of patients treated with olanzapine from the study described in* ***Chapter 5*** *showed that utilisation of serum H-FABP as a biomarker could increase the response rate from 57-59% to 69-75%.*

H-FABP and monocyte CD36, were associated with response to olanzapine treatment in recent-onset antipsychotic-free schizophrenia patients. Therefore, these markers could be used for identifying those patients who are less likely to respond to olanzapine and assigning them to treatment with different drugs with less severe side effects or to combination treatments with adjunctive drugs targeting these markers. This could lead to improved treatment response rates to olanzapine (**Figure 1**). These findings also indirectly implicate COX activity in response to olanzapine treatment. H-FABP is a critical factor for arachidonic acid transport and metabolism (299), especially in the brain (289). Also CD36 is known to mediate arachidonic acid transport (300). Arachidonic acid is a direct substrate for COX-1 and COX-2, which convert the former to prostaglandins and thromboxanes (325).

The two forms of cyclooxygenase differ in their tissue distribution and activity. COX-1 is constitutively expressed in all tissues of the human body (326), while COX-2 is induced by inflammatory stimuli at places of inflammation (327) and by synaptic activity (328). COX-2 is known to regulate astrocyte and microglia cell function in the central nervous system (329). It has been linked to schizophrenia through its effects on kynurenic acid, which is an antagonist at excitatory amino acid receptors (12, 330, 331). Specific COX-2 inhibition decreases kynurenic acid levels, while inhibition of COX-1 increases the concentrations of kynurenic acid and may lead to psychiatric symptoms (332). Compounds

targeting COX-2, such as the selective COX-2 inhibitor celecoxib, have already been tested for their therapeutic efficacy as add-on treatment to antipsychotic medication in schizophrenia. In the most recent quantitative meta-analysis of these studies (36), celecoxib did not show an overall significant effect on symptoms, with results from 5 individual studies varying from strong positive (190) to strong negative efficacy (333). However, this heterogeneity could be explained by the fact that the effects of COX-2 inhibition differ at distinct disease stages and show positive effects mainly at the onset of schizophrenia (189, 334), especially in the first 2 years (12). One study suggested that response to adjunctive celecoxib treatment may also depend on the initial inflammatory status since patients with lower soluble TNF-α receptor-1 concentrations responded better to supplementation with this drug (12). Also our study indirectly supports the concept of patient stratification for adjunctive treatment with drugs targeting COX by showing that patients with altered levels of markers upstream from the COX pathway differ in their response to antipsychotic treatment. Beside this relationship, the optimal dosage and duration of celecoxib supplementation still needs to be determined in order to potentiate therapeutic response and reduce the risk of serious cardiovascular side effects of this drug (335, 336). Among other drugs targeting COX, only aspirin add-on has been tested for antipsychotic efficacy. Aspirin inhibits COX-1 and modulates COX-2 activity. It has an overall significant beneficial effect on symptoms (36), with higher efficacy observed in patients with lower Th1/Th2 activity measured by evaluating the IFN-γ/IL-4 ratio (37). However, only two studies have been carried out to date investigating this drug as adjunctive treatment for schizophrenia. Therefore, further investigations are required to validate these findings. The possibility of using COX-2 inhibitors for prevention of psychosis in at-risk individuals should also be explored, as suggested previously (337, 338).

ACE as a biomarker of antipsychotic-induced cardiovascular side effects

As shown in **Chapter 5**, the most robust biomarkers altered after antipsychotic treatment were prolactin and ACE. While changes in the prolactin levels can be attributed to the antagonistic activity of antipsychotic medication at dopamine D_2 receptors in the pituitary and are linked with therapeutic efficacy (339), we showed that changes in ACE are more likely to be related to the cardiovascular side effects of neuroleptic drugs. A previous retrospective study of 90,000 schizophrenia patients and 190,000 healthy individuals showed that the risk of sudden cardiac death is increased in patients taking antipsychotic medication, especially the second-generation compounds (109). Although this study did not assess the underlying mechanisms, the authors hypothesised that this may be related to blockade of potassium channels and prolongation of cardiac repolarisation by antipsychotic drugs. However, they did not exclude the possibility that other mechanisms may be involved. Although the sample size of our study was smaller, it may help to explain the cardiovascular mechanisms of action of antipsychotic medication, in particular those of olanzapine. ACE, which was increased after treatment, is an enzyme responsible for converting angiotensin I to angiotensin II, which constricts vessels. Therefore, our results suggest that cardiovascular side effects of antipsychotic medication may be related to vasoconstriction and increased blood pressure. Caregivers are now recommended to monitor blood pressure of schizophrenia patients receiving antipsychotic drugs on a yearly basis (340). Incorporation of ACE measurements into standard laboratory blood testing in schizophrenia could help to detect adverse cardiovascular effects before their clinical manifestation and aid in early therapeutic intervention, for example with ACE inhibitors, which are now first-line treatments for hypertension in the UK (341). In this context, our finding from **Chapter 4** that probiotic supplementation decreased serum levels of von Willebrand factor may be of importance. Von Willebrand factor is an acute phase protein positively correlated with cardiovascular risk factors in schizophrenia, which does not respond to standard second-generation antipsychotic treatment (224). Therefore, our results suggest that although probiotic supplementation does not improve symptoms, it may

reduce antipsychotic-induced cardiovascular side effects. However, future studies are required to assess the exact beneficial effects of probiotic supplementation on cardiovascular risk factors in schizophrenia by careful evaluation of reporter molecules such as triglycerides, cholesterol and low-density lipoprotein.

6.2. STRENGTHS AND LIMITATIONS

The strengths of the studies presented in this thesis include the well-characterised patient populations and respective controls matched for age, gender, body mass index and other parameters that could affect biomarker levels. Patients investigated in **Chapters 3** and **5** had a disease onset within the preceding 5 years and were antipsychotic-naïve or antipsychotic–free at time of recruitment. All of our studies included follow-up measurements, which added valuable information about the longitudinal effects of antipsychotic treatment. Moreover, all findings reported in **Chapters 3** and **5** were validated in independent patient cohorts from different clinical centres. The research described in **Chapter 3** constitutes the biggest study to date investigating cytokine alterations in neuroleptic-naïve schizophrenia patients and the study outlined in **Chapter 4** was the first biomarker study to investigate the effects of probiotic supplementation in schizophrenia.

On the other hand, certain limitations should be considered. Although we used several proteomic and cytometric methods, our analyses were based predominantly on a multiplexed immunoassay profiling platform. This technology allowed us to analyse relatively large numbers of analytes in small sample volumes, but on the other hand the capacity of the platform restricted the number of investigated molecules to approximately 250. Therefore, some important analytes might have been omitted from our analyses. Also, the sensitivity was not sufficient to detect certain markers relevant for schizophrenia, such as IL-6. This also limited our conclusions, especially in **Chapter 4**, as no results of a similar study have been published to date. In the same chapter, we analysed chronically ill and medicated schizophrenia patients, which in the light of recent evidence might have affected response to immunomodulatory add-on treatments and this could explain the lack of therapeutic response to probiotic supplementation. Also, the sample size of the cohorts used to

investigate drug effects in **Chapter 3** and for the cytometric study in **Chapter 5** were limited and therefore results from these analyses should be considered as preliminary and need to be critically evaluated in follow-up investigations. Finally, our studies focused on the identification of peripheral biomarkers for schizophrenia and treatment response in an attempt to link the findings with disease symptoms and drug action mechanisms. However, we did not investigate the causal relationship between the peripheral and central nervous system alterations in schizophrenia.

6.3. FUTURE DIRECTIONS

The results presented in this thesis add to the evidence for peripheral alterations in schizophrenia and show that blood biomarkers may be a valuable source of information about the disease pathophysiology and drug response mechanisms, with the potential for personalised medicine applications in the future. However, the field of biomarker discovery for neuropsychiatric disorders is still in its infancy and further research is required for a successful translation of the candidate biomarkers into tools that can be used in psychiatric clinics. Our results encourage further studies on adjunctive immunomodulatory interventions in schizophrenia. We suggest that approaches targeted at the identified abnormalities in pro- and anti-inflammatory processes should displace indiscriminate immune system suppression in schizophrenia patients. This should be combined with the measurements of immune and metabolic markers in order to identify relevant biomarker-drug interactions as well as distinct subgroups of patients with differential response to treatment. At present, our research group participates in a biomarker study of a clinical trial targeting microglia activation in schizophrenia with adjunctive simvastatin treatment. Simvastatin is a lipid-lowering compound used in patients and individuals with hypercholesterolemia. Animal studies have shown that it may regulate microglia and astrocyte function in the brain and therefore affect glutamatergic neurotransmission (342). Further benefits of simvastatin treatment include lowering the risk for cardiovascular events, which is relevant from the perspective of the results presented in this thesis. Results of the simvastatin study are expected in 2016. We are also going to carry out further studies to

investigate the role of fatty acid metabolism in response to antipsychotic treatment. In light of our findings involving H-FABP and CD36 in response to olanzapine treatment, it would be worthwhile to measure serum arachidonic acid levels and correlate these to therapeutic response as well as to COX-1/2 activity. Finally, our preliminary results from schizophrenia patient-derived monocytes suggested that blood cells may serve as a surrogate model of the changes observed in the brain of schizophrenia patients. Therefore, these could be used for profiling the cell function in schizophrenia in order to identify patient subgroups or relevant drug targets in a personalised manner. They could also be used to screen for responses to different drugs *in vitro* in order to tailor the treatment to a specific patient. We have recently performed a proof-of-concept high-content screening study in the blood cells from schizophrenia patients and we are planning to extend this analysis to include patients with other psychiatric disorders such as major depression, bipolar disorder and autism spectrum disorder within the next year.

6.4. Conclusion

Our results indicate that mixed peripheral pro- and anti-inflammatory responses are present at the onset of schizophrenia and that cytokines such as IL-1RA, IL-10 and IL-15 may be involved in the aetiology of the disease. Second-generation antipsychotic treatment shows limited effects on immune markers, with only the anti-inflammatory cytokine IL-10 changing in parallel with symptom improvement. We showed that the metabolic markers H-FABP and CD36, known to regulate nervous and immune system functions, can predict response to olanzapine treatment. These results suggest that adjunctive immunomodulatory treatments and patient stratification approaches may help to achieve better therapeutic outcomes in schizophrenia. Furthermore, we showed that treatment with the second generation antipsychotics, olanzapine and quetiapine, increased levels of a known cardiovascular risk factor, ACE, which could be used for early diagnosis and prevention of antipsychotic-induced side effects. We also found that supplementation of beneficial probiotic microorganisms in schizophrenia patients remaining on a stable, long-term antipsychotic treatment, reduced levels of another immune and

cardiovascular marker, von Willebrand factor, although it did not reduce psychotic symptoms. Taken together, our results contribute to the framework on which current attempts to utilise peripheral immune and metabolic biomarkers in targeting specific groups of schizophrenia patients are based. Further work in this area could lead to the development of biomarker tests which could be used for better stratification of patients for personalised medicine approaches and could also lead to work on new drug targets for better treatment of patients with schizophrenia.

SUMMARY

Schizophrenia is now considered as an "umbrella" term for a group of conditions with similar manifestation of symptoms and various genetic and environmental aetiologies. Distinct molecular alterations in metabolic, immune and hormonal pathways are present in the blood of schizophrenia patients and the patterns of these changes can vary in different patients. This suggests the possibility that different subtypes of patients exist with distinct biological aetiologies and that the molecular status of the patient may be informative from a clinical and therapeutic perspective. The objective of this thesis was to study schizophrenia based on molecular signatures in the blood related to deregulation in inflammatory, metabolic and hormonal pathways as well as their relevance to the disease pathophysiology and drug response in the context of personalised medicine.

In **Chapter 2** we evaluated the evidence of altered immune function in schizophrenia and described the link between central and peripheral immune changes. We also discussed the potential role of these mechanisms for more targeted drug interventions and the development of companion diagnostics, which may lead to novel, personalised therapeutic approaches and deliver more effective treatments for schizophrenia patients.

Since previous studies on the role of the immune system at the onset of schizophrenia did not yield conclusive results due to limitations such as small sample size or dissimilarities in the clinical status of patients, in **Chapter 3** we attempted to account for such factors and carried out the biggest study to date investigating cytokine alterations in the blood of antipsychotic-naïve first-episode schizophrenia patients. Our results indicated changes in mixed pro- and anti-inflammatory responses in the blood at the onset of the disease, suggesting a role of cytokines such as IL-1RA, IL-10 and IL-15 in the aetiology of schizophrenia. We also found that serum levels of IL-10 changed following antipsychotic treatment and correlated with symptom improvement and may therefore be a potential treatment response biomarker for future studies of antipsychotic drug efficacy.

Immune changes constitute a promising target for novel treatment interventions in schizophrenia. In **Chapter 4**, we described the results of a double-blind placebo-controlled clinical trial, in which the immune changes in schizophrenia patients were targeted with adjunctive probiotic treatment. Probiotic microorganisms are known to modulate immune and brain function. Although the supplementation did not lead to improved symptoms compared with placebo, it showed a specific effect in lowering an immune and cardiovascular risk marker, von Willebrand factor, which may be of benefit to schizophrenia patients. However, further work will be needed to test this possibility.

In **Chapter 5**, we describe the first molecular profiling study to identify and validate blood biomarkers in drug-free recent-onset schizophrenia patients associated with response prediction and molecular effects of specific antipsychotic treatments. We identified robust serum and cellular biomarkers, H-FABP and CD36, that could aid in improving the treatment response rate in schizophrenia patients and help to reduce side effects of antipsychotic medication. These proteins are both components of fatty acid transport and metabolism pathways. We suggest that these markers could be used for patient stratification and as candidate drug targets for add-on or novel antipsychotic interventions. We also found that ACE may be a biomarker of antipsychotic-induced cardiovascular side effects and could be used for their early diagnosis and prevention.

Taken together, the findings described in this thesis add to the evidence for peripheral alterations in schizophrenia and suggest that distinct molecular endophenotypes in schizophrenia patients may be associated with the disease pathophysiology or response to treatment. This research contributes to de-convoluting the complexity of schizophrenia and drug response mechanisms. Potential applications of our findings in personalised medicine approaches include identification of patient subgroups more likely to respond to a particular antipsychotic drug or adjunctive treatment, and developing decision rules for continuation or termination of a selected treatment.

SAMENVATTING

Schizofrenie is een verzamelnaam voor een groep aandoeningen met een etiologie gedicteerd door genetica en omgevingsfactoren die leiden tot een overeenkomstige manifestatie van symptomen. In schizofreniepatiënten zijn op moleculair niveau aantoonbare afwijkingen gevonden in metabole, inflammatoire en hormonale netwerken. De mate van afwijking van de afzonderlijke netwerken kan per patiënt verschillend zijn, hetgeen suggereert dat er verschillende subtypes van schizofreniepatiënten bestaan met allen een uniek biologisch kenmerk. Het detecteren van een subtype, ofwel de moleculaire status van een patiënt, kan een leidraad vormen in de klinische en therapeutische diagnose.

Het doel van dit proefschrift was de bestudering van schizofrenie aan de hand van moleculaire signaturen in het bloed, verband houdende met verstoorde inflammatoire, metabole en hormonale netwerken. Tevens werd het verband van deze signaturen met de ziektemanifestaties en effecten van het medicijngebruik bestudeerd.

In **Hoofdstuk 2** wordt de bewijslast voor een afwijkende immuunfunctie in schizofrenie besproken en het verschil tussen centrale en perifere immunologische veranderingen beschreven. Daarnaast wordt de potentiële rol van deze mechanismes besproken met betrekking tot een meer gericht medicatiegebruik en de ontwikkeling van bijbehorende diagnostiek. Dit zou uiteindelijk kunnen leiden tot een nieuwe therapeutische benadering voor de behandeling en zorg van schizofreniepatiënten.

In het verleden verricht wetenschappelijk onderzoek heeft geen concrete antwoorden op de vraag over de rol van het immuunsysteem in schizofrenie geboden, veelal doorbeperkte groepsaantallen of de inclusie van patiënten met een te grote diversiteit in klinische status. Rekening houdende met deze beperkingen voerden wij in **Hoofdstuk 3** het tot op heden grootste onderzoek uit naar veranderingen in cytokines in het bloed van antipsychotica-naïeve schizofreniepatiënten die hun eerste ziekte-episode hebben ondergaan. Onze resultaten laten zien dat bij aanvang van de ziekte een combinatie van pro- en anti-

inflammatiereacties plaatsvindt in het bloed, met een rol voor cytokines zoals IL-1RA, IL-10 en IL-15. Tevens toonden we aan dat het serumniveau van IL-10 veranderde na een anti-psychotische behandeling tegelijk met een correlatie aan de symptomen. Deze bevinding maakt IL-10 een potentiële biomarker voor de behandelingrespons en kan gebruikt worden in verdere studies naar de anti-psychotische medicijnefficiëntie.

De veranderingen die optreden in het immuunsysteem gedurende het ziekteverloop zijn een goede kandidaat voor nieuwe behandelmethodes in schizofrenie. In **Hoofdstuk 4** wordt het onderzoek beschreven van een *double-blind* placebo gecontroleerde klinische studie waarin werd gekeken naar het effect van een probiotische behandeling op het immuunsysteem in schizofreniepatiënten. Probiotische micro-organismen staan bekend als modulatoren van het immuunsysteem. Ondanks dat de probiotische behandeling in het algemeen niet leidde tot een verbetering van de symptomen, was een verlaging van de von willibrandfactor, een immunologische en cardiovasculaire risicofactor, één van de belangrijkste opgetreden verandering in schizofreniepatiënten. Verder onderzoek op dit gebied is nodig om het resultaat te kunnen bevestigen.

In **Hoofdstuk 5** wordt de eerste moleculaire *profiling* studie beschreven van medicatievrije schizofreniepatiënten die recentelijk een episode hebben doorgemaakt. In deze patiënten werd gekeken naar bloedbiomarkers die de reactie op antipsychotische medicijnen kunnen verklaren en het effect hiervan op moleculair niveau. De geïdentificeerde serum en cellulaire biomarkers in deze studie zijn H-FABP en CD36. Deze biomarkers dragen bij aan het succes van een behandeling van schizofreniepatiënten en de verlaging van bijwerkingen van antipsychotische medicatie. Beide biomarkers zijn eiwitten die onderdeel zijn van het vetzuurtransport en metabool netwerk. Wij suggereren dat deze biomarkers gebruikt kunnen worden als medicatie-*target* voor een *add-on* of een nieuwe antipsychotische behandeling. Een andere geïdentificeerde biomarker is ACE die als indicatie kan dienen voor de anti psychotische geïnduceerde cardiovasculaire bijwerkingen. Deze biomarker kan gebruikt worden voor de vroege diagnose en preventie van schizofrenie.

In dit proefschrift tonen we aan dat er verschillende moleculaire endophenotypen bestaan in schizofrenie die geassocieerd kunnen worden met de ziektepathofysiologie of reactie op een behandeling. Dit onderzoek draagt bij aan de ontrafeling van de complexiteit van schizofrenie en de medicatierespons van deze patiënten. Daarnaast draagt dit onderzoek bij aan *personalised medicine* door de identificatie van patiëntsubgroepen die een verhoogde kans hebben op het slagen van een anti-psychotische medicatie behandeling of andere aanvullende behandelingen. Bovendien dragen de bevindingen bij aan de beslissing of een specifieke behandeling voor een patiënt moet worden stopgezet of gecontinueerd.

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LIST OF PUBLICATIONS

Tomasik J, Yolken RH, Bahn S and Dickerson FB (submitted) *Immunomodulatory effects of probiotic supplementation in schizophrenia patients: A randomized, placebo-controlled trial*.

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* The list of authors and titles of these publications may change.

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