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Investigating the Role of Inflammation in Schizophrenia



A thesis submitted to University of Dublin, Trinity College
for the degree of
Doctor of Philosophy

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August 2013

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Declaration and Statement of Plagiarism

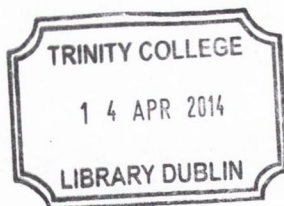
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List of Abbreviations

AA	Amino acid	FGA	First generation antipsychotics
ADHD	Attention deficit hyperactivity disorder	GABA	Gamma-aminobutyric acid
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	GFAP	Glial fibrillary acidic protein
ANOVA	Analysis of variance	GTP	Guanosine triphosphate
BBB	Blood brain barrier	GWAS	Genome-wide association studies
BMI	Body mass index	H	Histamine
BPRS	Brief Psychiatric Rating Scale	HDL	High density lipids
cAMP	Cyclic AMP or cyclic adenosine monophosphate	ICD-10	International Classification of Diseases 10
CASP1	Caspase 1	IDF	International Diabetes Federation
CD4	Cluster of differentiation 4	IFN- γ	Interferon-gamma
CNS	Central nervous system	IL	Interleukin
CNTF	Ciliary inhibitory factor	LDL	Low density lipids
CNV	Copy number variant	LIF	Leukaemia inhibitory factor
COMT	Catechol-O-Methyltransferase	LSD	Lysergic acid diethylamide
CSF	Cerebrospinal fluid	M	Muscarini
CT-1	Cardiotropin-1	MGSA	Melanoma growth stimulatory activity
D	Dopamine	MIP	Macrophage inflammatory protein
DISC	Disrupted in schizophrenia	mRNA	Messenger ribonucleic acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorders V	MS	Multiple sclerosis
DTNBP1	Dysbindin	MHC	Major histocompatibility
EDSS	Kurtzke's Expanded Disability Status Scale	MMP16	Metalloproteinase
EDTA	Ethylenediaminetetraacetic acid	NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
EGF	Epidermal growth factor	NGRN	Neugrin
EPSEs	Extra-pyramidal side effects	NMDA	N-Methyl-D-aspartate
ERK	Extracellular-signal-regulated kinase	NPN	Neuropoietin
FFA	Free fatty acid	NRG1	Neuregulin
		NSAIDs	Non-steroidal anti-inflammatory drugs

OSM	Oncostatin
PANSS	Positive and Negative Syndrome Scale
PBMC	Peripheral blood mononuclear cell
RAGE	Receptor for Advanced Glycation Endproducts
SANS	Scale for the Assessment of Negative Symptoms
SGA	Second generation antipsychotics
SNP	Single nucleotide polymorphism
S100B	S100 calcium binding protein B
TCF4	Transcription factor 4
TG	Triglycerides
Th	T helper
TLP	Toll like receptor
TNF- α	Tumour necrosis factor-alpha
VRK2	Vaccina-related kinase 2
WHO	World Health Organisation
ZNF804A	Zinc finger protein 804A
5-HT	5-hydroxytryptamine or serotonin

Scientific Abstract

Schizophrenia is a psychiatric illness that has more recently been described as a neurodevelopment disorder. The cause of this disorder is currently unknown and likely involves both genetic and environmental factors. Several studies have suggested that the genesis of this ill health is derived from aberrant neuronal function. This viewpoint, however, appears to somewhat isolate the brain from other systems, in particular overlooking the impact that altered metabolic and immune systems may have on this disorder. Moreover, in comparison to the funded research focused on studying the dysfunction of neurons in schizophrenia, very little advancements have been made to investigate the role of glia, and in particular astrocytes, in this disease. Thus, the aim of this thesis was to investigate the role of astrocytes, immune cells and adipocytes in schizophrenia (*Chapter 1*). This aim was achieved by examining a number of factors released by these cells (*Chapter 2*).

The first results chapter (*Chapter 3*) focused on investigating the levels of the neurotrophic factor S100 calcium binding B (S100B) in the serum of patients with schizophrenia and healthy controls. S100B is a well agreed upon astrocyte marker that is released by astrocytes and can be found in both the cerebrospinal fluid (CSF) and blood. In addition, S100B is also thought to be released from adipocytes, making it an interesting 'brain/metabolic' marker. Importantly, S100B has been previously shown to be altered in schizophrenia, however, many of these studies have been contradictory. In this chapter, the levels of S100B were measured in patients with schizophrenia and the impact of age, gender, medication and illness severity were analysed. The data showed that levels of serum S100B were raised in female patients with schizophrenia, but not male patients, in comparison to healthy controls. Furthermore, the levels of S100B were also found to positively correlate with body mass index (BMI). This data further supports the hypothesis that altered function and numbers of adipocytes and astrocytes may result in altered levels of S100B.

In order to further investigate the role that the immune system plays in schizophrenia, additional markers of inflammation, six cytokines, were examined in the serum of patients with schizophrenia (*Chapter 4*). Previous studies have shown that cytokines are altered in this illness, however this data has been somewhat contradictory. In this chapter, the pro-inflammatory cytokines interleukin-1 β (IL-1 β), IL-6, IL-8, IL-17, IL-23 and tumour necrosis factor-alpha (TNF- α) were investigated. The levels of these cytokines were examined individually and in combination, where all 6 cytokines were summed, to generate a 'cytokine signature'. The purpose of generating a 'cytokine signature' was in the hope of achieving two outcomes: (i) firstly, to generate a more robust and reproducible signal that would help resolve controversy in the field and (ii) secondly, to better separate patients from healthy controls with better fidelity. The data showed that the levels of pro-inflammatory cytokines were, in general, raised in patients with schizophrenia compared to control. The data also showed that the 'cytokine signature' analysis showed less scatter in the data compared to analysis of the individual cytokines. This chapter, therefore, confirmed that the immune system, by way of examining cytokine levels, is altered in patients with schizophrenia and moreover, demonstrated that the analysis of 'cytokine signatures' is an improved method over the analysis of individual markers.

The idea of using 'cytokine signatures' as an improved method to separate patients from healthy controls was further investigated in the last results chapter of this thesis (*Chapter 5*). For this purpose an autoimmune illness, multiple sclerosis (MS), was investigated. Analysis of the same cytokines (S100B, IL-1 β , IL-6, IL-8, IL-23 and TNF- α) were conducted in the serum of MS patients treated with interferon and natalizumab medications as well as in drug naïve patients and compared to healthy controls. The 'cytokine signature' showed the following results in rank order: control > drug naïve = interferon > natalizumab. Notably, natalizumab treated patients were separated from controls by almost 100% fidelity. The data further supported the idea that the analysis of cytokine signatures, when investigating immune function in disease, is likely better than the analysis of single cytokines. It was interesting to note that MS patients showed

a more obvious separation from healthy controls compared to schizophrenia patients, which showed less segregation from healthy controls. This observation is unsurprising given that an altered immune response plays a paramount role in MS. In contrast, alterations in the immune response in schizophrenia are likely subtle and part of a collection of factors that are dysfunctional in this illness.

In conclusion (*Chapter 6*), while we still do not know the exact origin of schizophrenia, it is likely that altered function within (and crosstalk between) the central nervous, immune and metabolic systems contributes to this ill health. Thus, the evidence in favour of continued investment of resources to examine neuronal dysfunction alone, is now questionable. In contrast, studies investigating the role of other cell types, such as astrocytes (*Chapter 8*), adipocytes and immune cells, in this disease are of paramount importance. Future research in this direction, may lead to the identification of novel avenues of discovery in the understanding of this disease and in the development of new therapies for this disorder.

Lay Abstract

Schizophrenia is a life-long mental illness, affecting about one in every one hundred people. We have yet to uncover exactly why some people fall ill with this illness, however, we know schizophrenia usually appears in adolescence or early adult life. People with this illness often hear voices, have false beliefs and withdraw from society and family. The discrimination and stigmatization people with schizophrenia face, in turn, leads to dependency and marginalisation and with poorer access to employment and housing opportunities.

Changes in the immune system, our body's defence against infections and germs, may play a part in why some people become ill with schizophrenia. Thus, in this study, we centered on examining if the immune system is altered in people with schizophrenia. We looked to see if there were altered levels of immune molecules, called cytokines, which regulate the function of our immune system. Interestingly, we found that the levels of these molecules are altered in patients with schizophrenia supporting the idea that the immune system plays a part in this illness.

The immune system also plays a role in diabetes, heart disease and related illness, known as the metabolic syndrome. These physical illnesses are present in a large number of people with schizophrenia and account for a significant cause of early death in this mental disorder. In this study we also examined links between metabolic illnesses, the immune system and schizophrenia. We work on this area of research as it may lead to the discovery of new medicines for this illness, and in the long term help decrease the impact of this ill health and increase longevity in this vulnerable patient group.

Aims and Hypothesis

Background

It is hypothesised that multiple factors such as hereditary and genetic influences underlie the pathophysiology of schizophrenia, furthermore, in conjunction with immune dysfunction a developmental model of neuroinflammation has been proposed (Meyer, 2013). It is thought that if a genetic predisposition is present, perinatal infection or similar insult may impair neuronal development and this is followed by a further insult to the brain in adolescence or early adulthood such as psychological stressors or trauma which manifests the symptoms of schizophrenia (Brown et al., 2009; Meyer et al., 2011). The relapsing remitting course of schizophrenia leads to neural degradation and it is thought that this period of the illness is crucial to the future prognosis. If prompt and appropriate treatment does not occur at this stage of the illness a further insult occurs in the brain leading to an accelerating disease progression (Iritani, 2013).

Aims and Hypothesis

In this study we hypothesize that the immune system plays a role in schizophrenia. In order to help determine this role in schizophrenia, we investigated levels of S100B, an astrocytic marker, and the pro-inflammatory cytokines, TNF- α , IL-1 β , IL-6, IL-8, IL-17, IL-23 in patients with schizophrenia and compared these levels to healthy controls. Furthermore we analysed this data to obtain an additive 'cytokine signature' for patients with schizophrenia, with the aim to further segregate patients from controls. We also aimed to determine if this method of cytokine signature can separate patients from controls in multiple sclerosis (MS), an illness with known immune dysfunction. In addition, we hypothesized that altered immune function in schizophrenia may be linked to immune dysfunction that occurs in the metabolic syndrome in schizophrenia. As such, we aimed to measure these metabolic parameters and correlate them to those cytokine levels obtained from participating subjects.

Value of Research

Three of the major benefits of research investigating immune function in schizophrenia are listed below:

Development of diagnostics

This research will further our knowledge regarding immune dysfunction in schizophrenia. This, and future studies, investigating cytokine levels and ‘cytokine signatures’ in schizophrenia may also help identify and develop a biomarker signature for this illness. Improvements in sensitivity and specificity of such biomarkers may help develop a diagnostic tool for this illness.

Discovery of novel drug targets

Investigating the role of the immune system in schizophrenia may also lead to the discovery of new molecular targets and a better understanding of immune pathways that could aid in the development of drug treatments for schizophrenia. Furthermore, immune biomarkers, if proven to be linked to disease state in schizophrenia, may also be useful in determining the efficacy of novel drug treatments.

Patient stratification and tailored medicine

The hypothesis that schizophrenia is an endophenotype disorder and that there are ‘subgroups’ within schizophrenia may be further determined using immune-based markers. The use of, for example cytokine signatures, may support current evidence that subgroups within the spectrum of schizophrenia have specific neurobiological and genetic architecture. This potential segregation of patients into subgroups within the umbrella of schizophrenia may have profound effects on future drug treatment choices. These efforts may help stratify patients, with the prospect of individual tailored pharmacological treatment based upon their subgroup within this illness.

Outputs

The outputs of this PhD Thesis are listed below

Papers

K. O'Connell, J. Thakore, K.K. Dev. Levels of S100B are raised in female patients with schizophrenia BMC Psychiatry (2013) 13,146 (1-9). (*Chapter 3 of Results*)

K. O'Connell, J. Thakore, K.K. Dev. Analysis of cytokine signatures: an enhanced method to demonstrate altered immune response in schizophrenia. Psychoneuroendocrinology (2013) submitted (*Chapter 4 of Results*)

Presented Talks

K. O'Connell, J. Thakore, K.K. Dev. Investigating the role of S100B and Astrocytes in Schizophrenia. Global Engagement of Graduate Education. University of Michigan (2012).

K. O'Connell, J. Thakore, K.K. Dev. Investigating Abnormal Immune Response in Schizophrenia. Lundbeck Neuroscience (2012).

K. O'Connell, J. Thakore, K.K. Dev. Abnormal Immune Response In Schizophrenia. Royal Academia of Medicine, Ireland (2011)

Presented Posters

K. O'Connell, J. Thakore, K.K. Dev. The expression of pro-inflammatory cytokines in patients with Schizophrenia. Neuroscience Ireland (2012) 7, P59

K. O'Connell, J. Thakore, K.K. Dev. The Expression of Pro-Inflammatory Cytokines In Patients With Schizophrenia. Schizophrenia International Research Society Conference (2012) 3rd Biennial SIRS Conference, 3, P260

K. O'Connell, J. Thakore, K.K. Dev. Investigation of immune response in patients diagnosed with schizophrenia. TBSI 1st TCD Postgraduate day (2012) 1, PNS3.

K. O'Connell, J. Thakore, K.K. Dev. The role of immune response in Schizophrenia. 5th School of Medicine postgraduate research day (2012) 5, PX.

K. O'Connell, J. Thakore, K.K. Dev. An investigation into inflammatory markers in patients with Schizophrenia treated with clozapine. The College of Psychiatry Ireland (2011) P41

K. O'Connell, J. Thakore, K.K. Dev. An investigation into S100B levels in patients with Schizophrenia treated with clozapine. Neuroscience Ireland (2011) 6, P1.1

Chapter 1.
Introduction

1. Introduction to Schizophrenia

1.1. The incidence and aetiology of schizophrenia

Schizophrenia is a lifelong illness, typified by perceptual abnormalities, false beliefs and neurocognitive impairment (Hegarty et al., 1994). The median incidence of schizophrenia has been observed to be 1.52%, with the rate ratio for males:females being 1.4:1. It is noteworthy that people with this illness experience a median lifetime morbid risk of 0.72%, which translates to a two- to three- fold increased risk of dying compared to the general population. This median point prevalence of schizophrenia is reported as 0.46% and does not vary by gender (McGrath 2008). Moreover, factors that influence prevalence of this illness include migrant status, economic status and geographical position (McGrath 2008).

Despite decades of research into schizophrenia the underlying aetiology remains unclear. It is now accepted that this debilitating disorder has a neurodevelopmental origin with involvement from both gene and environment effects (Stevens et al., 1997). It has been traditionally observed that a family history of schizophrenia relates to a major risk factor in acquiring this disorder. Twin and adoption studies have validated the existence of a robust genetic component in schizophrenia. Overall, the estimated heritability of risk lies at about 80% and study reviews have indicated that probandwise concordance rates in monozygotic twins are between 41-65% and in dizygotic twins between 0-28% (Sullivan et al., 2003). First-degree relatives of affected individuals are at a ten times greater relative risk of developing schizophrenia compared to the general population (Austin and Peay, 2006). If one parent has schizophrenia the lifetime risk for each child is 10-15%, if both parents have schizophrenia, the risk increases to 35-46% (Gottesman II, 1991).

Given that the relative risk of developing the disorder is quickly reduced as genetic distance from the affected individual increases, it can be concluded that combinations of genes are implicated (O'Donovan et al., 2009). However, identification of specific

molecular genetic variation has been difficult. Genetic effects in schizophrenia have proven to be complex with recent genome-wide association studies (GWAS) unable to identify one causative gene. These GWA studies have left us with the suggestion that interplay between the numerous genes involved only lowers the threshold for developing the disorder. The high heritability of schizophrenia is not solely due to genetic influences. Genes that affect schizophrenia also affect some of the heritable traits underlying schizophrenia, for example, neuro-cognitive functioning, brain volume measures, neurophysiological information processing traits and sensitivity to stress. (Boos et al., 2007; Bramon et al., 2005; Myin-Germeys et al., 2001).

1.2. Symptoms of schizophrenia

The debilitating symptoms of schizophrenia manifest in multiple domains of behaviour and can be grouped together to fall within three categories. Positive symptoms embody classic psychosis, such as sensory hallucinations, delusions or disorganized thinking. Negative symptoms are observed to be absent from the normal population and include blunted affect (lack of emotional response), alogia (lack of unprompted speech) and avolition (lack of motivation). The last group of symptoms are those of cognitive dysfunction, the severity of which may vary, but are typically characterized by impairment of attention and executive function; they also display non-specific emotional and behavioural difficulties with intellectual and language alterations, as well as subtle motor delays (Welham 2009).

Schizophrenia is a clinical syndrome with peak onset occurring in late adolescence or early adulthood. While some patients may only experience one psychotic episode, many will have the disorder lifelong and of those patients, many will follow a relapsing course over their lifetime (Robinson et al., 1999). Despite continued advances in treatments, particularly pharmacological treatments, there is little evidence to suggest that changes in diagnostic and therapeutic practices over time have altered the chronic course of this illness (Hegarty et al., 1994). The lifespan of those patients with schizophrenia is shortened by up to 25 years earlier than those within the normal population

([Kilbourne et al., 2009](#)). These patients have a two-three fold increased risk of dying prematurely. The cause for this premature mortality includes suicide and other mortality causes seen in the general population ([Saha et al., 2007](#)). However, the management of these comorbidities, while similar to the general population, is complicated in this patient group, namely due to the illness itself, access to healthcare and attitudes towards these medical comorbidities.

Global mortality risk factors such as hypertension, smoking, raised glucose, lack of physical activity, obesity and raised cholesterol ([WHO, 2009](#)) are significantly increased in those patients with schizophrenia, but there is no definitive evidence showing that these factors contribute to all-cause and cardiovascular mortality in this patient group ([Kilbourne et al., 2009](#), [Wildgust and Beary, 2010](#)). Clinical diagnosis is made using criteria specified in the Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V) and International Classification of Diseases-10 (ICD-10). Structural brain abnormalities are not sensitive enough (approximately 40-50% patients) or specific enough (seen in approximately 10% of normal controls) to be of diagnostic benefit ([Allen et al., 2009](#)). At present, there is no evidence-based biomarker to diagnose schizophrenia currently in practice and the biological entity of schizophrenia is based on patient introspection and clinical observation. There have been a number of recent studies attempting to identify blood-based biomarkers for psychiatric disorders such as schizophrenia, however, as yet none have proven to be robust.

1.3. Current treatment options in schizophrenia

The current mainstay pharmacological treatments for schizophrenia are antipsychotic medications (please see **Table 1**). However, less than 50% of patients with schizophrenia respond to an initial treatment with these medications ([Buckley et al., 2001](#), [Chakos et al., 2001](#)). Antipsychotic treatments for schizophrenia, the first being chlorpromazine, were first discovered in the 1950's and helped to uncover the dopamine hyperactivity hypothesis for schizophrenia. These medications, called first generation antipsychotics (FGAs) were efficacious, but came with the additional burden of extrapyramidal side

effects and tardive dyskinesia (Casey, 1991). To enhance compliance, FGAs were developed to be administered as long-acting depot injections, which remain a popular route of administration (Citrome et al., 2010). However, extrapyramidal side effects (EPSEs), a lack of efficacy in negative and cognitive symptoms and a population of treatment resistant patients has led to the advent of newer second-generation (atypical) antipsychotics (SGAs). These group of medications have relatively good efficacy for the positive symptoms of schizophrenia compared with older generation medications, and they do not carry the same burden of incidence of motor side effects. They do, however, tend to bring an increased incidence of metabolic side effects, such as weight gain and lipid abnormalities. These adverse effects occur in addition to the known abnormal metabolic profile seen in non-pharmacologically treated patients with schizophrenia (Ryan et al., 2003).

Clozapine, the first atypical medication discovered, remains a mainstay treatment for treatment resistant schizophrenia. It lacks the unpleasant EPSEs and motor dysfunctions of FGA and has provided a template for future antipsychotic development (Tandon et al., 2010). Clozapine has been shown to be more effective in patients where other antipsychotic medications have failed. A serious side effect of clozapine is agranulocytosis, which occurs in approximately 1-4% of patients treated with this medication and as such patients need to be continuously monitored (Atkin et al., 1996). Clozapine is only a weak inhibitor of dopamine receptors; which, in light of previous antipsychotic drug mechanisms, is an unexpected phenomenon. However, it's adverse side effects have spurred the development of additional SGAs, 12 of which are currently on the market (Tandon et al., 2010) (please see **Table 1**). Initial optimism, however, has been adjusted as more recent reviews suggest equal efficacy between first and second generation antipsychotics, with the exception of treatment resistant patients (Bonham and Abbott, 2008). As such, the overall message is that treatment should be tailored to the patient based on the pharmacological profile of the antipsychotic and the clinician's own experience.

Drug Name	Dose Range (mg/day)	Main Side Effects	Receptor Profile
Typical Antipsychotics			
Chlorpromazine	75-1000	Extrapyramidal side effects (akathisia, parkinsonism, dystonias) Tardive dyskinesia Muscle stiffness Weight gain	Antagonist: D ₁ , D ₂ , D ₃ , D ₅ , 5-HT ₁ , 5-HT ₂ , H ₁ , α ₁ -adrenergic, α ₂ -adrenergic, M ₁ , M ₂
Haloperidol	20-39		Antagonist: D ₂ , D ₃ , D ₄
Pimozide	4-20		Antagonist: D ₂ , D ₃ , α ₁ -adrenergic, 5-HT _{2A} , D ₁ , D ₄ , α ₂ -adrenergic
Trifluoperazine	5-20		Antagonist: D ₁ , D ₂
Sulpiride	200-800		Antagonist: D ₂ , D ₃
Depot Antipsychotics			
Haloperidol decanoate	50*	As for typical antipsychotics	Antagonist: D ₂ , D ₃ , D ₄
Flupenthixol decanoate	40*		Antagonist: D ₂ , D ₃ , 5-HT _{2A}
Fluphenazine decanoate	12.5*		Likely dopamine antagonist
Pipothiazine palmitate	50*	As for atypical antipsychotics	Likely dopamine antagonist
Zuclopenthixol decanoate	200*		Antagonist: D ₁ , D ₂ , α ₁ -adrenergic, 5-HT ₂
Risperidone	37.5*		Antagonist: D ₁ , D ₂ , D ₃ , D ₄ , 5-HT _{1B} , 5-HT _{2A} , 5-HT _{2C} , 5-HT ₇ , α ₁ , α ₂ , H ₁ . Partial Agonist: 5-HT _{1A}
Atypical Antipsychotics			
Amisulpride	50-800	Sedation Weight gain	Antagonist: D ₂ , D ₃ , 5-HT ₇ , α ₂
Aripiprazole	10-30	Sexual dysfunction Lipid abnormalities	Antagonist: D ₁ , 5-HT _{1B} , 5-HT _{2A} , 5-HT ₆ , α ₁ , α ₂ , H ₁ Partial Agonist: D ₂ , D ₃ , D ₄ , 5-HT _{1A} , 5-HT _{2C} , 5-HT ₇
Olanzapine	5-20	Tardive dyskinesia Akathisia	Antagonist: D ₁ , D ₂ , D ₃ , D ₄ , 5-HT _{1B} , 5-HT _{2A} , 5-HT _{2C} , 5-HT ₆ , 5-HT ₇ , α ₁ , α ₂ , M ₁ , M ₃ , H ₁ . Partial Agonist: 5-HT _{1A}
Quetiapine	150-750	(Specific to clozapine: agranulocytosis, seizures, myocarditis, constipation)	Antagonist: D ₁ , D ₂ , D ₃ , D ₄ , 5-HT _{1B} , 5-HT _{2A} , 5-HT _{2C} , 5-HT ₆ , 5-HT ₇ , α ₁ , α ₂ , M ₁ , M ₃ , H ₁ . Partial Agonist: 5-HT _{1A}
Risperidone	4-16		Antagonist: D ₁ , D ₂ , D ₃ , D ₄ , 5-HT _{1B} , 5-HT _{2A} , 5-HT _{2C} , 5-HT ₇ , α ₁ , α ₂ , H ₁ . Partial Agonist: 5-HT _{1A}
Sertindole	12-24		Antagonist: D ₂ , D ₃ , D ₄ , 5-HT _{1B} , 5-HT _{2A} , 5-HT _{2C} , 5-HT ₆ , 5-HT ₇ , α ₁ , α ₂ , H ₁ . Partial Agonist: 5-HT _{1A}
Clozapine	12.5-900		Antagonist: D ₁ , D ₂ , D ₃ , D ₄ , 5-HT _{1B} , 5-HT _{2A} , 5-HT ₆ , 5-HT ₇ , α ₁ , α ₂ , M ₁ , M ₃ , H ₁ . Partial Agonist: 5-HT _{1A} Inverse Agonist: 5-HT _{2C}

Table 1: Major compounds used for management of schizophrenia. The table shows major drugs used as therapies for schizophrenia. *Usual 2-week equivalent dose (mg).

Ref.<http://www.rcpsych.ac.uk/expertadvice/treatmentswellbeing/antipsychoticmedication.aspx>
Royal College of Psychiatrists, UK.

There are currently no successful pharmacological treatments that have been repeatedly shown to be effective in the treatment of negative symptoms in patients with schizophrenia (Stahl et al., 2007). Negative symptoms include decreased motivation, which can have a negative effect with regard to treatment adherence, sustaining employment and social relations. Social and psychological supports are important in improving functional outcomes in individuals with schizophrenia. The remission criteria (proposed by Andreasen, 2005) focuses on seven characteristic signs and symptoms associated with schizophrenia and the corresponding item as validated by the Positive and Negative Syndrome Scale (PANSS) and the Scale for the Assessment of Negative Symptoms (SANS) (Andreasen et al., 2005). A large meta-analysis has suggested that a substantial proportion (approx. 27%) of patients can achieve remission within a four week treatment period (Beitinger et al., 2008). Outcome studies of those with a first episode of psychosis have shown that heterogeneity is common, with 42% having a good long-term outcome, 35% an intermediate outcome and 27% having a poor outcome (Menezes et al., 2006).

2. Genetics of Schizophrenia

2.1. Genes associated with schizophrenia

While many genes have been implicated in schizophrenia (please see **Table 2**), there has been no definitive evidence linking any particular gene to the development of this disorder. However, various dysregulated genes have been found in this illness and they can be classified into (i) those that primarily affect the synapse, (ii) those with neuronal/glial functions and (iii) those with metabolic functions (Lin et al., 2012). Moreover, the proteins synthesized by most of the strongest candidate genes play a role in dopamine or glutamate signalling, two of the signalling systems implicated in schizophrenia. Other candidate genes include growth factors indicative in nerve growth and development. For example, (i) Neuregulin (NRG1) and Dystrobrevin-binding Protein 1 (DTNBP1, dysbindin) that is widely distributed in brain tissue and plays a role in biogenesis (ii) Catechol-O-Methyltransferase (COMT), which aids in the degradation of

neurotransmitters, and (iii) DISC-1 which is proposed to be “disrupted in schizophrenia” leading to abnormal neurodevelopment and intracellular signalling pathways within the brain (Buonanno et al., 2010; Hikida 2012). Furthermore, genome-wide association studies (GWAS) have identified both common and rare variants showing consistent association with schizophrenia compared to traditional genetic epidemiological research (Corvin et al 2010). Single nucleotide polymorphisms (SNPs) are observed in at least 1% of the general population, and are known to convey small effects which taken together give rise to an odds ratio of approximately 1.1 (Rethelyi 2013). Taking into account this small effect, it is deemed that resultant disease is due to a multiple, additive effect.

Results emerging from these GWAS further implicate a role for neurodevelopment, neuroplasticity and immune dysfunction in schizophrenia (Zhao et al., 2013; Jia et al., 2010; Valiente et al., 2011). Of particular interest, are studies which have shown extended Major Histocompatibility Complex (MHC) on chromosome 6, due to MHC’s role in neuronal development, synaptic plasticity and immune response (Stefansson et al., 2009). Other SNPs in genes also of interest include those encoding for microRNA 137 (MIR137) (Kwon et al., 2013, Ripke et al., 2011), transcription factor 4 (TCF4) (Stefansson et al., 2009; Steinberg et al., 2011), Neurogranin (NGRN) which plays a role in neuronal differentiation, CUB and Sushi multiple domains (CSMD1), Matriz metalloproteinase (MMP16) (Ripke 2011), Zinc finger protein 804A (ZNF804A), Vaccinia-related kinase 2 (VRK2) (Steinberg et al., 2011; O’Donovan et al., 2008) and multiple genes on chromosome 10 (Ripke et al., 2011). Rare variants (copy number variants, CNVs) have also been identified in schizophrenia and these microdeletions or duplications form polymorphisms in less than 1% of patients.

Gene Name	Function of Protein	Association
Neuregulin (NRG1)	Glutamate receptor function Synaptic function	+++
Dysbidin (DTNBP1)	Synaptic function	+++
Catechol-O-Methytransferase (COMT)	Degradation of dopamine, adrenaline, noradrenaline	+++
DISC-1	Development Intracellular signalling	+++
Regulator of G protein signalling 4 (RGS4)	Regulates G protein signalling	+
Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform (PPP3CC)	Phosphorylation	+
RAC-alpha serine/threonine-protein kinase (AKT1)	Development of nervous system	+

Table 2: Major genes associated with schizophrenia. The table shows genes primarily associated with schizophrenia. A number of other genes have been suggested but tend not to have been verified or well accepted and are often from single studies. +++, well accepted, ++, newly accepted, + not yet clear

2.2. Gene-environment interactions

Despite advances in identifying the genes that play a role in schizophrenia, these variants only explain a small proportion of the overall phenotypic variance observed in this illness. It is also thought that genes moderate environmental effects coining the term gene-environment interaction (van Os et al., 2008). These epigenetic factors that are susceptible to environmental influences may also affect heritability estimates giving rise to an increased risk of illness or symptomatology. Established environmental factors include prenatal infection, maternal stress, malnutrition, obstetric complications, urbanicity, cannabis and trauma; these factors may play a role in the development of, at least some cases, of schizophrenia (Brown et al., 2006; Cannon et al., 2002).

An association has also been established between minority group position for both first and second-generation migrants and the incidence of psychotic syndrome, including schizophrenia (Cantor-Graae et al., 2005). Furthermore, studies have demonstrated a dose-response association between urban environment and an increased incidence of schizophrenia (March et al., 2008; Krabbendam and van Os, 2005). It has steadily been reported that cannabis use can cause an exaggerated psychotic syndrome response in individuals with a genetic predisposition for psychosis (D'Souza et al., 2005). Moreover, although inconsistent, a number of studies have suggested that those patients with psychosis, with no history of previous cannabis use, have a predicted future use of cannabis, suggesting a bi-directional association between cannabis and psychosis (Ferdinand et al., 2005).

3. Structural & Network Abnormalities in Schizophrenia

3.1. Structural abnormalities

Schizophrenia has been extensively studied over the past century, providing a rudimentary understanding of the underlying genetics, mechanism of action and structural abnormalities associated with this illness. Serendipitous discovery has led to the treatment of this disorder and to several hypotheses concerning the origins of its

symptoms. Schizophrenia is associated with various structural abnormalities, in particular, an overall reduction in whole brain volume, with a decrease in grey matter, alteration of white matter tracts and an increase in ventricular volume (Keshavan et al., 2008, Vita et al., 2006). Key regional abnormalities include, a decrease in cortical thickness and altered cortical cytoarchitecture (possibly cell pruning) with bilateral reductions in the size of the hippocampus, left thalamus, temporal lobes, and right nucleus accumbens (Buckley et al., 2005; Rimol et al., 2010; Harrison, 1999; Harrison and Weinberger, 2005; Hof and Schmitz, 2009; Iritani, 2007). With support accumulating for these identified structural abnormalities in schizophrenia, uncertainty over the underlying cause of these abnormalities persists, with evidence for both neurodevelopmental and neurodegenerative processes (Buckley, 2005).

Approximately 80% of those diagnosed with schizophrenia show enlarged lateral ventricles (Shenton et al., 2001), however, the use of structural abnormalities as a diagnostic marker appears limited as many changes are subtle and estimates average at about a 3% overall reduction in volume (Steen et al., 2006). Additionally, attempts at providing a diagnosis based on structural abnormalities alone are also impeded as other psychiatric disorders, such as bipolar disorder, exhibit an overlap in similar abnormalities (Nieuwenhuis et al., 2012). Neurocognition studies, based on patients diagnosed with schizophrenia, have found that increased left ventricular volume and bilateral putamen reductions correlate with decreased motor speed and executive functioning respectively (Hartberg et al., 2011). Furthermore, abnormalities in white matter tracts, particularly the splenium (located at the posterior end of the corpus callosum) and posterior cingulum, have been linked with deficits in working memory (Sugranyes et al., 2012).

3.2. Synaptic and Network dysfunction

It is deemed likely that schizophrenia is a disorder of neurodevelopmental origin and that normal brain development and function is impaired long before the first symptoms of psychosis. In infancy there are about 150% the number of synapses compared to

that observed in adulthood, with a progressive decline in synaptic density between the ages of 2-16 (Huttenlocher 1979). The concept of abnormal synaptic pruning contributing to schizophrenia was proposed by Feinberg in 1982 and this hypothesis was further elaborated by Hoffman and Dobscha in 1989 who proposed that it was hyperpruning of collateral axons in the prefrontal cortex that lead to the clinical manifestation of schizophrenia. To date these hypothesis have yet to be disproven and there is some supporting evidence that abnormal synaptic evidence plays a role in the aetiology of schizophrenia. Studies have revealed a decrease in dendritic spine density in the dorsolateral prefrontal cortex layer 3 pyramidal cells in people diagnosed with schizophrenia (Glantz and Lewis, 2000). Moreover, these striatal spines are decreased in size by about 30% compared to non diseased individuals (Roberts et al, 1996). It has also been demonstrated that deficient pre-synaptic mRNA or protein levels are unable to distinguish whether the mechanism is due to reduced number or reduced expression in the pre-synaptic terminal. Eitherway, it is likely that this phenomenon has a notable effect on brain function.

4. Aberrant Neurotransmitter Signalling in Schizophrenia

4.1. Altered dopamine regulation

There are many strands of evidence to suggest that the dopaminergic system plays a role in the aetiology of schizophrenia. For example, all effective antipsychotics used in the treatment of schizophrenia act partly, or wholly, on the dopaminergic system, namely D₂ receptors (Rao et al., 2013). In addition, stimulants, such as amphetamines, which act by releasing dopamine from the presynaptic terminal, give rise to symptoms akin to those positive symptoms observed in schizophrenia (Rabe-Jabłońska et al., 2012). Moreover, neuroimaging studies have demonstrated an increase in resting-state synaptic dopamine concentration, synthesis, release and a raised number of dopaimne receptors in patients with schizophrenia during episodes of psychosis (Hietala et al., 1995; Abi-Dargham et al, 1998; Laruelle 1998). These findings are especially relevant as many current antipsychotic medications used in the treatment of schizophrenia block

dopamine receptors. However, it remains unclear how an excess of dopamine in the brain leads to the clinical symptoms of schizophrenia, such as delusions and hallucinations. Further imaging studies, using functional MRI, have demonstrated abnormal network response in patients with schizophrenia, showing hyperactivity and hypoactivity within specific brain regions (Callicott et al., 2000). Moreover, event-related potential studies have also shown decreased brain response in this patient group, suppressing brain activation in response to repeated stimuli (Patterson et al., 2008). Therefore, taken together, a combination of altered dopamine regulation and over inclusive attention to external stimuli in addition to cognitive scheme dysfunction, may lead to altered attribution and the development of psychotic symptoms (Kapur and Mamo, 2003).

As mentioned, the dopamine hypothesis owes its serendipitous discovery to findings that chlorpromazine attenuated the positive symptoms of schizophrenia (Kapur and Mamo, 2003). An increase in dopamine transmission between the substantia nigra and the caudate nucleus-putamen (neostriatum) has been noted; while in the mesolimbic forebrain and the tubero-infundibular system, dopamine transmission is decreased. Furthermore, in addition to the finding that dopamine receptor antagonists could reduce psychosis and that dopamine agonists, such as DOPA and amphetamines, produced psychosis-like symptoms, it has been concluded that dopamine hyperactivity was central to the pathophysiology of schizophrenia (Carlsson, 1977). In addition, the mechanism of action of most antipsychotic drugs hinges on their ability to perform as dopamine D₂ receptor antagonists (Tandon 2010). This hypothesis is further supported by studies demonstrating increased D₂ receptor densities and decreased dopamine reuptake transporter in patients with schizophrenia compared to the normal population (Zakzanis and Hansen, 1998; Rao et al., 2011). Indeed, over time, the dopamine hypothesis has evolved from that of hyperdopaminergia to specifically subcortical hyperdopaminergia with prefrontal hypodopaminergia, and more recently to a more comprehensive hypothesis including epigenetic factors leading to increased presynaptic striatal dopaminergic function (Howes and Kapur, 2009). However, in light of the fact

that antipsychotics have conclusively only attempted to address one category of symptoms, the dopamine hypothesis is merely one piece of a much larger puzzle.

Dopamine is a catecholaminergic neurotransmitter and a metabolite of the amino acid tyrosine. It exerts its actions on neuronal circuitry by slow modulation of the fast neurotransmission that is facilitated by glutamate and GABA. Dopamine innervations are the most prominent in the human brain and four major dopaminergic pathways have been identified, the nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular systems. These neurons are collectively involved in a number of human functions such as voluntary movement, feeding, affect, reward, sleep, attention, working memory and learning (Schultz, 2007). Abnormal dopaminergic signalling has also been implicated in other psychiatric disorders such as ADHD, Tourette's syndrome, bipolar disorder, depression, dyskinesias and various somatic disorders (Shen et al., 2012).

When it is released from the presynaptic terminal, dopamine activates G protein-coupled dopamine receptors, named D₁ to D₅ (please see **Table 3**) (Neve et al., 2004). An extensive list of pharmacological compounds have been developed for the treatment of various disorders to target these dopamine receptors, altering their function. It has been shown that D₁ and D₅ dopamine receptors activate the G α_s family of G proteins postsynaptically, which in turn stimulate cAMP production by adenylyl cyclase (Neve et al., 2004). In contrast, the D₂ and D₃ dopamine receptors are expressed both pre and post synaptically on dopamine target cells or neurons. The D₂, D₃ and D₄ dopamine receptors couple to the G $\alpha_{i/o}$ family of G proteins, inhibiting adenylyl cyclase (Sokoloff et al., 2006; Rondou et al., 2010; Rankin and Sibley, 2010). In the brain, D₁ receptors are expressed in high numbers in the nigrostriatal, mesolimbic and mesocortical areas; with D₂ receptors found in particularly high levels within the striatum, the nucleus accumbens, and the olfactory tubercle (Beaulieu and Gainetdinov, 2011).

Receptor Subtype	Locus	G-protein coupling	Signalling
D1	5q35.1	G _s	Increase cAMP and active PKA
D2	11q23	G _{i/o}	Decrease cAMP and active PKA
D3	3q13.3	G _{i/o}	Decrease cAMP and active PKA
D4	11p15.5	G _{i/o}	Decrease cAMP and active PKA
D5	4p16.1	G _{s/q}	Increase cAMP and active PKA

Table 3: Dopamine Receptor Subtypes. Adapted from Shin Hisahara and Shin Shimohama Dopamine Receptors and Parkinson's Disease International Journal of Medicinal Chemistry.

It has been reported that patients with schizophrenia have an enhanced sensitivity of postsynaptic D₂ dopamine receptors (Seeman, 2013). This idea is supported by evidence suggesting that patients with schizophrenia are supersensitive to dopamine-related psychostimulants and that the brains of these patients show an increase in the proportion of high-affinity D₂ dopamine receptors (Seeman, 2013). In addition, in vivo studies have shown no consistent changes in D₁ dopamine receptor binding but a number of studies have shown a higher level of D₂ dopamine receptor density in the basal ganglia of patients with schizophrenia compared to healthy controls (Frankle and Laruelle, 2002; Nikolaus et al., 2009b). Variants in the D₂, D₃ and D₄ dopamine receptor genes have also been linked to schizophrenia and to the response of pharmacological treatment in patients with schizophrenia (Parsons et al., 2007; Bertram, 2008; Rondou et al., 2010).

4.2. Altered serotonin pathway

In schizophrenia, the upregulation of the two key serotonergic pathways, projections from the dorsal raphe nuclei into the substantia nigra and the projections from the rostral raphe nuclei ascending into the cerebral cortex, limbic regions and basal ganglia, leads to hypofunction of the dopaminergic system (Kapur and Remington, 1996). This effect may be responsible for the negative symptoms of schizophrenia (Bleich et al., 1988). However, the serotonergic nuclei in the brainstem that give rise to descending serotonergic axons remain unaffected (Joyce, 1993). Serotonin has been implicated in a variety of behaviours and somatic functions, many of which are disturbed in schizophrenia, such as cognition, perception, attention, sleep, endocrine function, energy levels, appetite, sexual drive, aggression and mood (Murphy et al., 1998).

The serotonin receptors (5-hydroxytryptamine receptors or 5-HT receptors) are a group of receptors found in both the central and peripheral nervous systems. All serotonin receptors are GPCR except 5-HT₃, which is ligand-gated ion channel, and they mediate both excitatory and inhibitory neurotransmission. Serotonin receptors can modulate the release of a number of neurotransmitters such as glutamate, GABA, dopamine,

adrenaline/noradrenaline and acetylcholine as well as many hormones (Kroeze et al., 2002). The serotonin receptors are subdivided into seven subtypes, 5-HT₁ to 5-HT₇ (please see **Table 4**). Within these general classes of serotonin receptors lie a number of subtypes isoforms, for example 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}. Studies have shown that alteration of serotonergic neurotransmission using antipsychotic agents which are 5-HT_{2A} receptor antagonists can affect the psychopathology and cognitive dysfunction in schizophrenia (Meltzer, 1999). In addition, a number of direct and indirect 5-HT agonists (e.g. tryptophan, fenfluramine) may exacerbate the symptoms of schizophrenia (Dean, 2001). Moreover, stimulation of 5-HT_{2A}, or possibly 5-HT_{2C}, receptor is the basis for the hallucinogenic effect of indoleamines, such as LSD or psilocybin (Aghajanian and Marek, 2000). Furthermore, clozapine is a potent antagonist at both 5-HT_{2A} and 5-HT_{2C} receptors (Meltzer, 1989).

The “serotonin-dopamine” hypothesis of schizophrenia suggests an enhanced dopaminergic and serotonergic neurotransmission in subcortical areas of the brain (giving rise to positive symptoms of schizophrenia) and a decrease in dopaminergic and serotonergic activity in the prefrontal cortex (leading to negative symptoms of schizophrenia) (Kapur and Remington, 1996). This hypothesis has stemmed from clinical observations regarding the effectiveness of the antipsychotics such as clozapine which act as antagonists of both 5-HT_{2A} and D₂ receptors (Meltzer, 1989). An increased activity of mesolimbic dopaminergic neurons is essential in the aetiology of positive symptoms of schizophrenia with deficits in dopaminergic activity in the mesocortical and nigro-striatal systems proposed to be related to negative symptoms and extra-pyramidal symptoms (Kapur and Remington, 1996). There is also evidence to suggest that serotonin modulates neuronal dopaminergic activity, occurring at the level of cell bodies in the ventral tegmentum, substantia nigra and medial and dorsal raphe (Joyce, 1993). In the mesolimbic system, tonic release of dopamine is reliant upon stimulation of 5-HT receptors, therefore, blockade by 5-HT antagonists may contribute to their ability to possibly decrease negative symptoms.

Receptor Subtype	G protein coupling	Signalling	Activation potential
5-HT ₁	G _{i/o}	Decrease cAMP	Inhibitory
5-HT ₂	G _q	Increase IP ₃ and DAG	Excitatory
5-HT ₃	Ligand-gated	Na ⁺ and K ⁺ channel	Excitatory
5-HT ₄	G _s	Increase cAMP	Excitatory
5-HT ₅	G _{i/o}	Decrease cAMP	Inhibitory
5-HT ₆	G _s	Increase cAMP	Excitatory
5-HT ₇	G _s	Increase cAMP	Excitatory

Table 4: Serotonin Receptor Subtypes. The seven subtypes of 5HT receptors are shown. All are GPCRs except the ion channel 5-HT3.

In the dorsal raphe neurons, serotonin may also regulate dopaminergic neurons. Of interest, the 5-HT_{1A} receptor on astrocytes and glial cells regulates the release of the neurotrophic factor S100B, which plays a role in regulating the development of astrocytes and cortical neurons (Azmitia, 2002). If 5-HT_{1A} is decreased in the neurodevelopment setting this would have a downstream effect on cortical development, leading to altered development of cortex wiring. In light of the heterogeneity of schizophrenia it is likely that no single type of abnormality of the serotonergic or other neurotransmitter system is likely to emerge as characteristic for all patients (Meltzer and Fatemi, 1996).

4.3. Altered glutamate neurotransmission

The prominent glutamatergic pathways in the brain are usually the cortico-cortical pathways, the pathways between the thalamus and the cortex, and the extrapyramidal pathway (the projections between the cortex and striatum). Other glutamate projections exist between the cortex, substantia nigra, subthalamic nucleus and pallidum. The glutamatergic pathways are hypoactive in the brains of those diagnosed with schizophrenia and this is thought to cause the confusion and psychosis associated with the disorder (Goff and Coyle, 2001). Glutamate receptors are subdivided into ionotropic and metabotropic. Ionotropic glutamate receptors comprise of AMPA, Kainate and NMDA receptors, whereas metabotropic glutamate receptors comprise of subtypes mGluR1-8 (please see Table 5).

The NMDA receptor is a specific type of ionotropic glutamate receptor and is central to the control of memory and synaptic plasticity (Martin et al., 2000). Activation of these receptors results in the opening of a nonselective cation ion channel which allows the flow of Na⁺ and lesser amounts of Ca²⁺ into the cell and K⁺ out of the cell in a voltage dependent manner. The NMDA receptor is both ligand gated and voltage dependent and it requires activation by two ligands: glutamate and either D-serine or glycine. The receptor forms a heterotetramer between two NR1 subunits and two localised NR2 subunits. Due to a lack of specific subunit drugs it has been difficult to determine the

role of the different subunits in the pathophysiology of schizophrenia (Goff and Coyle, 2001). Agonists of the NMDA receptor bind glutamate and D-serine or glycine to the cell membrane; depolarisation of the cell membrane also makes it more likely that the ion channel will open.

Hypofunctioning of glutamate receptors have been reported as an influencing factor in the development of schizophrenia with a relative deficit of presynaptic glutamate receptors (Mechri et al., 2001). From a neurochemical perspective, NMDA antagonists such as phencyclidine (PCP) or ketamine reproduce the symptomatic, neurocognitive and neurochemical aspects of schizophrenia, which implies that regardless of the underlying aetiology of schizophrenia NMDA dysfunction gives rise to a final common pathway for this disorder (Kornhuber et al., 1989). Proposed glutamatergic models of schizophrenia can account for both the positive and negative symptoms of schizophrenia. Studies investigating the effects of ketamine (NMDA receptor antagonist) have shown an increase in both positive and negative symptoms as well as cognitive changes, mirroring that of schizophrenia (Lorrain et al., 2003). Moreover, when patients with a diagnosis of schizophrenia are given ketamine they show an increase in both positive and negative symptoms (Krystal et al., 1994). Also of interest are findings which show that amphetamine may reverse ketamine-induced working memory disturbance, suggesting that augmentation of frontal dopaminergic systems may be beneficial in schizophrenia (Krystal et al., 2005). Furthermore, previous studies have suggested that amphetamine treatment may indeed improve cognitive impairment (Barch et al., 2005).

Neurocognitive deficits are seen with NMDA antagonists although these deficits differ from those seen in bilateral hippocampal damage (Parwani et al., 2005). Whereas dopamine receptors are circumscribed with relatively few innervations of the primary sensory cortex, NMDA receptors in contrast are distributed throughout the cortex (Conti, 1997). In light of this, studies have concentrated on whether information processing deficits occur in higher cortical regions (pre-frontal cortex) or throughout the brain (involving primary sensory regions).

Name	Subunits/Subtype	Signalling	Activation potential
Ionotropic			
AMPA	GluR1-4	Na+, (Ca2+)	Excitatory
Kainate	GluR5-7, KA1-2	Na+, (Ca2+)	Excitatory
NMDA	NR1 (a-g); NR2 (A-D), NR3	Na+, Ca2+	Excitatory
Metabotropic			
Group 1	mGlu1 (a-d), mGlu5 (a,b)	Increase PLC, IP3	Excitatory
Group 2	mGlu2, mGlu3	Increase AC, cAMP	Excitatory
Group 3	mGlu4 (a,b), mGlu6, mGlu7 (a,b), mGlu8 (a,b)	Decrease AC, cAMP	Inhibitory

Table 5: Glutamate Receptor Subtypes. The ionotropic and metabotropic subdivisions of glutamate receptors are shown.

This concludes that impaired dopaminergic regulation and GABAergic neurotransmission observed in schizophrenia may be accounted for by NMDA dysfunction (Stone et al., 2007). New potential molecular contributors to NMDA dysfunction are being developed and studied and these drugs may offer some treatment hope for the negative symptoms of schizophrenia (Mullasseril et al., 2010).

5. Metabolic Syndrome and Schizophrenia

5.1. The basis of metabolic syndrome

The International Diabetes Foundation (IDF) definition of metabolic syndrome states that to have metabolic syndrome one must have central adiposity defined and two or more of the following four factors: elevated concentrations of triglycerides, elevated blood pressure, decreased concentrations of HDL cholesterol, and dysglycemia (IDF 2005). Based on this definition, the prevalence of metabolic syndrome in the adult population in the United States was 39% +/- 1.1% (Ford et al., 2005). It is associated with numerous clinical morbidities aside from cardiovascular disease and type 2 diabetes mellitus, such as chronic low grade inflammation, oxidative stress, hyperuricemia, hypertension, dyslipidemia, hyperandrogenism and polycystic ovary syndrome, hepatic steatosis and nonalcoholic fatty liver disease, impaired glucose tolerance, obstructive sleep apnoea, hypergonadism, vascular dementia and Alzheimer's disease and some forms of cancer (Cornier et al., 2008). Our current understanding of the metabolic syndrome implies the role of insulin resistance in clinical illnesses and the interrelation between this insulin resistance and hypertension, type 2 diabetes mellitus and cardiovascular disease. This observation was first described by Reaven in 1988, and while different definitions of metabolic syndrome have subsequently emerged the paradigm remains the same.

5.2. Metabolic syndrome and insulin resistance

Due to the clustering of type 2 diabetes mellitus, hypertension, dyslipidaemia and cardiovascular disease this has lead to the hypothesis that the phenotypes of metabolic

syndrome arise from a common predecessor, which is thought to be insulin resistance (Haffner, 1999; Alberti, 1998). From a pathophysiological viewpoint, it is proposed that mitochondrial overload leads to lipogenesis, insulin resistance, reactive oxygen species formation, peroxisomal dysfunction and endoplasmic reticulum stress and an unfolded protein response (Nisoli et al., 2007). Insulin resistance infers whole body decreased glucose uptake in response to physiological insulin levels and as such it's effect of glucose and other metabolic pathways involving insulin (Abed et al., 2012). The liver regulates substrate metabolism and is the primary target of insulin action (please see **Figure 1**). During insulin resistance subjects experience a net effect of diminished hepatic glucose output and an increase in free fatty acid flux within the liver which impairs hepatic insulin action and hepatic glucose output (Boden, 2003).

5.3. Obesity, adipocytes and cytokines

Obesity is accompanied by expanded adipose tissue mass which leads to increased lipolysis and free fatty acid (FFA) turnover. Insulin, in normal circumstances, inhibits adipose tissue lipolysis, however this process is increased in the presence of insulin resistance, leading to an increase in FFA release, with visceral adipocytes more sensitive than subcutaneous adipocytes, leading to even more FFA flux (Large and Arner, 1998). In addition to an insulin resistant liver and increased plasma FFA levels disrupting the glucose-fatty acid cycle, insulin-mediated glucose uptake by skeletal muscle is altered, leading to hyperglycaemia (Roden et al., 1999; Petersen et al., 2007). The impact of obesity depends upon lipid partitioning and the distribution of body fat. The majority of fat stored in subcutaneous tissue, while other fat is stored intraabdominally and in the liver and muscle. It is thought that the aetiology of metabolic syndrome may involve this pattern of lipid partitioning which determines the secretion pattern of adipocytokines and it's effect on circulating cytokines and FFA flux (Matsuzawa, 2006). While metabolic syndrome may appear to be of complex aetiology that correlates with obesity it still remains to be distinct in it's presentation. It is likely that obesity is an initiating factor in metabolic syndrome and that environmental factors and western diet also play a role (Weiss et al., 2013).

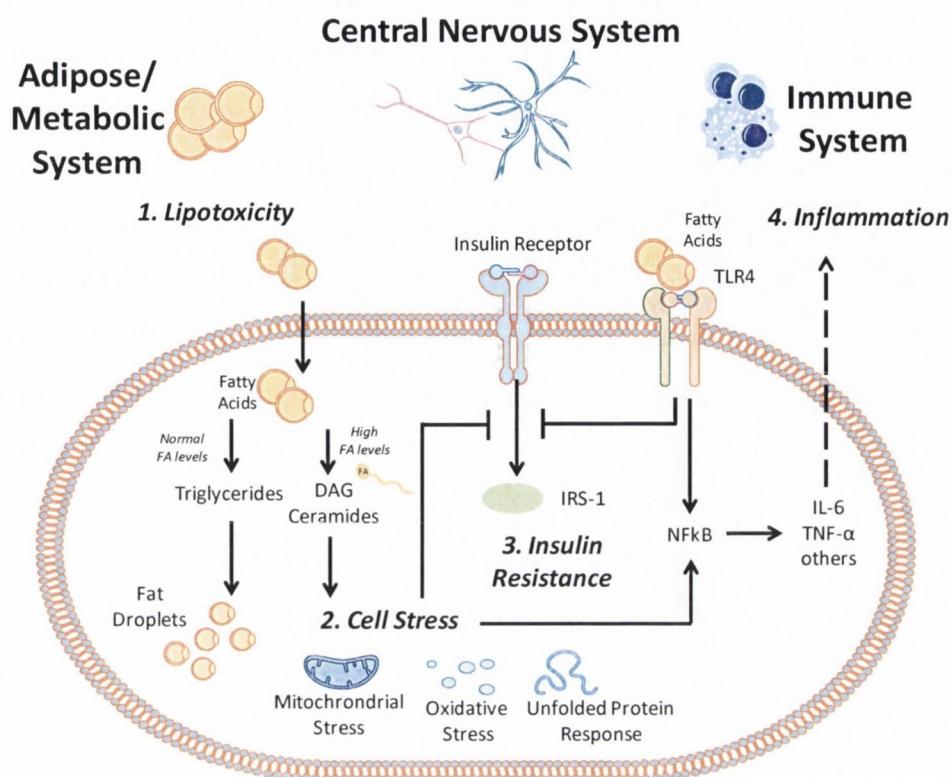


Figure 1: insulin Resistance pathway. The diagram depicts how (1) lipotoxicity induces (2) cell stress (mitochondrial stress, oxidative stress and unfolded protein response stress), which attenuates (3) insulin receptor function inducing insuling resistance and (4) activates immune pathway signalling, such as Nfkb, to promote release of cytokines and induce inflammatory response. Thus, the diagram aims to show cross-talk between adipose, brain and immune tissues and systems at the cellular and signalling level.

5.4. Links between metabolic syndrome in schizophrenia

The metabolic syndrome has consistently been identified in patients with schizophrenia (Aguilar et al., 2012). Studies have shown increased prevalence of 10-year coronary heart disease risk, HDL and total cholesterol scores and increased prevalence of diabetes mellitus in patients with schizophrenia (Osborn et al., 2006; Goff et al., 2005). The presence of metabolic abnormalities and conditions such as cardiovascular disease can have a serious impact on the physical health of this patient group. This increased risk is reflected by a high rate of premature mortality (De Hert et al., 2011; Mitchell, 2009; Brown, 1997; Brown et al., 2000). The exact prevalence of metabolic syndrome in patients with schizophrenia has been difficult to determine, although its point prevalence, according to IDF criteria, is thought to be around 35% (Suttajit et al., 2013).

The aetiology of metabolic syndrome in patients with schizophrenia is multifactorial although it likely involves antipsychotic treatment, high levels of stress and unhealthy lifestyle such as poor diet and exercise. Studies have shown that patients with schizophrenia have a poor diet, characterised by a high intake of saturated fat and decreased fibre, the cause of which is unknown (Dipasquale et al., 2013). It is known that patients treated with atypical antipsychotic medications are at an increased risk of metabolic syndrome. This is partly related to their propensity to induce weight gain (~>7%), although this weight gain differs among antipsychotic agents (Newcomer, 2005; Haddad and Sharma, 2007). Moreover, it has also been shown that those patients taking typical antipsychotic medications and drug-naïve patients, diagnosed with schizophrenia, also have increased risk factors for cardiovascular disease and type 2 diabetes mellitus (Ryan et al. 2003; Ryan et al., 2004). This suggests that metabolic syndrome in this patient group may be independent of atypical antipsychotic treatment.

6. The role of Inflammation in schizophrenia

6.1. The 'multiple hit' hypothesis

In recent times, a developmental model of neuroinflammation has been seen to provide a more succinct explanation for the underlying aetiology of schizophrenia (Meyer, 2013). It is hypothesized that an individual who has a genetic predisposition experiences an induction of inflammatory processes at a very early stage in life (Iritani, 2013). Maternal influenza, toxoplasmosis and prenatal malnutrition in the first and second trimesters have been linked to an increased risk for developing schizophrenia in later life (Brown et al., 2009; Shen et al., 2008). Early prenatal infections and insults are hypothesized to detrimentally alter the developing immune system causing downstream effects that may ultimately affect brain maturation and prime an individual for a second hit later in life (Iritani, 2013). It has also been suggested that in-utero exposure to infection may lead to the production of pro-inflammatory cytokines and, in turn, oxidative stress and hypoferremia (Meyer et al., 2011). This foetal neuroinflammation can lead to the priming of microglia and astrocyte abnormalities which, ultimately, alter neurodevelopment and brain pathology in the long-term. A second hit, such as excessive stress or substance use, occurs in adolescence or early adulthood and this may then lower the threshold enough for the manifestation of clinical symptoms. It is thought that the relapsing remitting course of this illness, and the accompanied negative symptoms, may lead to the development of neural degeneration and intervention at this stage of the illness is crucial (Iritani, 2013). If intervention does not occur, or is inappropriate, during this period of the illness, this has been termed the 'third hit', and the progression of the illness is subsequently thought to be accelerated (Iritani, 2013). Please see **Figure 2**.

6.2. Anti-inflammatory therapy in schizophrenia

Of interest, and perhaps most pertinent to the developmental neuroinflammation hypothesis of schizophrenia are the results of a small number of studies concerning the use of non-steroidal anti-inflammatory drugs (NSAIDs). Celecoxib, a selective

cyclooxygenase-2 inhibitor, reduces the production of an inflammatory immune response and is a first-line treatment for arthritis (Garner et al., 2002). When administered as an adjuvant to an antipsychotic treatment, patients report an increased reduction in scores on the Positive and Negative Symptom Scale compared to those prescribed antipsychotics alone (Müller et al., 2010; Akhondzadeh, et al., 2007). Similar results from a Dutch study were reported with aspirin as an adjuvant in a cohort of patients with schizophrenic spectrum disorders (Laan et al., 2010).

6.3. Crosstalk between metabolic, immune and central nervous system

A number of studies have supported a communication between both nervous system and immune system. The immune system can achieve this cross talk with the nervous system via signalling molecules such as cytokines. It is likely that peripheral molecules such as cytokines communicate with the CNS at locations in the body such as the cerebral vascular epithelium, which in turn may affect blood brain barrier permeability (Konsman et al., 2002). Therefore, when signalling molecules, including cytokines, are present in the bloodstream they can partially penetrate the blood brain barrier and bind to receptors on neurons and glial cells (Yarlagadda et al., 2010). Immune organs, such as spleen and thymus, are also now known to have nervous system innervations (Mignini et al., 2003). In addition, immune cells, such as B cells, natural killer-T cells and monocytes have also been shown to express receptors for neurotransmitters, such as β 2-adrenoreceptor. Activation of β 2-adrenoreceptor can affect the differentiation of naïve CD4+ T-cells, providing evidence that neurotransmitter molecules can affect the immune system (Kohm et al., 2002). Thus, cytokine and neurotransmitter signalling likely coalesce to play a concerted role in bidirectional communication between immune and central nervous systems. Moreover, hormonal and cytokine signalling likely creates a tripartite communication between the metabolic, immune and central nervous systems; of which a disrupted equilibrium likely occurs in schizophrenia.

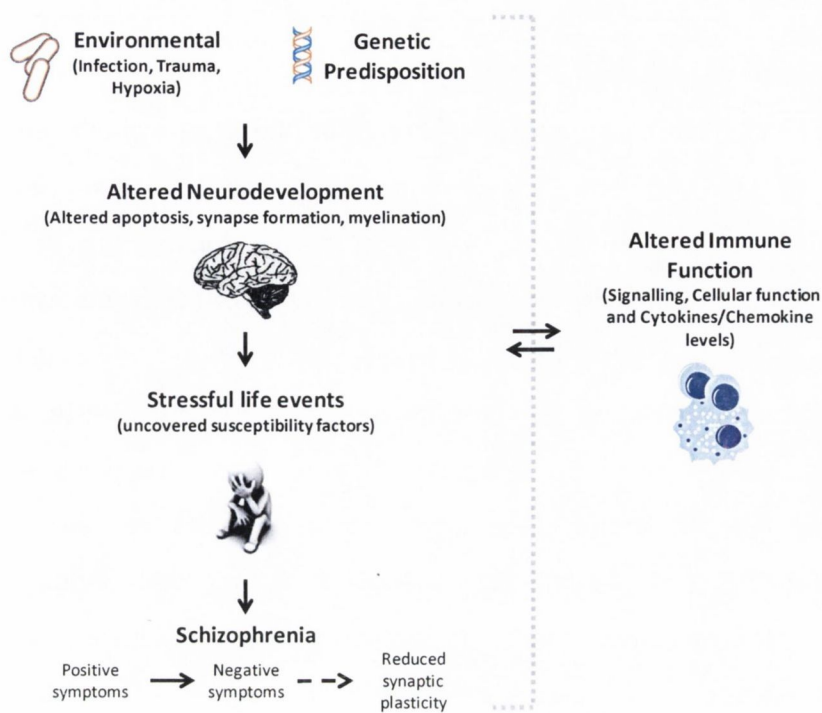


Figure 2: The hypothetical neuroinflammation hypothesis of schizophrenia. The diagram suggests that (i) initial genetic and/or environmental impacts, (ii) followed by altered brain neurodevelopment and (iii) subsequent stress (life, drug abuse, or other) faced in adolescence or early adulthood contribute, in a concerted manner, to the onset and development of schizophrenia. Notably, each of these events likely alters immune function, which is hypothesized to play a role in this illness.

7. Cytokines in fat, immune and glial cells

7.1. Cytokine-mediated signalling

Cytokines mediate and regulate all aspects of immunity and are released by immune cells and glial cells during inflammation. There are more than 100 identified genes encoding for cytokine-like functions and these cytokines can be divided into five subfamilies: interleukins, chemokines, interferons, colony-stimulating factors and growth factors and tumour necrosis factors (TNFs) (Steinke and Borish, 2006; Dinarello, 2007) (Please see **Figure 3**). The effects of cytokines are diverse, with one cytokine exerting multiple biological effects on many different cell types and similarly many cytokines can have the same action. Cytokines also can have a synergistic or antagonistic effect on each other (Arend and Dayer, 1990). Cytokines usually act on kinase linked receptors, regulating phosphorylation cascades and subsequently, gene expression. Secretion of cytokines is usually in response to the recognition of microbes via cell receptors or in response to inflammatory cytokines, such as TNF and IL-1. The chemokine receptors are guanosine triphosphate (GTP)-binding GPCR superfamily of receptors.

7.2. The classification of cytokines

Cytokines can be anti-inflammatory or pro-inflammatory in nature. Pro-inflammatory cytokines (for example IL-1, IL-6 and TNF- α) tend to recruit immune cells and initiate fevers and responses to infections (Fassbender et al., 1997). Anti-inflammatory cytokines (for example IL-4, IL-10 and TNF- β) downregulate the differentiation and proliferation of type 1 helper T-cells (TH1 cells), which can produce pro-inflammatory actions (Elenkov et al., 2004). As previously mentioned, cytokines can be categorised into five subfamilies: interleukins, chemokines, interferons, colony-stimulating and growth factors, and tumour necrosis factors (Steinke and Borish, 2006) (Please see **Figure 3**). The chemokines subfamily are classified dependent upon the number and location of N-terminal cysteine residues. They are divided into two major families: the CC chemokines, in which the cysteine residues are adjacent to each other, and the CXC

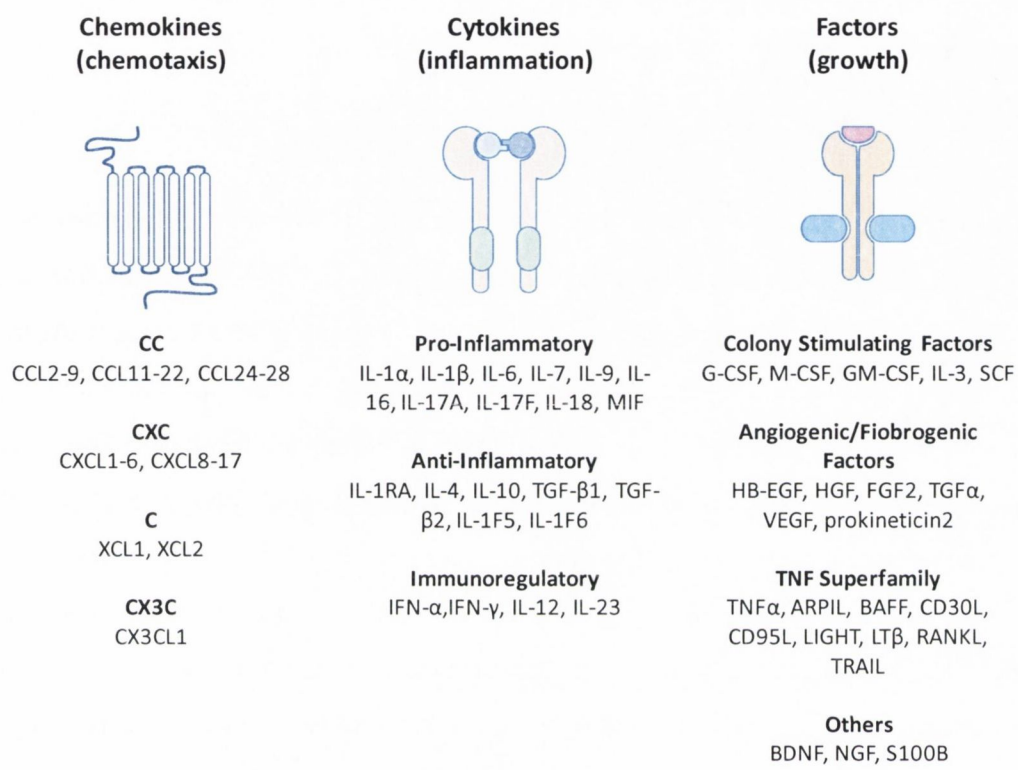


Figure 3: The families of chemokine, cytokine and growth factors. The diagram shows division of signalling molecules into three superfamilies, namely chemokines primarily involved in cell chemotaxis, cytokines that play a role in regulating inflammatory response and growth factors that promote growth and/or survival.

family, where these residues are separated by one amino acid. Most of the CC chemokines mediate recruitment of neutrophils and lymphocytes and most of the CXC chemokines recruit monocytes and lymphocytes (Abbas et al., 2012). These subfamilies of CC and CXC are produced by leucocytes and several cell types such as endothelial cells, epithelial cells and fibroblasts (Abbas et al., 2012).

7.3. Synthesis of cytokines by fat, immune and glial cells.

Cytokines are synthesized by new gene transcription as a result of cellular activation and are not usually stored in preformed molecules (Abbas et al., 2012). This transcriptional activation is transient and, therefore, cytokine synthesis is also transient. As indicated above, cytokines can be produced by a number of cell types including adipocytes, of which aberrant function is associated with metabolic alterations. The main cells involved in modulation of immune response are T helper (Th) lymphocytes, which also produce cytokines and bring together the function of other immune cells. In addition, natural killer cells, macrophages, natural killer T cells and dendritic cells also produce cytokines. The immune response can be subtyped as type-1, type-2 or type-17 and this categorisation is specific depending on cytokine profile involved. The type-1 immune response is followed by the secretion of interferon-gamma (IFN- γ) and interleukin-2 (IL-2); the type-2 response involves interleukin-4, interleukin-5, IL-9 and IL-13 (Fort et al., 2001). Cytokines may also be synthesized and released within the CNS. Many cytokines within the CNS are produced by glial cells (both astrocytes and microglial) and under certain conditions neurones have been shown to produce cytokines (Freidin et al., 1992). Microglia are macrophagocytic cells within the CNS. They can act as both antigen presenting cells and as recruiters of immune cells within the CNS. This produces an immune response, protecting the brain from foreign antigens. The role of astrocytes is discussed in more detail in *Chapter 8*.

7.4. Cytokines and brain disease

Cytokines have been implicated in a number of acute and chronic disease states associated with injury and dysfunction within the central nervous system. For example, animal (mouse) models of ischaemic stroke have shown a reduction in brain damage, following the introduction of IL-1 receptor antagonist (Relton and Rothwell, 1992). Moreover, IL-1 receptor antagonist can also reduce the incidence of motor seizures and delay onset in patients with epilepsy (Vezzani et al., 2002). In addition, Interferon- β , an immuno-active drug, elevates levels of IL-1 receptor antagonist and is used in the treatment of multiple sclerosis (Coclet-Ninin et al., 1997). Taken together, evidence strongly suggests a reciprocal communication between the immune system and the nervous system and this has lead to the 'cytokine hypothesis' of schizophrenia (Reale et al., 2011). Metabolic syndrome has consistently been identified as part of the schizophrenic illness and is also associated with cytokine mediated inflammation. Macrophages 'infiltrate' adipose tissue and play a role in both adipocyte hypertrophy and cytokine release (Gustafson et al., 2007; Heilbronn et al., 2008). Please see **Table 6**.

S100B	IL-1	IL-2	IL-6	IL-8	TNF- α	IL-17	IFN- γ	IL-23	IL-4	IL-10
Schizophrenia										
<i>Plasma/serum</i>										
↑	↑ 1, 2, 18, 26, 41 ↔ 69, 34, 25, 40, 43 ↓ 20	↑ 23-25 ↔ 20-22 ↓ 19, 26	↑ 1,2,23,27,28,35-39 ↔ 19, 32, 34, 40, 41	↑ 1,2	↑ 2, 18, 26 ↔ 32, 34, 52-54, 19	↓ 3	↑ 50, 51 ↔ 21, 10, 11, 18, 19	n.d.	↔ 19	↑ 55 ↔ 19
<i>Ex vivo production</i>										
n.d.	↑ 71 ↔ 25, 59	↑ 66-68 ↔ 69 ↓ 22, 25, 56-65	↔ 25, 20 ↑ 115	n.d.	↔ 54	n.d.	↑ 66, 78 ↓ 56, 69, 72-77	n.d.	n.d.	↔ 56, 66, 67
<i>CSF</i>										
↑ 117	↔ 44, 79 ↓ 20	↑ 24, 70 ↔ 20, 79, 44	↔ 38, 43	n.d.	n.d.	n.d.	↔ 118	n.d.	↑ 118	↔ 118
Metabolic syndrome										
↑ 92, 93	↑ 5, 80, 81, 83, 84, 85, 87	↑ 88	↑ 5, 6, 7, 89, 96, 108	↑ 98 ↔ 7	↑ 5, 6, 82, 88, 89	↑ 14, 106	↑ 99-103	↑ 106, 17	↓ 107	↓ 110-114
Obesity										
↑ 9, 92, 94	↑ 12, 81, 84, 85	n.d.	↑ 7, 8, 89, 90, 96	↑ 97	↑ 7, 8, 89, 90	↑ 15, 106	↑ 10, 104	↑ 106	↓ 107	↓ 111-112
Cardiovascular disease										
↑ 10	↑ 13, 81, 86, 87	↑ 94 ↔ 95	↑ 8, 11, 95	↑ 11	↑ 8, 95	↓ 16, 109 ↑ 109	↑ 105	n.d.	n.d.	↓ 115

Table 6: Altered cytokines in schizophrenia and metabolic disease

↑ indicates studies showed an increase, ↓ studies showed a decrease; ↔ no change observed; n.d. no reported studies were noted. The numbers refer to the cited references for these studies, see below.

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8. Cytokines in schizophrenia

In schizophrenia, there have been a number of studies correlating cytokine levels, however many of these are contradictory (**Table 6**). For example, several studies have concluded that some cytokines are raised in un-medicated patients with schizophrenia; these cytokines include IL-2, IFN- γ , IL-6 and IL-8 (Cazzullo et al., 2001; Zhang et al., 2002; Ganguli et al., 1994). However, these studies are contradicted by further reports, specifically a meta-analysis of 62 studies, including both medicated and unmedicated patients, which found elevated expressions of the cytokines IL-2, IL-6 and IL-1RA in patients with schizophrenia compared to healthy controls (Potvin et al., 2008). In addition, a more recent meta-analysis of cytokine alterations in schizophrenia showed that levels of IL-1 β , IL-6 and TGF- β may be markers of acute psychosis in schizophrenia (Miller et al., 2011). Altered cytokines have also been reported in other psychiatric disorders, for example in depressive states, elevated levels of IL-1 β , IL-6 and TNF α have been noted (Levine et al., 1999). As previously mentioned, serotonin is thought to be involved in the mechanistic pathway of major depressive disorder. Interestingly, IFN- α , IL-1 β , TNF- α and IFN- γ can upregulate the expression of serotonin transporters, thereby decreasing available serotonin (Zhu et al., 2006). Furthermore, in bipolar disorder, a recent meta-analysis found that levels of IL-2R, TNF- α , IL-6R and IL-4 were also significantly raised in patients compared to healthy controls (Munkholm et al., 2013).

8.1. TNF- α

8.1.1. Signalling and Function

TNF- α is also known as cachectin and TNF-SF1A, and it is the prototypic ligand of the TNF superfamily, consisting of a 35 amino acids cytoplasmic domain, a 21 amino acid transmembrane segment and a 177 amino acid extracellular domain (Tang et al, 1996). This pleiotropic molecule plays a central role in inflammation, apoptosis and the immune system development (Baud and Karin, 2001). It is produced by a variety of cells including immune and epithelial cells (Baud and Karin, 2001). TNF acts via two receptors, TNFR1 (TNF Receptor-1) and TNFR2 (TNF Receptor-2). TNF- α binds to both

TNFR1 and TNFR2; of note, TNFR1 is expressed by all human tissues and TNFR2 is mostly expressed in immune cells (Baud and Karin, 2001). TNF- α controls apoptosis to regulate lymphoid tissue development (Naylor et al., 1993). It also promotes inflammation by inducing the activation of macrophages and vascular endothelial cells (Baud and Karin, 2001). Furthermore, it is known to play a role in insulin resistance and fatty acid metabolism contributing to the development of type 2 diabetes (Seijkens et al., 2013). In addition, it has been suggested that of those inflammatory cytokines, TNF- α especially plays a role in alterations in neuroplasticity, cell resilience and neuronal survival (Brietzke et al., 2008; Potvin et al., 2008).

8.1.2. Association with schizophrenia

Altered levels of TNF- α have been noted in patients with schizophrenia (Song et al 2009; Di Nicola et al., 2013; Dunjic-Kostic et al., 2013). A meta-analysis performed on forty studies, including those for acutely relapsed patients and first episode psychosis, showed that TNF- α levels were raised and remained elevated following antipsychotic treatment (Miller et al., 2011). As anti-psychotic treatment does not appear to alter TNF- α levels, this implies that TNF- α may be an important factor in the pathogenesis of schizophrenia. In turn, adjunctive pharmacological treatment with anti-inflammatory agents (specific to TNF- α) may have a role in the treatment of schizophrenia.

8.2 IL-1 β

8.2.1. Signalling and function

IL-1 β is a member of the interleukin 1 cytokine family, which consists of two agonists, IL-1 α and IL-1 β , and one antagonist, IL-1ra (Dinarello, 1997). The IL-1 β molecule binds to the type 1 IL-1 receptor (IL-R1) and type 2 IL-1 receptor (IL-R2) (Dinarello, 2009). It is produced as a pro-protein by activated macrophages, which is converted to an active form by caspase 1 (CASP1/ICE) (Dinarello, 2009). The IL-1 gene is located on chromosome 2q14 (Dinarello, 2009). IL-1 is a pro-inflammatory cytokine that influences neurodegenerative and neuroprotective processes in the brain (Eder, 2009). IL-1 β

strongly promotes the processing and release of a number of signalling molecules such as epidermal growth factor (EGF), heparin-binding EGF (HB-EGF) and neuregulin 1 (NRG-1) (Eder, 2009). Within the CNS, IL-1 β plays a role in cell proliferation, differentiation and apoptosis and also in modulating synaptic plasticity (Rothwell and Luheshi, 2000). The signalling of IL-1 β occurs via binding and dimerisation of the IL-1 receptors; recruitment of the adapter proteins MYD88 and IRAK 4 subsequently occurs, with eventual activation of many transporter factors, such as NF- κ B (Weber et al., 2010; Rothwell and Luheshi, 2000).

8.2.2. Association with schizophrenia

Abnormal levels of the cytokine IL-1 β have been found in both the brain and peripheral blood of patients with schizophrenia, although results have been inconsistent (Söderlund et al., 2009; Fatjó-Vilas et al., 2012; Di Nicola et al., 2013; Barak et al., 1995). It has been reported that the protein and mRNA levels of the IL-1RA are decreased in the prefrontal cortex, whereas levels of IL-1 β were not affected in patients with schizophrenia, suggesting that down-regulation of IL-1RA production in the prefrontal cortex occurs in schizophrenia (Toyooka et al 2003; Fatjó-Vilas et al., 2012). Additionally, it has also been shown that serum levels and peripheral blood mononuclear cell (PBMC) mRNA expression of IL-1 β are increased in antipsychotic naïve first episode patients with schizophrenia (Song et al 2009; Liu et al., 2007). This increase suggests that IL-1 signalling may be involved in the proposed inflammatory process that occurs in schizophrenia (Katila et al., 1999).

8.3. IL-6

8.3.1. Signalling and function

The IL-6 family of cytokines has members which include: IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary inhibitory factor (CNTF), cardiotropin-1 (CT-1), cardiotrophin-like related cytokine and neurotrophin-1/B-cell stimulating factor 3 (NNT-1), neuropoietin (NPN), IL-27 and IL-31. IL-6 is a glycosylated protein of 21-28 kDa and

has the four-helix bundle structure characteristic for those IL-6 type cytokines (Mihara et al., 2012). The receptor for IL-6 is a hexameric protein complex consisting of distinct receptor-binding sites: an IL-6 receptor subunit (IL6R) and interleukin 6 signal transducer glycoprotein 130 (Mihara et al., 2012). Although IL-6 is mostly regarded as a pro-inflammatory cytokine, it also has many regenerative or anti-inflammatory activities (Heinrich et al., 2003). IL-6, along with IL-1 and TNF- α , is elevated in almost all inflammatory states and has been recognised as a target for therapeutic intervention (Shimamoto et al., 2013). Dysregulation of IL-6 has been implicated in the pathogenesis of a number of diseases such as multiple myeloma, autoimmune disease and prostate cancer (Ishihara and Hirano, 2002). Moreover, it also plays a role in regulating brain development, synaptic plasticity and a number of behaviours such as feeding, sleeping and stress (Bauer et al., 2007).

8.3.2. Association with schizophrenia

Levels of IL-6 in patients with schizophrenia have been more consistently raised compared to controls, although there have also been inconsistent findings in this regard (Akiyama 1999, Maes et al, 2000, Barak et al., 1995, Baker et al., 1996; Di Nicola et al., 2013; Borovcanin et al., 2013; Dunjic-Kostic et al., 2013; Zhang et al., 2004). Nevertheless, a meta-analysis reported an increase in levels of IL-6 in the blood of patients with schizophrenia but with no alteration of sIL-6R (Potvin et al., 2008). Serum and CSF levels of sIL-6R have also been correlated to severity of positive symptoms of schizophrenia (Muller et al, 1997). Animal studies have demonstrated that a single maternal injection in IL-6 during pregnancy leads to schizophrenia-like symptoms and have also indicated a link between hyper-IL-6 signalling and oxidative stress which may be associated with GABAergic dysfunction within the brain in schizophrenia.

8.4. IL-8

8.4.1. Signalling and Function

IL-8 is a non-glycosylated protein consisting of 72 amino acids, 8 kDa in size and a member of the CXC chemokine family (Holmes et al., 2009). It is a chemokine produced

by macrophages and cells such as epithelial cells, airway smooth muscle cells and endothelial cells (Zeng et al., 2003). In addition, the human IL-8 gene is located on the 4q12-q21 chromosome (Holmes et al., 2009). The IL-8 receptor is a dimeric glycoprotein consisting of a 59 kDa and 67 kDa subunit (Holmes et al., 2009). It is a G-protein-coupled receptor protein and there are at least two IL-8 receptor types, type 1 receptor which specifically binds IL-8 and the type 2 receptor, binding to IL-8 related factors such as melanoma growth stimulatory activity (MGSA) and macrophage inflammatory protein (MIP) (Holmes et al., 2009). Moreover, IL-8 can be secreted by cells with toll-like receptors (TLRs) which play a role in the innate immune response (Holmes et al., 2009). IL-8 has two primary functions, it induces chemotaxis in target cells such as neutrophils and induces phagocytosis upon arrival at the site of infection (Zeng et al., 2003). Furthermore, IL-8 can also act as a potent promoter of angiogenesis (Li et al., 2003).

8.4.2. Association with schizophrenia

As IL-8 is associated with inflammation and studies have linked raised levels of IL-8 during pregnancy to an increased risk of schizophrenia in the offspring (Brown et al., 2004). What's more, increased levels of IL-8 have also been reported in first-episode psychosis (Di Nicola et al., 2013). Importantly, these increased levels of IL-8 have been reported to reduce the likelihood of a good response to antipsychotic medications in schizophrenia (Zhang et al., 2004).

8.5 IL-17

8.5.1. Signalling and Function

The IL-17 family of cytokines includes six members, IL-17/IL-17A, IL-17B, IL-17C, IL-17D, IL-17E/IL-25 and IL-17F and they are produced by multiple cell types (Korn et al., 2009). Most IL-17 proteins are secreted as disulfide-linked dimers (except IL-17B). Activation of the IL-17 receptor family (IL-17 R/IL-17 RA, IL-17 B R/IL-17 RB, IL-17 RC, IL-17 RD and IL-17 RE) triggers intracellular pathways that lead to the production of pro-inflammatory cytokines and anti-microbial peptides (Korn et al., 2009; Song and Oian, 2013). Moreover, IL-17A, IL-17F and IL-17 A/F are produced by activated T cells and signal via

IL-17 RA and IL-17 RC receptor complex (Korn et al., 2009). Ligand binding of this complex recruits intracellular proteins and leads to downstream activation of transcription factors such as, NF- κ B and AP-1 (Korn et al., 2009). In addition, IL-17C signals in an autocrine fashion in epithelial cells producing anti-microbial peptides and pro-inflammatory cytokines and overexpression may lead to the development of autoimmune disorders, similar to IL-17A (Song and Oian, 2013). However, the majority of target cells and effects of IL-17B, IL-17D and IL-17 RD have yet to be reported (Song and Oian, 2013).

8.5.2. Association with schizophrenia

Previous studies in schizophrenia have been controversial with regard to the predominance of type-1 or type-2 cytokine levels in patients, and studies of IL-17 in particular have been limited. IL-17 is one of the major proteins involved in inflammation (Gabay and McInnes, 2009) and a recent study has suggested that levels of IL-17 are significantly lower in patients with first episode schizophrenia compared to healthy controls (Borovcanin et al., 2012). Furthermore, a previous study which assessed the effect of antipsychotics on stimulated blood cells from patients with schizophrenia, found that antipsychotics may contribute to an increase in the production of IL-17 (Himmerick et al., 2011).

8.6 IL-23

8.6.1. Signalling and Function

IL-23 is produced by dendritic cells and macrophages (Välimäki et al., 2013). This interleukin is a heterodimeric cytokine composed of two disulfide-linked subunits, a p19 subunit that is unique to IL-23, and a p40 subunit that is shared with IL-12. The IL-23 receptor complex consists of two receptor subunits, the IL-12 receptor beta 1 subunit (IL-12 R beta 1) and the IL-23-specific receptor subunit (IL-23 R) (Hölscher, 2005). IL-23 and IL-12 both induce proliferation and IFN- γ production by human T cells (Croxford et al., 2012). IL-23 is an important inflammatory component in infection, promoting the upregulation of the matrix metalloprotease MMP9, increasing angiogenesis and

reducing CD8+ T-cell infiltration (Tang et al., 2012; Tan et al., 2009). It stimulates naïve CD4+ T cells to differentiate into a subset of cells called Th17 cells (Tang et al., 2012). Th17 cells play a role in producing IL-17 which is a pro-inflammatory cytokine that stimulates the production of other pro-inflammatory molecules (IL-1, IL-6, TNF- α , NOS-2) and also enhances T cell priming (Tang et al., 2012). Studies have shown that knockout mice deficient in either p40 or p19 have a decreased inflammatory response in inflammatory diseases such as multiple sclerosis (Kikly et al., 2006). This evidence supports the role of IL 23 in the inflammatory response (Tang et al., 2012).

8.6.2. Association with schizophrenia

No studies involving the direct measurement of IL-23 in patients with schizophrenia were found to be reported to date. It is noteworthy, however that IL-23 promotes differentiation of Th17 cells (Tang et al., 2012) as indicated above. In this thesis, therefore, we included IL-23 analysis, given it's role in inflammation and link with IL-17/Th17-mediated response.

8.7. S100B

8.1.1. Signalling and Function

S100B is a calcium-binding protein of 92 residues, with its gene located on 21q22.3 (Steiner et al., 2011; Rothermundt et al., 2009; Rothermundt et al., 2004a). Within the central nervous system (CNS), S100B is thought to be a marker for astroglial activation, linking astrocyte dysfunction to schizophrenia (Steiner et al., 2011; Rothermundt et al., 2009; Rothermundt et al., 2004a; Schulte-Herbrüggen et al., 2008). In addition to astrocytes, S100B is released from many cell types, such as, adipocytes, chondrocytes, cardiomyocytes and lymphocytes (Muller et al., 2007; Beumer et al., 2012; Schwarz et al., 2012; Leonard et al., 2012; Steiner et al., 2010a; Steiner et al., 2010c; Streitbürger et al., 2012; Steiner et al., 2012; Muller and Schwarz, 2006). S100B can act in a paracrine and autocrine manner, where low concentrations regulate proliferation and differentiation of neurons and glia. S100B also acts as a neurotrophic factor, regulating dopaminergic and glutamatergic synaptic function, in addition to synaptogenesis

(Steiner et al., 2011; Rothermundt et al., 2009). In contrast, excessive levels of S100B promote the expression of inducible nitric oxide synthase or pro-inflammatory cytokines causing neuronal dysfunction and apoptosis. In S100B knockout mice, increased brain-derived neurotrophic factor and decreased noradrenaline further suggest a role for S100B in regulating the levels of neurotrophic factors and neurotransmitters (Schulte-Herbrüggen et al., 2008).

8.1.2. Association with schizophrenia

Studies have shown that copy number variations in S100B as well as polymorphisms in its gene and promoter region are associated with altered S100B protein levels in schizophrenia (Zhai et al., 2012; Zhai et al., 2011; Dagdan et al., 2011; Suchankova et al., 2011; Saus et al., 2010; Hohoff et al., 2010; Roche et al., 2007; Liu et al., 2005). In addition, polymorphisms in the receptor for S100B, RAGE (receptor for advanced glycation end products) have been linked with schizophrenia, as have increased levels of the soluble version of RAGE (Suchankova et al., 2012; Steiner et al., 2009). At the protein level, findings suggest that S100B is increased in schizophrenia, and that these protein levels are correlated with medication, gender, age and illness severity (Dagdan et al., 2011; Steiner et al., 2009; Falcone et al., 2010; Zhang et al., 2010b; Steiner et al., 2008; Ryoum et al., 2007; Rothermundt et al., 2007; Steiner et al., 2006; Rothermundt et al., 2004b; Wiesmann et al., 1999; Zhang et al., 2010a; Qi Ly et al., 2009; Schroeter and Steiner, 2009a; Schroeter et al., 2009b; Pedersen et al., 2008; Ehrenreich et al., 2007; van Beveren et al., 2006; Schmitt et al., 2005; Rothermundt et al., 2004c; Schroeter et al., 2003; Lara et al., 2001; Rothermundt et al., 2001; Gattaz et al., 2000).

Importantly, astrocytes express dopamine receptors (DA2Rs) and antipsychotic medications regulate the cellular release of S100B (Liu et al., 2008; Nardin et al., 2011; Herrmann et al., 2012). Of interest, S100B is also expressed in immune cells (including T cells and natural killer cells) and in adipocytes suggesting it may have a role in altered immune response and metabolic activity in schizophrenia (Rothermundt et al., 2009; Muller et al., 2007; Beumer et al., 2012; Schwarz et al., 2012; Leonard et al., 2012; Steiner et al., 2010a; Steiner et al., 2010c; Streitbürger et al., 2012; Steiner et al., 2012; Muller and Schwarz, 2006). Indeed, findings have

suggested that altered S100B levels in schizophrenia may be due to metabolic disorder, visceral obesity, diabetes and/or immune dysfunction in this illness ([Beumer et al., 2012](#); [Leonard et al., 2012](#); [Steiner et al., 2010a](#); [Schroeter et al., 2009a](#); [Sen et al., 2007](#)). Here, serum levels of S100B in patients, diagnosed with schizophrenia, were investigated and relationships between age, gender, illness severity, type of medication, treatment time and various metabolic factors, were examined. In this study, focus was given toward patients treated with clozapine, which has been previously shown to increase the incidence of hyperglycaemia, diabetes and metabolic syndrome ([Koller et al., 2001](#)).

Chapter 2.

Methods and Materials

1. Clinical Studies

1.1. Study population (schizophrenia)

Ethical approval for this study was obtained from the St. Vincent's Hospital Fairview Ethics Committee, Dublin, Ireland. Patients attending an urban mental health clinic who met the DSM-IV-TR ([Diagnostic and Statistical Manual of Mental Disorders, 4th edition, Revised, 2000](#)) diagnostic criteria for schizophrenia were recruited. These patients were treated with clozapine (n = 91) or depot antipsychotic medication (n = 36). Healthy controls were recruited from the same urban area with matched demographics (n = 50). Informed, written consent was gained from all participants. Exclusion criteria for both groups included (i) other psychiatric and neurological diagnoses, (ii) a co-morbid diagnosis of substance abuse disorder and (iii) those with an IQ < 70. Healthy controls recruited did not have a significant physical disability or disease, or a personal or family history of psychiatric illness. While psychometric testing was not performed for individual participants, all participating subjects were educated to at least secondary school level in Ireland or equivalent. The Demographic data (male:female ratio, age range, treatment duration, daily dose, BPRS, and SANS), biochemical data (fasting glucose, triglycerides, HDL, LDL, total cholesterol), and cardiovascular disease risk factor and lifestyle data (smoking status, diabetes mellitus status, body mass index, hypertension treatment, systolic blood pressure, and Framingham score) were collected for each group.

1.2. Study population (multiple sclerosis)

Ethical approval for this study was obtained from the Cork University Hospital Ethics Committee, Cork, Ireland. Informed, written consent was gained from all participants. These patients were treated with interferon beta 1a or 1b (Rebif or Betaferon) (n=11), natalizumab (n=14) or were drug naive (n=12). The patient group treated with interferons were as follows: Rebif (n=5) and Betaferon (n=6). The patient group treated with natalizumab received from between 4-60 infusions. Controls were recruited from the same urban area (n=18). All confirmed MS patients had a relapsing-remitting form

of MS as defined by the revised McDonald criteria. Disease severity was scored at time of collection using the Kurtzke's Expanded Disability Status Scale (EDSS). MS patients treated with interferon or natalizumab were recruited from outpatient clinics, while the drug naive MS group were recruited from an inpatient setting. Controls were recruited from medical staff as well as healthy volunteers. All participants were caucasians resident in south/south east Ireland. Details of the patient demographics are presented in results *Chapter 5. Please note that samples were collected by Dr. Tzehow Mok, Cork University Hospital, Cork, Ireland.*

1.3. Treatment conditions

The range of treatment duration for patients with schizophrenia, treated with clozapine, was 0.25-19 years (with mean treatment duration $\pm$ standard deviation as 7.07 $\pm$ 4.57 years). The range of daily dose for clozapine medication was 125-900 mg per day (with mean daily dose $\pm$ standard deviation as 432.1 $\pm$ 185.7 mg per day), $n=91$. For patients treated with depot medications (I.M.) the range of weekly dose and the mean weekly dose $\pm$ standard deviation for (i) zuclopentixol was 50-100mg per week and 95 $\pm$ 15.8 mg per week, $n=10$; (ii) flupenthixol decanoate was 12.5-100 mg per week and 42.3 $\pm$ 32.9 mg per week, $n=10$; (iii) risperidone was 12.5-37.5 mg per week and 23.6 $\pm$ 7.0 per week, $n=11$; (iv) flupheazine decanoate was 25-50 mg per week and 41.7 $\pm$ 14.4 per week, $n=3$; and (v) haloperidol was 25-62.5 mg per week and 43.8 $\pm$ 26.5 per week, $n=2$. The chlorpromazine equivalent doses expressed as mean mg per day were as follows: clozapine 864 mg per day, zuclopentixol 95 mg per day, flupenthixol decanoate 423 mg per day, risperidone 188.8 mg per day, flupheazine decanoate 833 mg per day, and haloperidol 292 mg per day (Woods, 2003).

1.4. Illness severity and clinical variables

To assess illness severity within the patient group, the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962) and Scale for The Assessment of Negative Symptoms (SANS) (Andreasen, 1982) was administered by a trained clinical psychiatrist. The BPRS assesses severity of psychopathology in patients with schizophrenia, where values range

between 18 - 126. The SANS scale was used for assessing and rating the severity of 'negative symptoms' in patients with schizophrenia, where patients scored between 0 - 125. To determine subject demographic and metabolic syndrome risk profile, clinical variables such as fasting cholesterol, HDL, LDL, triglycerides, and fasting glucose were determined for all participating subjects. Data including body mass index (BMI), blood pressure (BP), smoking status, a known diagnosis of diabetes and hypertensive treatment was obtained for all subjects. This enabled calculation of the Framingham score for all individuals within the groups studied. The Framingham score is a predictive score, showing the percentage risk of a cardiovascular event occurring for that participant in the subsequent 10 years (Wilson, 1998).

2. Biochemical methods

2.1. Materials and equipment

The following materials were used: Ethanol (E7023-500ML, Sigma, St. Louis, MO), Methanol (34966-2.5LT, Sigma, St. Louis, MO), Isopropanol (I9516-500ml, Sigma, St. Louis, MO), Hydrochloric acid (258148-500ml, Sigma), Calcium phosphate (C7263-500G, Sigma, St. Louis, MO), Potassium chloride (KCl) (P9541-500G, Sigma, St. Louis, MO), Sodium chloride (NaCl) (S3014, Sigma, St. Louis, MO), Calcium chloride (C3306-100G, Sigma), MgSO₄ (M2643-500G, Sigma), Na₂HPO₄ (S3264, Sigma, St. Louis, MO), NaH₂PO₄ (S8282, Sigma, St. Louis, MO), Non-fat milk (Marvel, Ireland), PBS (20012-019, Gibco, Carlsbad, CA), Tris (0497-1KG, Amresco, Cochran Solon, OH), Tris-EDTA (TE) (T9285, Sigma, St. Louis, MO), Water (W4502-1L, Sigma, Louis, MO), Whatman papers (grade 5, 1003-919, Whatman, GE, UK), Whatman papers (grade 3, 1003-917, Whatman, GE, UK), Poly Vinylidene Difluoride Membrane (PVDF membrane) (P2938, Sigma, St. Louis, MO). The following equipment was used: Fujifilm LAS-3000 Intelligent Dark-box; Shaker (Excella E24 Incubator, New Brunswick Scientific, Mason Technology); Powerpack (Biorad); 50 ml centrifuge (Hettich Rotina 380R); Heating block (Grant); Small bench centrifuge (Hermle, Mason Technology, Ireland); Water bath (UAB 12 EU Grant). (For details of antibodies, please see **Table 1**).

2.2. Clinical biochemistry analysis

To aid in determining demographic and metabolic syndrome risk profile, a peripheral venous blood sample was obtained from all participating subjects using the Sarstedt Monovette system. Venous blood samples obtained for the measurement of HDL, LDL, TG and fasting cholesterol were collected in a bottle containing serum-separating tubes. Blood samples for the measurement of fasting glucose were collected in fluoride/oxalate containing bottles. All samples were sent to the Biochemistry Laboratory, Mater Misericordiae University Hospital, Dublin, and analysed within the same day as collection. This laboratory tested for the above named specific chemical analytes in the individual blood samples.

The lab biochemical test principles are as follows: Glucose was measured as glucose oxidase catalyses the oxidation of Beta D- glucose (in the plasma) to D glucono -1 ,5 - lactone with the formation of hydrogen peroxide. This lactone is then slowly hydrolysed to D-gluconic acid. A peroxidase enzyme breaks down hydrogen peroxide into oxygen and water. Oxygen then reacts with an oxygen acceptor which itself is converted to a coloured compound, the amount of which is measured colorimetrically. The levels of cholesterol were measured enzymatically via a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. A byproduct of this reaction (H_2O_2) is quantitatively measured in a reaction producing a colour. The absorbance of this colour is measured at 500nm and it's intensity is proportional to cholesterol concentration. The measurement of TG is also an enzymatic process whereby a series of coupled reactions hydrolyzes TG to produce glycerol. This glycerol is further oxidized and H_2O_2 , a reaction product, is measured as described above. HDL is measured using a apoB containing assay. Lipoproteins in the specimen react with a blocking agent thus excluding them and therefore only HDL-chol is detected. LDL-cholesterol is calculated from values of total cholesterol, TG and HDL-cholesterol: $[LDL\text{-}chol] = [total\ chol] - [HDL\text{-}chol] - [TG]/5$ (all values are expressed in mg/dL).

2.3. Cytokine analysis

All participating subjects were fasting for at least 12 hours prior to collection of blood sample for biochemical and cytokine analysis. Blood samples obtained for cytokine analysis were collected in monovette clotting activator serum tubes (Sarstedt, UK) and 2-3 h after collection were centrifuged to separate serum fraction. Serum samples were stored at -80 °C until used for analysis. Cytokine analysis was conducted using standard dot-immuoblotting method. Briefly, 2 µl of serum sample was blotted onto Amersham Hybond-C Extra nitrocellulose blotting membrane (Fisher Scientific, GZRPN203E) and left to dry for 1 h at room temperature. The nitrocellulose membranes were then (i) blocked, (ii) incubated with primary antibody, (iii) washed, (iv) incubated with horse raddish peroxidase (HRP) conjugated secondary antibody, and (v) washed again before (vi) exposing to development reagent. All blocking and antibody incubation steps were performed in PBST-block (PBS, 0.1% Tween-20 supplemented with 5% non-fat milk) for 1 h at room temperature, and all wash steps performed by 3 x 5 min incubation with PBST. Blots were developed by incubating in Immobolin chemiluminescent HRP substrate and imaged on a Fujifilm LAS-3000 Intelligent Dark-box. Densitometry measurement of band intensity was used for quantification (MCID Elite, Imaging research, Inc., 2003). The following primary antibodies against human cytokines were used: monoclonal mouse IgG1s for IL-6 (clone 6708, MAB206), CXCL8/IL-8 (clone 6217, MAB208), IL-17 (clone 41802, MAB3171) and IL23p19 (clone 727753; MAB17161) and polyclonal Ab goat IgGs for IL1 β /IL1F2 (AB-201-NA) and TNF α (AB-210-NA) (R&D systems). The secondary antibodies were peroxidase conjugated (HRP) goat anti-mouse or anti-goat IgG (Sigma).

3. Data Analysis

3.1. Two-dimensional (2D) normalisation analysis

Dot immunoblots were performed in two separate array sets (in duplicates) so that the data for individual cytokines could be subjected to 2-dimensional (2D) normalisation i.e. normalisation to remove both dot-to-dot and blot-to-blot variation, as we have

reported previously (Chatterjee et al., 2009). A scatter plot for each cytokine was obtained by plotting the cytokine signals in the first array against that obtained in the second array. The samples lying at the extremities in this analysis displayed highest cytokine signals. The scatter plots were also divided into 4 quadrants, where x- and y-line cut-offs excluded all control samples from patient groups.

Furthermore, to study the value of examining the levels of all 6 cytokines in combination (i.e. the level of 'cytokine signature') over individual cytokines, the data from all 6 cytokines was summed. The scatter plots were also divided into 4 quadrants, where x- and y-line cut offs excluded all healthy control samples from the right upper quadrant. Therefore, those samples lying in the right upper quadrant display high 'cytokine signature' levels and those in the left lower quadrant display low 'cytokine signature' levels.

3.2. Statistical analysis

All statistical analysis was performed using Prism 5 GraphPad Software package. Unless otherwise stated, statistical tests performed were either Student's unpaired t-test (two-tailed) or one-way ANOVA (with Bonferroni post-hoc test). Individual statistical tests are described in figure legends. All data is shown as mean $\pm$ SEM, where n = number of subject samples. The significance levels (or alpha levels) were set at $p < 0.05^*$, $p < 0.01^{**}$ and $p < 0.001^{***}$.

Primary Antibodies	Host	Dilution Used	Catalog	Clone
Human IL-6	Mouse IgG1	1:2000	MAB206	Clone 6708
Human CXCL8/IL-8	Mouse IgG1	1:2000	MAB208	Clone 6217
Human IL1b/IL1F2	Polyclonal Goat IgG	1:7000	AB-201-NA	
Human TNFa	Polyclonal Goat IgG	1:7000	AB-210-NA	
Human IL-17	Mouse IgG1	1:2000	MAB3171	Clone 41802
Human IL-23	Mouse IgG2A	1:2000	MAB17161	Clone 727753
Human S100B	Mouse IgG2A	1:2000	MAB1820	Clone 472806

HRP Secondary Antibodies	Host	Dilution Used	Catalog	Conjugate
Anti Goat	Rabbit	1:5000	A4174	HRP
Anti Mouse	Goat	1:5000	A8924	HRP

Table 1: Primary and Secondary Antibodies Used. A list of primary and secondary antibodies used in this thesis are shown. All primary antibodies were purchased from R&D systems. All secondary antibodies were HRP-conjugated and purchased from Sigma.

Chapter 3.
The role of S100B in schizophrenia

Aims

- to collect demographic data of patient cohort studied
- to analyse metabolic parameters altered in clozapine treated patient group
- to investigate altered BMI in clozapine treated patient cohort
- to demonstrate changes in serum levels of S100B in patients treated with clozapine and healthy control subjects
- to demonstrate if S100B serum concentration is related to age, treatment time or illness severity
- to investigate the effects of medication on S100B serum concentration
- to test whether S100B levels are elevated in female patients with schizophrenia
- to examine if levels of S100B correlate with BMI in female patients with schizophrenia

Abstract

Background

The neurotrophic factor, S100B, is released primarily from astrocytes, with serum and CSF levels of S100B reported as altered in schizophrenia. However, many of these reports are contradictory. Here, serum levels of S100B in schizophrenia and influence of age, gender, medication and illness severity were examined.

Methods

Serum S100B levels were measured in patients with schizophrenia treated with clozapine. Lifestyle, metabolic and illness severity parameters were correlated with S100B concentrations.

Results

Data showed raised serum levels of S100B in schizophrenia female patients, but not male patients, compared to controls. Correlation analysis demonstrated a positive association between S100B serum concentrations and BMI.

Conclusions

This study supports previous findings that adipocytes may contribute to S100B serum concentrations in females, in addition to astrocytes. This study also supports the hypothesis that metabolic effects of medication, lifestyle choices and the illness itself, may be contributing factors to altered levels of S100B.

1. Introduction

1.1. S100B Structure and domains

The gene encoding S100B is found on the long arm of chromosome 21 at 21q22.3. S100B is a calcium binding protein belonging to the S-100 protein family (**Figure 1**). It is a homodimer, consisting of two beta subunits with a molecular weight of approximately 21kDa (Beaudeau et al., 1999) (**Figure 1**). S100B has a number of functions, depending on its concentration; it can have tropic or toxic effects on cells. In micromolar concentrations (0.5-2.0 μ M) S100B causes cell changes typical of apoptosis and in nanomolar concentrations it is neuroprotective, stimulating neurite and astrocyte growth (Mariggio et al., 1994). S100B can increase intracellular concentration of calcium in both glial and neuronal cells (Barger et al., 1992). S100B also likely plays a role in cell-cell communication; gap junction permeability can be affected by intracellular calcium changes. In animal experiments using rats, antibodies to S100B block synaptic function such as long-term potentiation (LTP), reducing the rat's ability to negotiate a maze space tasks (Frazeli et al., 1990).

S100B is found in many brain cells, primarily in astrocytes and possibly also microglia, oligodendrocytes and neuronal cells (**Figure 2**). Intracellularly it is mainly found in the cytoplasm and vesicles in astrocytes within the CNS and Schwann cells within the PNS (Fano et al., 1995). S100B is a marker of structural damage, in that it is released from stimulated astrocytes into extracellular space and CSF, where it can then traverse the blood brain barrier into the peripheral circulation (Beaudeau et al., 1999) (**Figure 2**).

1.2 S100B function

The S100B proteins communicate signals from cell surface receptors to target molecules inside the cell, in the nucleus or cytoplasm. Extracellularly, S100 proteins bind to RAGE (receptor for advanced glycation end products) which in turn activates a number of intracellular signalling pathways (**Figure 3**). There are five identified intracellular

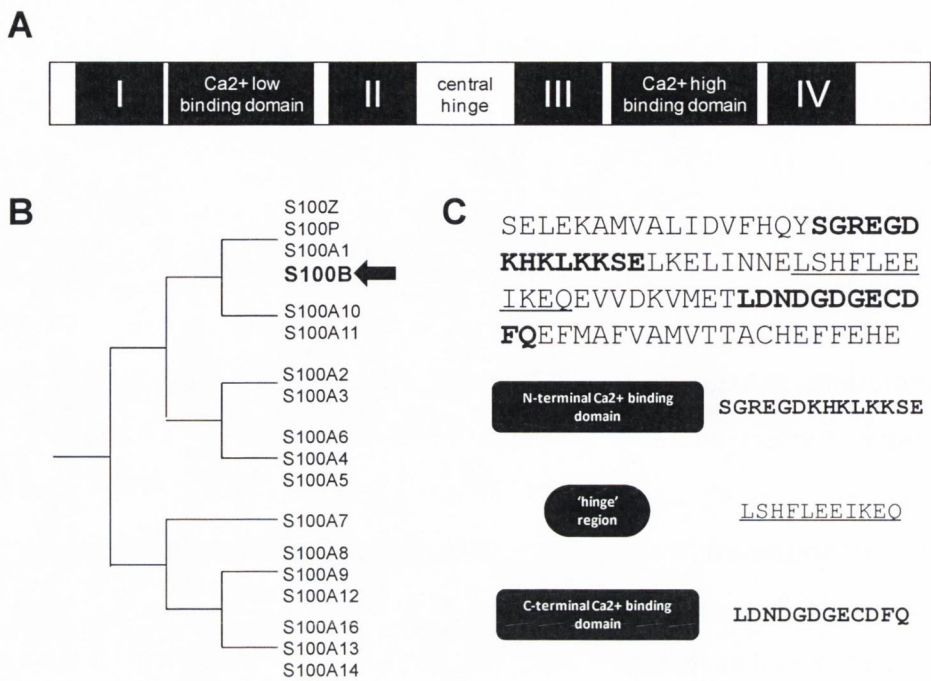


Figure 1: S100B structural domains. (A, C) S100B is composed of 91 amino acids and has a molecular weight of 11 kDa. S100B structural domains includes (i) four-helical segments (I – IV), (ii) two EF-hand calcium-binding domains (one binds calcium with low affinity, and with high) and (iii) a central hinge region. (B) S100B belongs to a large family of proteins and is closely related to S100Z, S100P and S100A1.

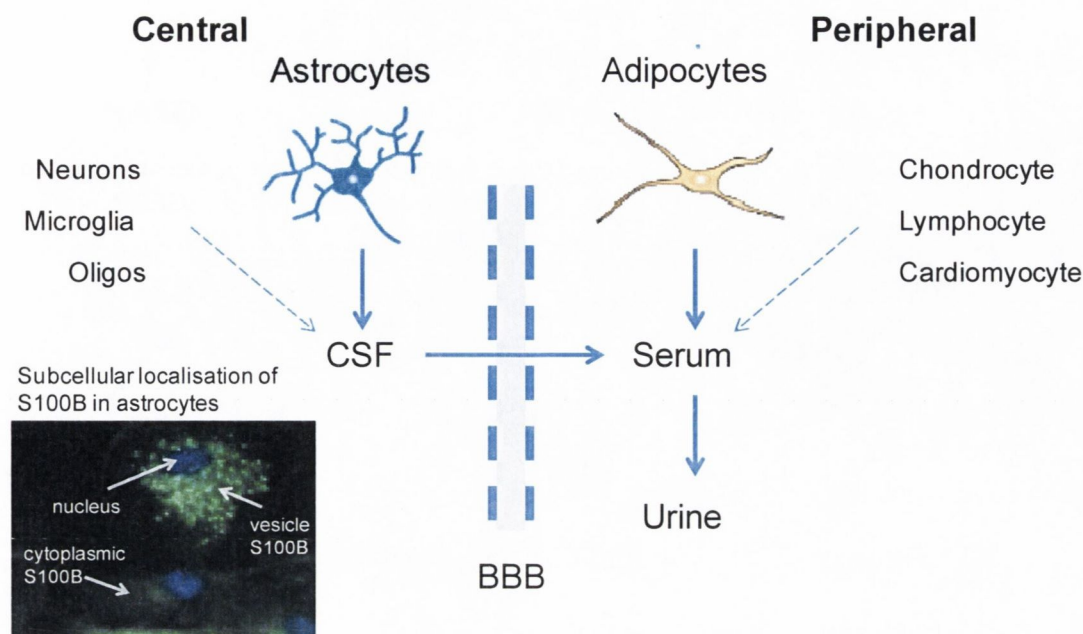


Figure 2: Cellular sources of S100B. In the CNS, astrocytes are the major source of S100B, where is also known as a marker for astrocytes. In the periphery adipocytes (fat cells) are thought a major source of S100B. The inset shows the subcellular localization of S100B in astrocytes, where it was found in vesicles and in the cytoplasm (*please note this inset was obtained from freely available images from the internet*).

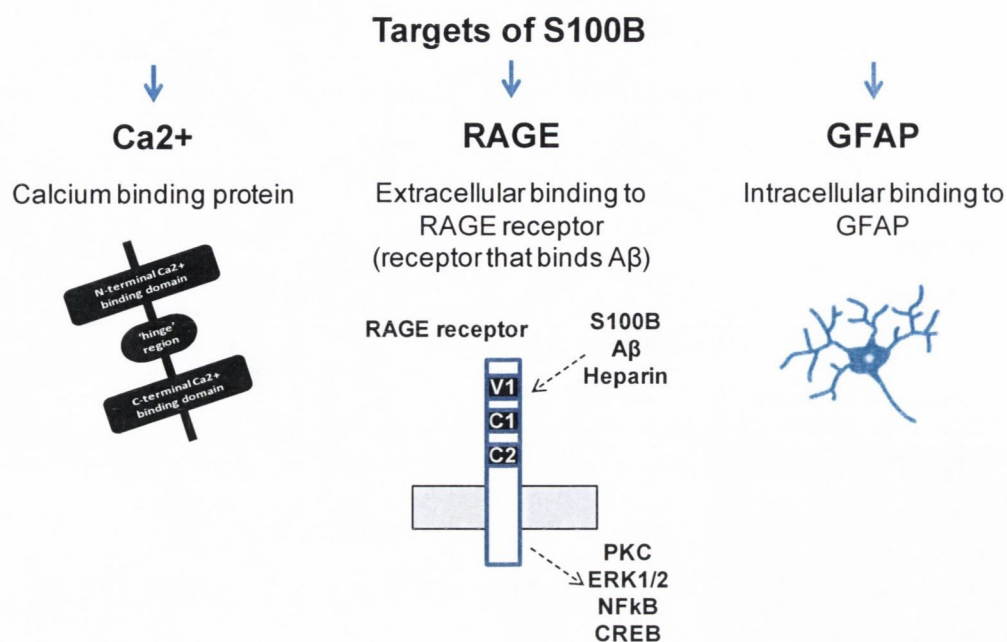


Figure 3: Targets of S100B. S100B is a calcium binding protein. S100B also binds to GFAP in astrocytes. Interestingly, S100B activates the RAGE receptor (receptor for advanced glycation end products). S100B binds to RAGE, causing RAGE receptor dimerisation and leading to activation. The RAGE receptor is composed of three Ig-domains (V, C1 and C2), a transmembrane domain and a cytosolic tail (for intracellular signalling). Signalling pathways include PKC, pERK and also the transcription factors NF- κ B and pCREB, allowing possible links to synaptic function and cytokine/chemokine production.

functions for S100 proteins: (i) firstly, to regulate phosphorylation mediated by protein kinases, (ii) secondly, the modulation of enzyme activity, (iii) thirdly, to maintain the cell shape and motility, (iv) fourthly, plays a role in signal-transduction pathways, and (v) finally, they promote calcium homeostasis. One of the prominent functions of S100B is in the regulation of calcium homeostasis and cell motility. S100B can have both trophic and toxic effects depending upon its concentration. At nanomolar concentrations S100B may stimulate neuronal growth and enhance neuronal survival during their development, and at micromolar concentrations S100B is thought to have deleterious effects by increasing the expression of the cytokine IL-6 (pro-inflammatory) and may play a role inducing apoptotic neuronal death.

1.3 Targets of S100B

In the brain S100B is released by astrocytes and its effects occur in part due to interaction with RAGE (**Figure 3**). RAGE is a multiligand receptor of the immunoglobulin superfamily and it interacts with distinct families of ligands acting as mediator for a broad range of functions in cell types such as cellular migration, proliferation, survival and apoptosis ([Donato et al., 2003](#)). At baseline levels RAGE is present in most tissues at low levels, however when pathology is present RAGE is upregulated ([Yan et al., 2007](#)). A number of intracellular mechanisms are activated when these ligands interact with RAGE, such as extracellular signal-regulated kinase (ERK)1/2, mitogen-activated protein kinases (MAPKs), p38, c-Jun NH₂-terminal kinase (JNK), and transcription factor nuclear factor kB (**Figure 3**).

1.4 Diseases associated with S100B

S100B concentrations have been examined in a number of diseases (**Table 1**). In Alzheimer's disease, levels of S100B are increased and further still correlate negatively with normalized brain volume, concluding that S100B is associated with brain atrophy. ([Petzold et al., 2002](#)). As the locus for S100B is on chromosome 21, trisomy 21 (Down's syndrome) leads to increased transcription of S100B. This prolonged overexposure to

Desease	Change	Reference
Schizophrenia	Increased	please see introduction
Bipolar Affective disorder		
- manic state	Increased	Andreazza et al 2007
- depressive state	Increased	Andreazza et al 2007
Alzheimer's disease	Increased	Petzold et al 2002
Down Syndrome	Increased	Royston et al 1999
Parkinson's disease	Increased	Marxreiter et al 2013
Neuromyelitis optic	Increased	Storoni et al 2012
Epilepsy	Increased	Griffin et al 1995
Intracerebral haemorrhage	Increased	Delgado et al 2006
HIV/AIDS dementia	Increased	Pemberton et al 2001

Table 1: Association of S100B with diseases. A summary of diseases in which S100B is known to be associated is shown. The mechanism of these increased S100B levels have, in most part, been attributed to altered astrocyte function.

S100B is associated with the progressive neurodegeneration seen in Alzheimer's disease. Individuals with Down's syndrome have higher incidence of Alzheimer's disease compared to the normal population. In Down's syndrome levels of activated S100B+ astrocytes are positively correlated to the number of amyloid and classical plaques (Royston et al., 1999). S100B and the number of S100B immunoreactive astrocytes found in the temporal neocortex of patients with intractable epilepsy are raised compared to normal controls (Griffin et al., 1995). When studying patients with intracerebral haemorrhage, a positive correlation was noted between plasma S100B levels and haemorrhage volume. S100B was also higher in patients with poorer prognostic outcomes (Delgado et al., 2006). In dementia associated with end stage HIV infection (AIDS dementia), levels were significantly higher in advanced disease, compared to the earlier disease stages. S100B is also positively correlated with rapid progression of illness (Pemberton et al., 2001). With regard to psychiatric illness, S100B is increased in active manic and depressive states in individuals with bipolar affective disorder compared to remission states (Andreazza et al., 2007).

1.5 S100B in schizophrenia

Reports of S100B in the literature have been inconsistent to date. Initial studies reported that S100B was raised in medicated patients compared with controls (Weismann et al., 1999). These findings were refuted in a further study, which found S100B levels to be decreased in medicated patients (Gattaz et al., 2000). Subsequent studies identified higher levels of S100B in patients with schizophrenia who were un-medicated, and a correlation was noted between S100B levels and length of medication free period (Lara et al., 2001).

Two possible explanations for these findings may be that the initial pathogenic insult to brain, at the onset of psychotic symptoms, leads to increased S100B release; however, schizophrenia is now viewed to be likely a developmental disorder and it seems likely that pathological structural brain changes occur long before the positive symptoms of schizophrenia materialize. Another explanation for this pattern of S100B findings is that

indeed at the point of sampling, during a psychotic episode, S100B levels may be raised, and that following treatment with antipsychotic medication, S100B levels decrease. This may be specific to certain antipsychotic medications, although replicated studies looking specifically at the effect of different antipsychotic medications are not extensive.

Previous studies have measured S100B over a number of short time points, and no reported longitudinal studies were noted. It has been reported that negative symptoms correlate with S100B levels ([Rothermundt et al., 2001](#)). Both Lara et al. and Rothermundt et al. found increased levels of S100B in un-medicated patients with schizophrenia. A further study contradicted these findings, stating that levels of S100B were higher in the first week after initiation of medication. These S100B levels then decreased over subsequent weeks following initiation of medication, as reported in previous studies ([Schroeter et al., 2003](#)). Our studies aimed to study serum S100B levels in patients with schizophrenia treated with the second-generation antipsychotic medication clozapine. We hypothesized that clozapine medication may induce immune silence in patients with schizophrenia.

2. Results

2.1. Demographic data of patient cohort studied

In order to determine changes in S100B levels in patients diagnosed with schizophrenia, the demographics of control and patient cohorts were initially analysed. In addition, the patient cohort was scored for both negative and positive symptoms using the SANS and BPRS rating scales, respectively. A total number of 97 subjects diagnosed with schizophrenia, treated with clozapine, and 27 healthy control subjects were recruited (**Table 2**). The age range of the healthy controls was 25–57 years of age (with mean age $\pm$ standard deviation as 42.4 ± 10.3) and for patients treated with clozapine medication was 22–78 years of age (with mean age $\pm$ standard deviation as 42.5 ± 12.2). No statistical difference was found in age between these two groups ($p = 0.962$, Student's unpaired t-test, two-tailed). While the male: female ratio was 57 % for the patient group, this ratio was 41 % for the control group. The BPRS and SANS rating for the patient group was 31 ± 9 and 34 ± 18 , respectively (**Table 2**). This data indicates a typical patient population treated with clozapine displaying both positive and negative symptoms.

2.2. Metabolic parameters altered in clozapine treated patient group

Cardiovascular disease is the leading cause of natural death in patients with schizophrenia (Osby et al., 2000). Furthermore, it has been reported that patients diagnosed with schizophrenia have abnormal metabolic parameters prior to treatment with psychotropic medications (Spelman et al., 2007). In addition, previous studies show that clozapine increases the incidence of hyperglycaemia, diabetes and metabolic syndrome (Koller et al., 2001). Here, a number of cardiovascular risk factors were investigated for both patient and control groups. The fasting glucose levels found in the patient group (5.52 ± 0.09 mMol/L) compared to control group (5.06 ± 0.16 mMol/L) was significantly raised ($p = 0.0167$, Student's unpaired t-test, two-tailed) (**Table 3**). Furthermore, statistically significant abnormalities in Triglyceride (2.33 ± 0.13 mMol/L vs. 1.11 ± 0.12 mMol/L) ($p = 0.0001$, Student's unpaired t-test, two-

tailed) and HDL (1.07 ± 0.03 vs. 1.41 ± 0.11 mMol/L) ($p = 0.0001$, Student's unpaired t-test, two-tailed) levels were observed between patient and control groups (**Table 3**). Notably, the levels of LDL (3.03 ± 0.11 vs. 3.20 ± 0.18 mMol/L) ($p = 0.4579$, Student's unpaired t-test, two-tailed) and total cholesterol (5.08 ± 0.13 vs. 5.11 ± 0.16 mMol/L) ($p = 0.9117$, Student's unpaired t-test, two-tailed) were not different between patient and control groups. These abnormal triglyceride and HDL levels likely predispose the patient group to an increased risk of cardiovascular and cerebrovascular disease. In addition, this data is in keeping with previous observations that clozapine may alter metabolic parameters (Koller et al., 2001).

2.3. Clozapine treated patient cohort display increased BMI

Given the observations that metabolic parameters were altered in the patient cohort (**Table 4**) and that previous evidence has linked clozapine to weight gain (Choong et al., 2012); further analysis on cardiovascular risk was determined. In the current study, the Framingham Score, a prediction of a cardiovascular event occurring over the next 10 years, was calculated in the patient population analysed. No significant difference in the Framingham Score between patients and controls ($7.01 \pm 0.62\%$ vs. $5.31 \pm 0.89\%$) ($p = 0.1797$, Student's unpaired t-test, two-tailed) was observed. In addition, an approximate 2-fold increase was observed in smoking rate between patient (61 %) and control (30 %) groups (**Table 4**). This finding has been well documented in patients with schizophrenia [60]. The data also showed a greater than 100-fold increase in diagnosis of type 2 diabetes within the patient group (6 %) compared to control group (0.04 %) was noted (**Table 4**). Importantly, a statistically significant difference in BMI (32.45 ± 0.62 vs. 27.53 ± 0.86) between patient and control groups ($p = 0.0002$, Student's unpaired t-test, two-tailed) was observed (**Table 4**). This increase in BMI for the patient group is in concordance with the previous known association between patients treated with clozapine medication and subsequent weight gain (Choong et al., 2012).

2.4. Serum levels of S100B in patients treated with clozapine and healthy control subjects

A number of previous studies have suggested that the levels of S100B protein are altered in patients with schizophrenia, with the general observation that S100B levels are increased (Dagdan et al., 2011; Hohoff et al., 2010; Steiner et al., 2006; van Engelen et al., 1992; Nygaard et al., 1997; Wiesmann et al., 1998). While some controversy exists, studies suggest that medication status may alter S100B concentrations (Zhang et al., 2010; Wiesmann et al., 1999; Schroeter et al., 2003; Rothermundt et al., 2001; Gattaz et al., 2000). Here, a study to further investigate the role of S100B in schizophrenia was conducted comparing patients treated with clozapine and healthy controls. The data showed no statistical significant difference in S100B serum concentration between patients treated with clozapine (79.48 ± 4.04 ng/L, $n = 97$) and control groups (67.78 ± 4.01 ng/L, $n = 27$) ($p = 0.1438$, Student's unpaired t-test, two-tailed) (Figure 4). These results suggest that S100B serum concentration in patients with schizophrenia, treated with clozapine, are not significantly altered when compared to healthy controls, when analysing the whole sample population.

2.5. S100B serum concentration is not related to age, treatment time or illness severity

Previous reports have suggested a correlation between the levels of S100B and age (Schmitt et al., 2005; van Engelen et al., 1992; Wiesmann et al., 1998), antipsychotic medication (Wiesmann et al., 1999; Qi et al., 2009; Schroeter et al., 2009; Schroeter et al., 2003; Lara et al., 2001; Rothermundt et al., 2001; Gattaz et al., 2000), illness severity and symptoms (Rothermundt et al., 2001; Rothermundt et al., 2004). However, these reports have in best part been conflicting. Thus, to further investigate these discordant findings, the age, treatment time and illness severity of participating subjects was recorded and plotted against S100B serum concentration. The data showed no significant correlation between the levels of S100B and age (Pearson's correlation; $r = -0.06026$; p value (two tailed) = 0.7653 for control, Pearson's correlation; $r = 0.09035$; p

value (two tailed) = 0.3763 for clozapine treated patients) (**Figure 5A**), treatment time (Pearson's correlation; $r = -0.02633$; p value (two tailed) = 0.7980) (**Figure 5B**), acute illness severity (BPRS) (Pearson's correlation; $r = 0.04655$; p value (two tailed) = 0.649) (**Figure 6A**) or negative symptoms illness severity (SANS) (Pearson's correlation; $r = 0.1874$; p value (two tailed) = 0.8547) (**Figure 6B**), where the alpha value = 0.05.

2.6. Effects of medication on S100B serum concentration

While some studies have reported the levels of S100B to be altered dependent on medication, these reports have been contradictory (Wiesmann et al., 1999; Qi et al., 2009; Schroeter et al., 2003; Schroeter et al., 2009; Lara et al., 2001; Rothermundt et al., 2001; Gattaz et al., 2000). Furthermore, little is known about the direct effects of antipsychotic medication on glial cells within the central nervous system. To determine whether choice of antipsychotic medication effects the levels of S100B, this study also compared patients treated with clozapine ($n=97$) and those treated with depot antipsychotic medications comprising zuclopentixol ($n=9$), flupenthixol decanoate ($n=10$), risperidone ($n=10$), flupheazine decanoate ($n=3$) and haloperidol ($n=2$). The results showed no statistical significant difference between clozapine treated patients (79.48 ± 4.04 ng/L, $n = 97$), depot antipsychotic treated patients (73.08 ± 6.64 ng/L, $n = 34$) and healthy controls (67.78 ± 4.01 ng/L, $n = 27$) ($p = 0.2706$, clozapine treated patients and antipsychotic treated patients vs. control, one-way ANOVA and Bonferroni post-hoc test) (**Figure 7**). Taken together these data suggest that choice of antipsychotic medication has no significant effect on S100B serum concentration in patients with schizophrenia.

2.7. S100B levels are elevated in female patients with schizophrenia

Given that previous studies have suggested that the levels of S100B may be dependent on gender (Nygaard et al., 1997; Wiesmann et al., 1998; Gazzolo et al., 2003), in the current study these levels were examined in male and female healthy control and patient groups. The data showed a statistically significant increase in levels of S100B in female patient group (97.78 ± 10.34 ng/L, $n = 29$) compared to male patient group

(73.24 $\pm$ 3.70 ng/L, n = 68) (p = 0.0101, one-way ANOVA and Bonferroni post-hoc test) (**Figure 8**). Additionally, a statistically significant difference was found in S100B serum concentration between female patients and female controls (68.24 $\pm$ 4.72 ng/L, n = 17) (p = 0.0101, one-way ANOVA and Bonferroni post-hoc test) (**Figure 8**). In contrast, no statistical difference was observed between male patient group and male control group (67.00 $\pm$ 7.61 ng/L, n = 10) (as determined by one-way ANOVA and Bonferroni post-hoc test) (**Figure 8**). The data is supportive of previous studies indicating the levels of S100B differ in male and female genders. Studies have suggested that adipocytes release S100B, in addition to astrocytes. Furthermore reports show that insulin reduces the levels of S100B in adipocytes and astrocytes (Steiner et al., 2010b). Of interest, overweight, visceral obesity and insulin resistance may be correlated with levels of S100B in schizophrenia (Steiner et al., 2010a). Therefore, in addition to S100B being a marker for aberrant astrocyte function, it may also be associated with altered adipocyte cellular function.

2.8. Levels of S100B correlate with BMI in female patients with schizophrenia

To further examine if metabolic factors are associated with increased serum levels of S100B in females, a correlation between BMI and levels of S100B were examined in male and female patients. As indicated above, the data showed a statistically significant difference in BMI (32.45 $\pm$ 0.62 vs. 27.53 $\pm$ 0.86) between patient and control groups (p = 0.0002, Student's unpaired t-test, two-tailed) (**Table 4**). Further analysis showed that female patients (35.31 $\pm$ 1.16, n = 29) displayed increased BMI compared to male patients (31.37 $\pm$ 0.69, n = 68) and compared to female controls (27.38 $\pm$ 1.01, n = 17) and male controls (27.24 $\pm$ 1.69, n = 10) (p = 0.0001, male patients and female controls vs. female patients, one-way ANOVA and Bonferroni post-hoc test) (**Figure 9**). In addition, S100B serum concentration plotted against BMI showed a statistically significant correlation (Pearson's correlation; $r=0.4868$; p value (two tailed) = 0.0117) in female patients with schizophrenia treated with clozapine (**Figure 10B**) but was not correlated in the male patient group (Pearson's correlation; $r=-0.2395$; p value (two tailed) = 0.0511) (**Figure 10A**). An increase in BMI in the female patient group

compared to male patient group and control groups is likely associated increased adipose tissue and is in agreement with the hypothesis that S100B protein in serum is influenced by not only altered release from astrocytes but also due to release from adipocytes in schizophrenia.

Demographic Data	Patient Group	Control Group
Subjects (Male:Female)	97 (68:29)	27 (10:17)
Age	43 ± 12	42 ± 10
BPRS (18 - 126)	31 ± 9	n/a
SANS (0 - 125)	34 ± 18	n/a

Table 2: Demographic Data. Demographics of patients treated with clozapine and healthy control subjects. Brief Psychiatric Rating Scale (BPRS), Scale for the Assessment of Negative Symptoms (SANS). All values reported as mean +/- standard deviation. The range of values is shown in brackets, where indicated.

Biochemical Data	Patient Group	Control Group	Significance	
Fasting Glucose	5.52 ± 0.09	5.06 ± 0.16	<0.001	***
Triglycerides	2.33 ± 0.13	1.11 ± 0.12	<0.001	***
HDL	1.07 ± 0.03	1.41 ± 0.11	<0.001	***
LDL	3.03 ± 0.11	3.20 ± 0.18	0.358	
Total Cholesterol	5.08 ± 0.13	5.11 ± 0.16	0.912	

Table 3: Biochemical Data. Biochemical data of patients treated with clozapine (n = 97) and healthy control subjects (n = 27). All biochemical values expressed as mMol/L. Statistical analysis was performed using Student’s unpaired T test (two-tailed).

CVD Risk Factors	Patient Group	Control Group	Significance
Smoking Status (+)	58/95 (61%)	8/27 (30%)	~2 fold increase
Diabetes Mellitus (+)	6/95 (6%)	1/27 (0.04%)	>100 fold increase
Body Mass Index (BMI)	32.45 ± 0.62	27.53 ± 0.86	<0.001 ***
Hypertension Treatment	24/95 (25%)	6/27 (22%)	no change
Systolic Blood Pressure	123 ± 1.5	122 ± 1.9	0.568
Total Cholesterol	5.08 ± 0.13	5.11 ± 0.16	0.912
Framingham Score (%)	7.01 ± 0.62	5.31 ± 0.89	0.180

Table 4: Cardiovascular Disease Risk Factor Data. Cardiovascular disease risk factors data and Framingham-10-year (%) risk of cardiovascular disease incident of patients treated with clozapine (n = 97) and healthy control subjects (n = 27). Data expressed as mean ± SEM. Statistical analysis was performed using Student’s unpaired T test (two-tailed).

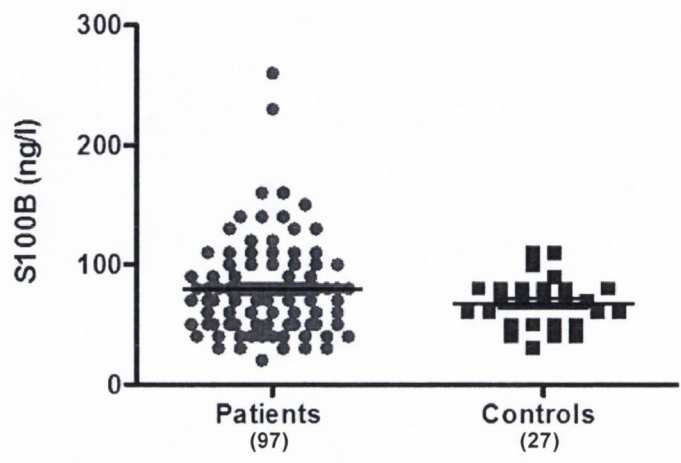


Figure 4: S100B concentration in patients treated with clozapine and healthy controls. Data shows S100B concentration for patients treated with clozapine (79.48 $\pm$ 4.04 ng/L, n = 97) and healthy control subjects (67.78 $\pm$ 4.01 ng/L, n = 27). The data showed no statistical significant difference in S100B serum concentration between patients treated with clozapine and control groups (p = 0.1438, Student's unpaired t-test, two-tailed). All statistical analysis was performed using Student's unpaired T test (two-tailed). Number of subjects in parenthesis (patients:controls).

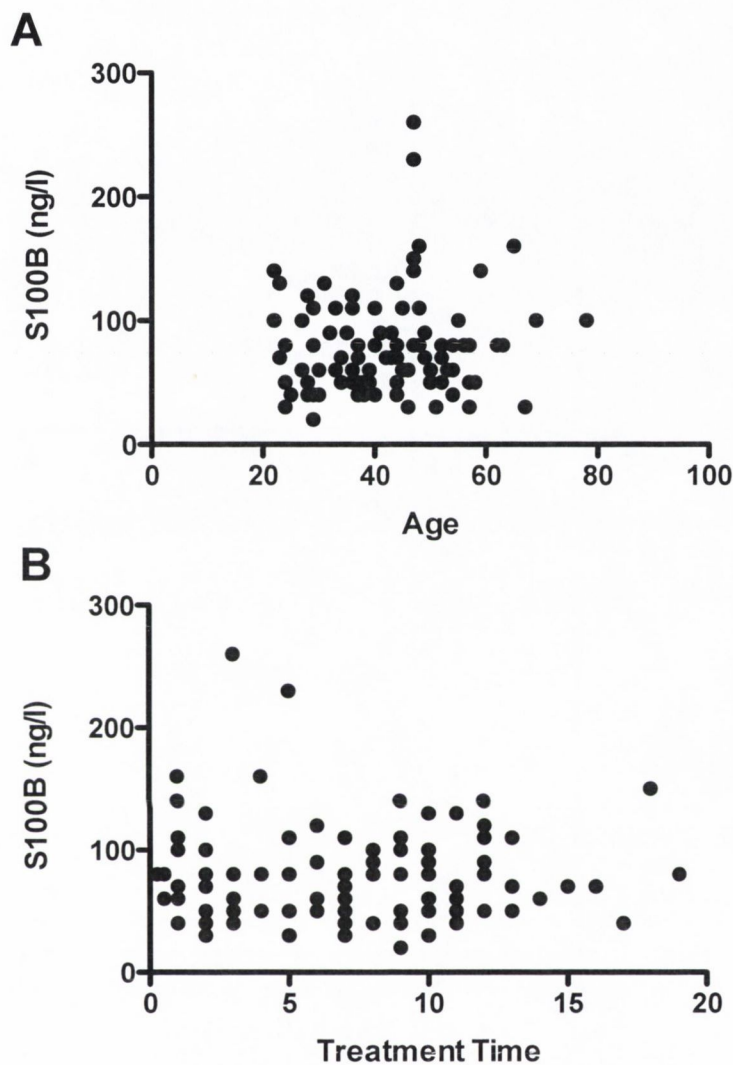


Figure 5: Correlation between S100B and age or clozapine treatment time. S100B concentration plotted against (A) age, (B) time on clozapine treatment in years. No notable correlations between S100B concentration any of these parameters in patient group were observed: age (Pearson’s correlation; $r = -0.06026$; p value (two tailed) = 0.7653 for control, Pearson’s correlation; $r = 0.09035$; p value (two tailed) = 0.3763 for clozapine treated patients) or treatment time (Pearson’s correlation; $r = -0.02633$; p value (two tailed) = 0.7980).

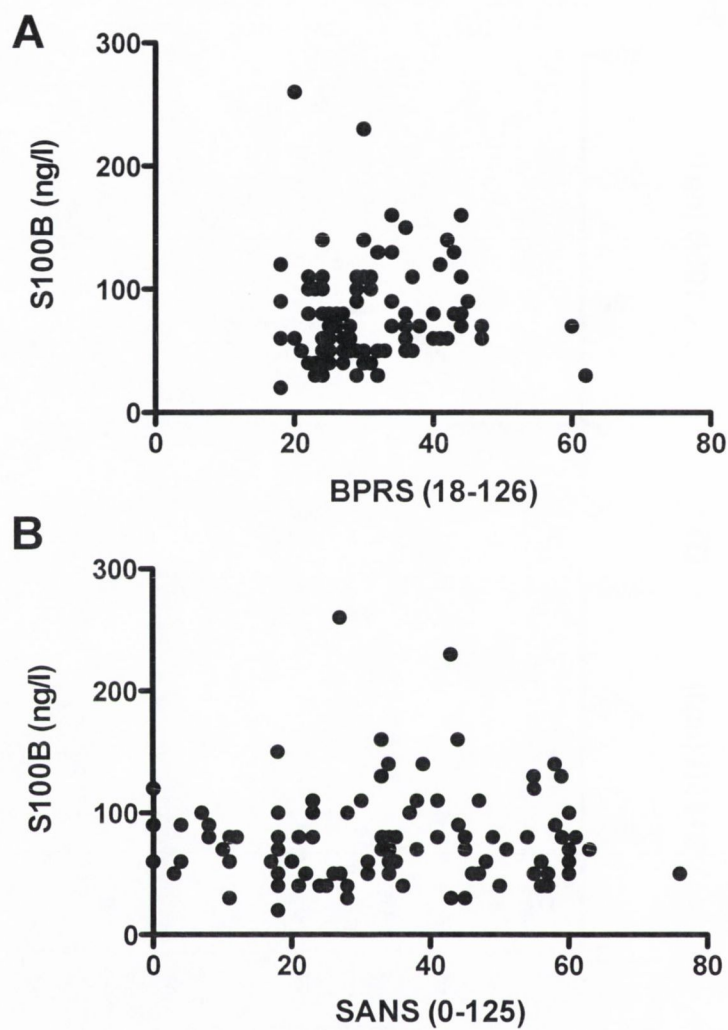


Figure 6: Correlation between S100B and BRPS or SANS. S100B concentration plotted against (A) BPRS and (B) SANS. No notable correlations between S100B concentration any of these parameters in patient group were observed: acute illness severity (BPRS) (Pearson’s correlation; $r = 0.04655$; p value (two tailed) = 0.649) or negative symptoms illness severity (SANS) (Pearson’s correlation; $r = 0.1874$; p value (two tailed) = 0.8547), where the alpha value = 0.05.

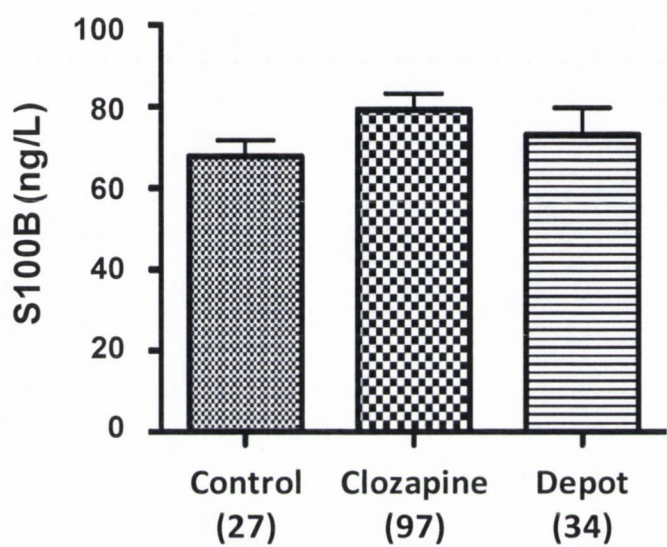


Figure 7: Levels of S100B in patients treated with depot medications. Graph shows S100B concentration for patients treated with clozapine and those treated with depot medication compared to healthy control subjects. No statistical significance was observed between the groups. Number of subjects in parenthesis. Patients treated with depot medications include those treated with zuclopentixol (n =9), flupenthixol (n = 10), risperidone (n = 10), flupenhazine (n = 3) and haloperidol (n = 2).

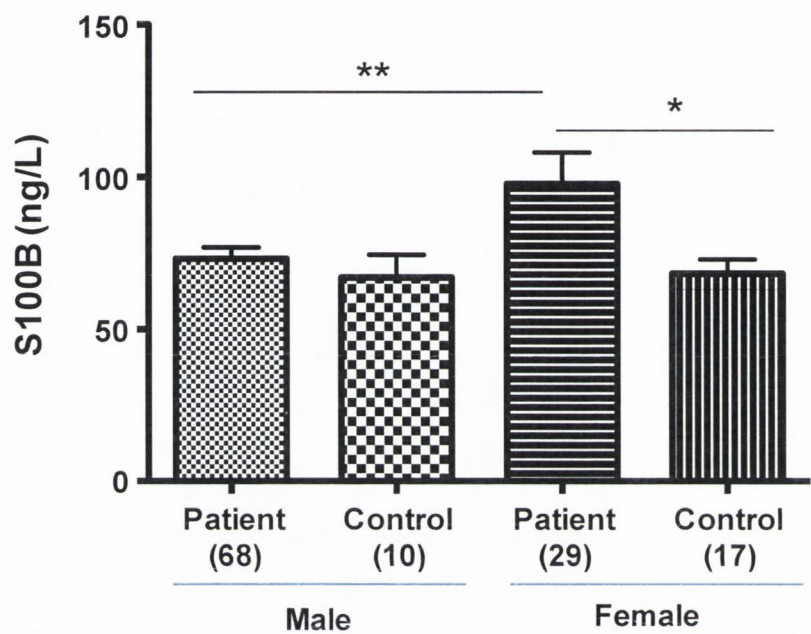


Figure 8: S100B levels in male and female patients treated with clozapine and healthy controls. Data shows significant statistical difference in S100B concentration between male (73.24 $\pm$ 3.70 ng/L, n = 68) and female (97.78 $\pm$ 10.34 ng/L, n = 29) patients treated with clozapine (p = 0.0101, one-way ANOVA and Bonferroni post-hoc test). S100B concentration in female patients showed significant statistical significance compared to female controls (68.24 $\pm$ 4.72 ng/L, n = 17) (p = 0.0101, one-way ANOVA and Bonferroni post-hoc test). No statistical difference was observed between male patient group and male control group (67.00 $\pm$ 7.61 ng/L, n = 10) (as determined by one-way ANOVA and Bonferroni post-hoc test). Data represented as mean $\pm$ SEM. Number of subjects in parenthesis.

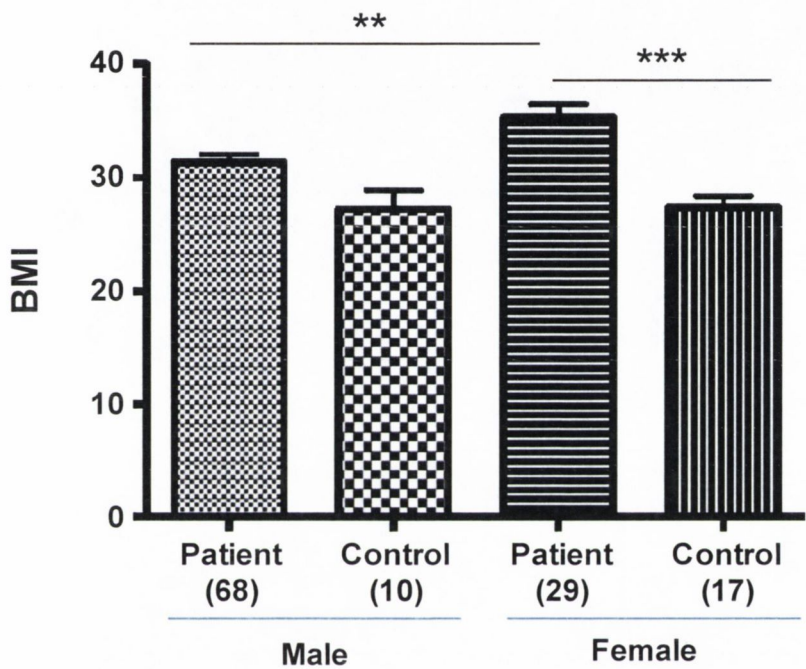


Figure 9: BMI levels in female patients treated with clozapine and healthy controls. Data shows significant statistical difference in BMI levels between male (31.37 ± 0.69 , $n = 68$) and female (35.31 ± 1.16 , $n = 29$) patients treated with clozapine. BMI levels in female patients also showed significant statistical significance compared to female controls (27.38 ± 1.01 , $n = 17$) and male controls (27.24 ± 1.69 , $n = 10$) ($p = 0.0001$, male patients and female controls vs. female patients, one-way ANOVA and Bonferroni post-hoc test). Data represented as mean $\pm$ SEM. Number of subjects in parenthesis.

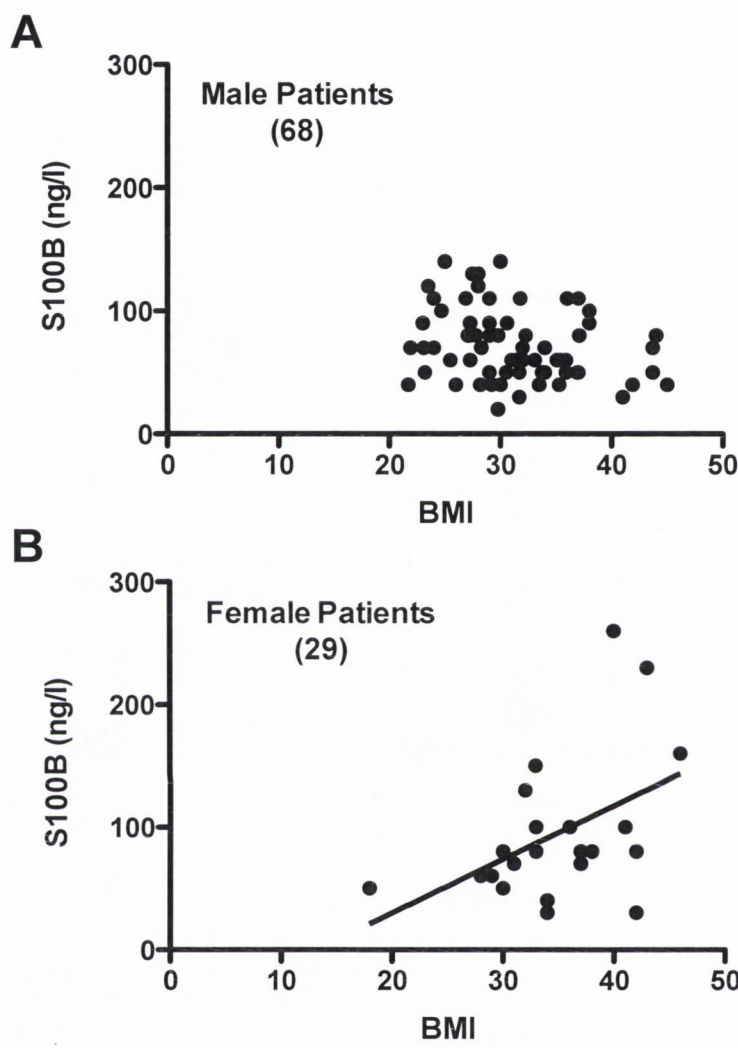


Figure 10: S100B concentration in female patients treated with clozapine and healthy control subjects. Correlation between S100B concentration and BMI for (A) male and (B) female patients treated with clozapine. A significant correlation was observed for S100B concentration and BMI in female patients treated with clozapine (Pearson $r = 0.4868$; p value (two tailed) = 0.0117). Number of subjects in parenthesis.

3. Discussion

In the current study 97 subjects diagnosed with schizophrenia, treated with clozapine, 34 patients treated with depot antipsychotic medications and 27 healthy control subjects were recruited. Fasting glucose levels, triglyceride and HDL were raised in the patients compared to healthy controls. As expected the patient group showed an increased percentage of subjects who smoked and increased numbers with a known diagnoses of type 2 diabetes compared to control. Patients diagnosed with schizophrenia also showed higher BMI levels compared to healthy controls. When comparing S100B levels, no statistical difference was found between patients and controls. The serum levels of S100B were also not correlated with age, treatment time or illness severity. Analysis of S100B serum concentrations in patients treated with the depot antipsychotic medications including zuclopentixol, flupenthixol, risperidone, flupheazine and haloperidol also showed no observable differences in S100B levels compared to control. Importantly, the data here showed that S100B levels were elevated in female patients with schizophrenia compared to male patients and to those female and male healthy control subjects. Of interest, BMI levels were also elevated in female patients compared to male patients and healthy control groups. A correlation analysis showed that levels of S100B increased with BMI in female patients with schizophrenia. Taken together, the data suggested that levels of S100B are altered in schizophrenia and these levels are also likely related to patients BMI, in addition to astrocyte dysfunction.

The question has emerged if S100B is a specific marker for astrocyte dysfunction in schizophrenia? The widespread cellular expression of S100B and the lack of disease specificity for this protein ([Schroeter et al., 2009](#); [Sen et al., 2007](#)), has questioned if S100B is a sole marker for astrocytes ([Steiner et al., 2011](#)). Astrocytes have diverse roles including (i) direct communication of astrocytic end-feet with endothelial cells that allows astrocytes to control the blood–brain-barrier; (ii) uptake of neurotransmitters, such as glutamate at the synaptic cleft, that allow astrocytes to regulate synaptic

transmission and excitotoxicity; and (iii) release of transmitters, growth factors, and cytokine/chemokines, allowing astrocytes to regulate cellular communication, migration and survival, for example of neurons and lymphocytes (Dev et al., 2008). It is significant that astrocytes also play a role in scar formation after CNS injury, thus having apparent opposing roles in physiology and pathophysiology. In disease, astrocytes have been suggested to play roles in a range of psychiatric, neurological and neurodegenerative disorders. Previous studies have focused on the role of S100B in astrocytes, and suggested that astrocyte dysfunction may increase release of S100B in schizophrenia. However, S100B is also released from many other cell types including adipocytes and a number of *in vitro* studies have shown that levels of S100B from these fat cells can be regulated by, for example, glucagon, adrenaline and insulin (Steiner et al., 2010b; Goncalves et al., 2010; Netto et al., 2006). Moreover, chronic fasting, weight gain and diet have also been shown to influence serum levels of S100B in patients and animal models (Netto et al., 2006; Holtkamp et al., 2008). The findings that S100B and BMI levels are elevated in female patients compared to male patients and to controls, and that S100B and BMI levels correlated in female patients (but not male patients), are in line with the hypothesis that visceral fat and altered adipocyte function could be a mechanism explaining elevated levels of S100B in schizophrenia (Steiner et al., 2010a; Barreira et al., 2012). On a more cautionary note, these results equally suggest that S100B can no longer be considered as a sole marker of astrocyte dysfunction in brain disease given its widespread distribution.

Previous studies have suggested that S100B levels in patients treated with clozapine are increased (Qi et al., 2009), which in part is in agreement with the data in the current study showing raised levels of S100B in female patients treated with clozapine. The effects of clozapine are thought to be mediated primarily via antagonism at 5HT_{2A}R and DA₄R, with weak DA₂R blocking activity (Meltzer et al., 1994). Astrocytes express DA₂Rs and activation of these receptors using apomorphine decreases the levels of S100B in these cells, via a signalling pathway that involves inhibition of adenylyl cyclase (Gazzolo et al., 2003). Moreover, antipsychotic medications (such as clozapine, haloperidol and

risperidone), which block DA2Rs, also decrease S100B levels (Nardin et al., 2011; Herrmann et al., 2012). Treatment of astrocytic C6 cells and oligodendrocytic OLN-93 cells with haloperidol and clozapine also decreases the levels of S100B (Steiner et al., 2010c). This data is in contrast with elevated levels of S100B observed in patients diagnosed with schizophrenia (Nardin et al., 2011). However, contradictory to the hypothesis that astrocyte derived S100B levels are raised in schizophrenia, is the finding that chronic antipsychotic medication, such as haloperidol or olanzapine, reduce astrocyte numbers in Macaque Monkeys (Konopaske et al., 2008). Recent data also demonstrated that insulin down regulates levels of S100B in adipocytes (in addition to astrocytes) suggesting that other cell types may determine the levels of S100B (Steiner et al., 2010a). Interestingly, molecular links between S100B, DA2Rs and schizophrenia have been suggested where S100B has been shown to interact with the third cytoplasmic loop of the DA2R, and to enhance receptor signalling to ERK and inhibition of adenylate cyclase (Liu et al., 2008; Stanwood et al., 2008; Hearst et al., 2010; Dempsey et al., 2011). In S100B transgenic animals, there is a decrease in the expression of DA2R suggesting crosstalk between S100B and DA2R function (Liu et al., 2011). Taken together, these studies support the S100B/DA2R protein complex as a molecular target for antipsychotic medications and possible aberrant S100B/DA2R-mediated signalling in schizophrenia.

4. Conclusions

This current study showed that levels of S100B are raised in female patients diagnosed with schizophrenia and correlate with BMI, which is possibly linked to higher levels of release from adipocytes. A limitation of this study was the unequal distribution of genders among the two groups (controls vs. patients). While ANOVA tests were performed, these assumed the sample size were equal and thus may have been too liberal. Another limitation of this study is the direct comparison of S100B levels between patients on medication and drug naive patients with schizophrenia. Previous studies have however reported that levels of S100B in drug naive patients with schizophrenia are raised (Zhang et al., 2010; Lara et al., 2001). Notably, however the levels of S100B were not correlated with BMI in these reports. In this current study we also did not investigate if the levels of S100B were associated with genetic mutations reported in schizophrenia. While it may be possible that our findings are explained, in part, by pathogenetic mechanisms, further studies would be required to determine this possibility, for example by investigating the levels of S100B in siblings of patients with schizophrenia. Moreover, further analysis of molecules that are more specific to adipocytes (rather than S100B) would be worthy of investigation. In addition, cellular studies demonstrating the effects of antipsychotic medications on the release of S100B in adipocytes would be warranted.

In closing, while a number of studies have demonstrated that levels of S100B are altered in schizophrenia, many of these reports are contradictory when suggesting that age, gender, medication and illness severity all have an impact (or not) on S100B levels. The study here suggested two important factors that may help unify these apparent contradictory findings. Firstly, that the serum S100B concentrations are likely influenced by metabolic activity. Secondly, that the levels of S100B are not solely dependent on astrocyte dysfunction and may involve altered fat cell (adipocyte) function. Thus, the data presented here supports previous studies that have suggested an association between metabolic syndrome, diabetes and immune response dysfunction in

schizophrenia. The clinical implications of this study are two-fold, firstly that S100B levels may no longer be considered as a biomarker that is dependent on neurological function alone, and secondly, that regulating metabolic dysfunction in schizophrenia may represent a novel drug target.

Chapter 4.
Investigating cytokines in schizophrenia.

Aims

- to investigate if levels of proinflammatory serum cytokines are raised in depot and clozapine treated patients with schizophrenia
- to conduct correlation analysis of cytokine serum levels with treatment time and participant age
- to determine if positive (BPRS) or negative (SANS) symptoms correlate with cytokine serum levels
- to demonstrate if cytokine serum levels are increased in female versus male patients
- to examine if altered BMI explains increased cytokine serum levels in female versus male patients
- to validate the use of 'cytokine signatures' in determining immune dysfunction in schizophrenia

Abstract

Background: The immune system is suggested to play a role in schizophrenia, as evidenced by altered serum and plasma cytokines levels in this illness. To date, however, the use of cytokines as markers for this illness is curtailed as they fail to separate patients from healthy controls with high enough fidelity.

Methods: Here, the levels of six cytokines (IL1 β , IL-6, IL-8, IL-17, IL-23, TNF α) were measured in serum samples obtained from patients with schizophrenia, treated with clozapine (n = 91) or depot medication (n = 36), and compared with healthy controls (n = 50). Individual cytokine levels were measured using dot-immunoblotting methods. Treatment time, patient age, gender, illness severity and metabolic parameters were correlated with these cytokine levels. In addition, a 'cytokine signature' was generated by summing all 6 cytokines and the benefit of this analysis, over single cytokine measurements, was examined.

Results: The levels of proinflammatory cytokines were raised, predominately in females, in depot and clozapine treated groups compared to healthy controls. Compared to individual cytokines, the 'cytokine signature' analysis showed less scatter of data. This 'cytokine signature' method thus improved separation between patient and control groups.

Conclusions: The use of analysing a 'cytokine signature' resulted in a greater than 2-fold increase in differentiation of patient from control groups compared to analysis of individual cytokines. This work supports the idea that 'cytokine signatures' may be better suited to investigate altered immune response in schizophrenia. Furthermore, the incorporating of additional cytokines to those analysed here, may lead to a 'cytokine signature' characteristic for schizophrenia.

1. Introduction

Schizophrenia occurs in approximately 1% of the population and presents as a well defined heterogenous set of clinical symptoms that affects cognitive function, perceptual experiences and belief systems. Although decades of research have been conducted we still know little about the cause of this illness and have been unable to elucidate why people acquire this disorder (Insel, 2010). Nevertheless, while having yet to attain full understanding of the biological mechanisms underlying the pathophysiology of schizophrenia, a neurodevelopmental model has been proposed with involvement of both environmental and genetic triggers (Barnett et al., 2007; Fatemi and Folsom, 2009; Kim et al., 2011).

Schizophrenia is also associated with a reduction in life expectancy, higher rates of cardiovascular disease (CVD) and increased rates of obesity, diabetes, hypertension, smoking and metabolic syndrome (Barnett et al., 2007). One potential explanation for these clinical observations is abnormal crosstalk between the immune, metabolic and central nervous systems (Müller and Schwarz, 2006). This altered crosstalk may potentially result from a dysfunction in cellular communication, hormonal signalling and/or neuronal networks (Müller and Schwarz, 2006). Previous studies have reported an increase in proinflammatory cytokines and a decrease in anti-inflammatory cytokines in schizophrenia (Müller and Schwarz, 2008). In addition, supportive evidence has demonstrated an anti-cytokine effect by some antipsychotic medications (Pollmacher et al., 2000; Sugino et al., 2009) and inflammation-related genes have been identified as up regulated in the brains of people diagnosed with schizophrenia (Saetre et al., 2007). Furthermore, several clinical trials have successfully used anti-inflammatory therapies such as COX-2 inhibitors (Müller et al., 2010), anti-TNF (Soczynska et al., 2009), aspirin (Laan et al., 2010) and anti-oxidants (Dodd et al., 2008) as adjuncts to antipsychotic medications.

With an increasing interest in looking beyond the central nervous system to explain the genesis of schizophrenia, the role of inflammation, unifying the behavioural and physical factors, is now gathering pace. A cytokine hypothesis has been suggested, where the serum concentrations of several pro-inflammatory cytokines are found increased in patients with schizophrenia (Kinney et al., 2010; Watanabe et al., 2010). These cytokines can alter neurotransmitter, neurodevelopmental and neural network activity and thus could be potential factors that induce psychiatric symptoms (Khairova et al., 2009). For example, elevation in pro-inflammatory cytokines (such as IL-1 β , IL-6, TGF- β and TNF- α) and chemokines (CCL2 and CCL4) have been found in patients with schizophrenia (Drexhage et al., 2010b). These raised levels of inflammatory markers in the serum of patients with schizophrenia have also been related to the disease state, metabolic factors and antipsychotic medication (Potvin et al., 2008; Miller et al., 2011b; Beumer et al., 2012). Studies linking single cytokines to schizophrenia have however been conflicting, possibly due to variations in the methods used and/or the clinical status and lifestyle of the patients. Another difficulty using serum-based biomarkers has been their inability to distinguish single individuals with schizophrenia from healthy controls with high reliability. In this regard, studies have reported the benefits of analysing a set of molecule signatures, rather than single molecules, found in the serum of patients with schizophrenia to distinguish them from controls (Schwarz et al., 2012).

We and others have examined the levels of a neurotrophic cytokine, S100B, in patients with schizophrenia, which is released from many cell types including those of the immune and central nervous systems, thus may represent a potential marker linking neuronal dysfunction and aberrant immune response in schizophrenia (Steiner et al., 2011; O'Connell et al., 2013). In addition, we have reported altered metabolic function and increased visceral fat in patients with schizophrenia (Thakore et al., 2002). Here, to further examine changes in immune function in patients diagnosed with schizophrenia, the levels of a set of cytokine levels were investigated, namely IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α . We chose these specific cytokines for analysis due to previous reports which have stated their altered levels associated with schizophrenia (*please see*

introduction chapter). While there is an increasing hypothesis that the 'immune-theory' of psychiatric disorders is related to a deregulated balance between pro- and anti-inflammatory cytokines, we choose to focus on pro-inflammatory cytokines in order to generate a combined 'cytokine signature' that would not be masked incorporating changes in anti-inflammatory cytokines? The relationships between levels of these six cytokines, gender, age, and treatment time and illness severity were examined. In addition, patients treated with clozapine and depot medication were investigated to determine the effects of medication. Finally, in order to distinguish patients with schizophrenia from healthy controls, the benefit of analysing a set of cytokines, generating a 'cytokine signature', compared to analysing individual cytokines was also demonstrated.

2. Results

2.1. Levels of proinflammatory cytokines are raised in depot and clozapine treated patients

To demonstrate altered immune function in schizophrenia, the levels of six proinflammatory cytokines, namely IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α , were examined in patients with schizophrenia compared to healthy controls (**Table 1**). In clozapine treated patients, a significant increase in levels of IL-1 β ($p<0.001$), IL-6 ($p<0.001$) and IL-8 ($p<0.001$), but not IL-17, IL-23 and TNF- α , compared to controls was observed (one-way ANOVA, Bonferroni post-hoc test) (**Figure 1**). In depot treated patients, a significant increase in levels of IL-1 β ($p<0.001$), IL-6 ($p<0.001$), IL-8 ($p<0.001$), IL-23 ($p<0.001$) and IL-17 ($p<0.05$), but not TNF- α compared to controls was also observed (one-way ANOVA, Bonferroni post-hoc test) (**Figure 1**). In addition, the levels of IL1 β ($p<0.05$) and IL-6 ($p<0.05$) in depot treated patients were raised in comparison to clozapine treated patients (one-way ANOVA, Bonferroni post-hoc test) (**Figure 1**). To study the value of examining the levels of all 6 cytokines in combination (i.e. the level of 'cytokine signature') over individual cytokines, the data from all 6 cytokines was summed. Significantly raised 'cytokine signature' compared to control (100%) was found in both clozapine (151%, $p<0.05$) and depot (196%, $p<0.001$) treated patients, with no difference between clozapine and depot treated patients (one-way ANOVA, Bonferroni post-hoc test).

2.2. The correlation of cytokine serum levels with treatment time and participant age

Analysis was also conducted to determine if treatment time and participant age had an impact on immune response. The mean treatment times were 7.03 $\pm$ 0.49 years (range 0.3-19 years) for clozapine and 6.06 $\pm$ 0.53 years (range 0.2-10 years) for depot treated groups, with no significant difference between the two groups ($p=0.35$, Student's unpaired t-test, two tailed). No significant correlation between levels of cytokines and treatment time was observed in either clozapine or depot treated patient groups

(**Figure 2, Table 2**). The mean age of healthy controls was 44.9 ± 1.6 years (range 22-63 years), for the clozapine group was 41.4 ± 1.2 years (range 22-69 years) and for depot groups was 49.6 ± 1.9 (range 28-70 years). Again, no significant difference in age was observed between control, clozapine or depot treated patients (one-way ANOVA, Bonferroni post-hoc test). Moreover, we deemed no noteworthy correlation between levels of cytokines and patient age (**Figure 3, Table 2**).

2.3. Positive (BPRS) or negative (SANS) symptoms did not correlate with cytokine serum levels

Given that levels of cytokines were altered in patients with schizophrenia versus healthy controls (**Figure 1**), a correlation between positive (BPRS) and negative (SANS) symptoms was investigated. The mean BPRS score of patients treated with clozapine was 30.3 ± 0.96 (range 0-62) and for depot medication was 26.4 ± 1.16 (range 18-48). A significant increase in BPRS score was observed between patients treated with clozapine compared to those on depot medication ($p=0.03$, Student's unpaired t-test, two tailed). A possible explanation for this observed increase may be that those patients on clozapine have a diagnosis of treatment resistant schizophrenia. Notably, no significant correlation between levels of cytokines and BPRS score was observed for patients treated with clozapine or depot medication (**Figure 4, Table 2**). The mean SANS score was 34.5 ± 1.88 (range 0-76) for the clozapine treated group and 39.8 ± 2.57 (range 0-70) for the depot treated group. In this case, no significant difference in SANS score was found between clozapine and depot patient groups ($p=0.12$, Student's unpaired t-test, two tailed). Again, no significant correlation between levels of cytokines and SANS score was observed for patients treated with clozapine or depot medication (**Figure 5, Table 2**).

2.4. Cytokine serum levels are increased in female versus male patients

Next the impact of gender on cytokine levels was examined. No significant difference was found in males vs. females within healthy controls, clozapine or depot patient

groups for any of the 6 cytokines examined (one-way ANOVA, Bonferroni post-hoc test). In addition, the levels of cytokines were not significantly different between male control, male clozapine or male depot groups (one-way ANOVA, Bonferroni post-hoc test). In contrast, the levels of some cytokines were raised in female clozapine patients (TNF α , $p < 0.05$) and female depot patients (IL-8, $p < 0.01$; IL-17, $p < 0.01$ and IL-23, $p < 0.01$) compared to female controls (one-way ANOVA, Bonferroni post-hoc test) (**Figure 6**). We also examined the summed levels of all 6 cytokines (i.e. 'cytokine signature'), where values were taken as a % of female healthy controls. In this analysis, we found the levels of 'cytokine signature' differed significantly in female clozapine and female depot groups as follows: control female (100%, $n=36$) < control male (172%, $n=14$) $\approx$ clozapine male (170%, $n=65$) $\approx$ depot male (188%, $n=28$) < clozapine female (257%, $n=26$, $p < 0.01$) < depot female (293%, $n=8$, $p < 0.001$) (one-way ANOVA, Bonferroni post-hoc test). This data is in agreement with our previous study suggesting raised levels of cytokines in patients with schizophrenia predominate in females (*please see previous chapter; O'Connell et al 2013*).

2.5. Altered BMI does not explain increased cytokine serum levels in female versus male patients

BMI is known to be significantly raised in patients with clozapine compared to first generation antipsychotic medications (Covel et al., 2004). Of interest, raised BMI is likely associated with increased levels of visceral fat and adipocyte number, that are a potential source of increased systemic cytokine levels (Fontana et al., 2007). As expected, we found patients on clozapine (32.4 ± 0.64) had significantly raised BMI levels compared to healthy controls (27.6 ± 0.64 , $p = 0.001$) and patients on depot medication (27.6 ± 0.75 , $p = 0.001$) (one-way ANOVA, Bonferroni post-hoc test). No significant difference was observed between BMI for healthy controls and patients treated with depot medication (**Figure 7**). The analysis of male and female BMI levels showed the following rank order: control male (28.4 ± 1.50) $\approx$ control female (27.3 ± 0.67) $\approx$ depot male (27.6 ± 0.93) $\approx$ depot female (27.6 ± 0.93) < male clozapine (31.7 ± 0.72) < female clozapine (34.0 ± 1.27) (**Figure 7**). Both male and female clozapine

treated groups showed a significant increase in BMI compared to control and depot groups (one-way ANOVA, Bonferroni post-hoc test) (**Figure 7**). A correlation between levels of cytokines and BMI was also conducted (**Figure 8, Table 2**). We deemed no noteworthy correlation between levels of cytokines and BMI in male or female healthy controls, clozapine or depot groups (**Figure 8, Table 2**).

2.6. The use of 'cytokine signatures' in determining immune dysfunction in schizophrenia

Population studies examining the levels of cytokines have revealed a great deal of information regarding the role of the immune system in disease. However, the correlation of cytokine/chemokine levels and illness state as a predictor of disease in individual patients has been more difficult limiting their use as *bone fide* biomarkers. To determine if disease state in single individuals is better predicted using a set of cytokines rather than single cytokines, the overlap in 'cytokine signatures' between healthy controls and patients with schizophrenia was investigated using 2-dimensional (2D) normalisation methods as we have reported previously ([Chatterjee et al., 2009](#)). Analysis of individual cytokines showed a poor separation of patients with schizophrenia from healthy controls in the upper right quadrant. Out of 127 schizophrenia patients (91 clozapine and 36 depot) the following number of patient samples were found separated from healthy controls in the upper right quadrant: IL-1 β (6 samples, i.e. 5%), IL-6 (2 samples i.e. 2%), IL-8 (3 samples i.e. 2%), IL-17 (1 sample, i.e. 1%), IL-23 (5 samples, i.e. 4%) and TNF- α (no patient samples, i.e. 0%) (**Figure 9**).

Next, a scatter plot using a 'cytokine signature' (i.e. sum of 6 cytokines signals for each patient) was generated (**Figure 10, signature face of graph**). In this case, less scatter along the x/y-axis was found compared to any of the individual cytokines analysed. Moreover, a greater number of patient samples were separated from healthy controls (17 samples, i.e. 13%) compared to the individual cytokines analysed (**Figure 10, signature face of graph**). These data suggest benefits in the use of 'cytokine signature' analysis, rather than single cytokines, to separate patients with schizophrenia from

healthy controls individuals. In an attempt to further separate individual patients from healthy controls, the 'cytokine signature' was also plotted against BMI (**Figure 10, signature x BMI face of graph**). The data showed that combined 'cytokine signature' and BMI analysis further separated individual patients from healthy controls, where 27 out of the 127 patients analysed (i.e. 21%) were clearly separated from healthy controls. In this 3D plot, we found three subpopulations, with controls in the lower quadrants, a number of clozapine treated patients (n = 19) in the high BMI quadrant and a number of depot treated patients (n = 8) in the high 'cytokine signature' quadrant. Taken together, these studies suggest there is better value in the use of cytokine signatures over individual cytokines as potential markers for schizophrenia, and the use of BMI further separates patients treated with clozapine vs. depot medication.

Data	Clozapine Group	Depot Group	Control Group	Significance
Subjects (Male:Female)	91 (65:26)	36 (28:8)	50 (14:36)	n/a
Age (Range) (Yr)	41.4±1.2 (22-69)	49.6±1.9 (28-70)	44.9±1.6 (22-63)	0.0001
Treatment Duration (Range) (Yr)	7.03 ±0.49 (0.3-19)	6.06 ±0.53 (0.2-10)	n/a	n/a
Daily Dose (Range) (mg/day)	432.1 ±185.7 (125-900)	various (see methods)	n/a	n/a
BPRS (Range 18 - 126)	30.3±0.96 (0-62)	26.4±1.16 (18-48)	n/a	n/a
SANS (Range 0 - 125)	34.5 ±1.88 (0.76)	39.8±2.57 (0-70)	n/a	n/a
Fasting Glucose	5.55 ± 0.91	5.37 ± 1.67	5.14 ± 1.42	0.1807
Triglycerides	2.36 ± 1.33	1.12 ± 0.57	1.12 ± 0.61	0.0001 ***
HDL	1.05 ± 0.10	1.48 ± 0.59	1.47 ± 0.44	0.0001 ***
LDL	2.95 ± 1.02	3.17 ± 0.93	3.30 ± 0.89	0.1131
Total Cholesterol	4.98 ± 1.19	5.16 ± 0.85	5.28 ± 1.05	0.2836
Smoking Status (+)	54/91 (59%)	10/36 (28%)	12/50 (24%)	~2 fold increase
Diabetes Mellitus (+ Medication)	6/91 (7%)	2/36 (6%)	2/50 (4%)	n/a
Body Mass Index (BMI)	32.4 ± 0.64	27.6 ± 0.75	27.6 ± 0.64	0.0001 ***
Hypertension Treatment	23/91 (25%)	4/36 (11%)	6/50 (12%)	~2 fold increase
Systolic Blood Pressure	123.9 ± 15.41	124 ± 12.8	123.5 ± 14.4	0.9682
Framingham Score (%)	7.00 ± 6.30	5.49 ± 4.70	6.04 ± 5.03	0.4086

Table 1: Demographic data, biochemical data & cardiovascular disease risk factor and lifestyle data. Demographics of patients treated with clozapine (n = 91), those treated with depot medication (n = 36) and healthy control subjects (n = 50). Brief Psychiatric Rating Scale (BPRS), Scale for the Assessment of Negative Symptoms (SANS). Cardiovascular disease risk factors data and Framingham-10-year (%) risk of cardiovascular disease incident. All values reported as mean +/- standard deviation. The range of values is shown in brackets, were indicated. All biochemical values expressed as mMol/L. Statistical analysis was performed using one-way ANOVA, Bonferroni post-hoc test.

		IL1β		IL6		IL8		IL17		IL23		TNFα	
		r	p	r	p	r	p	r	p	r	p	r	p
Treatment Time Correlation Values (Supplemental Fig. 1A)													
Clozapine		-0.08	0.47	-0.03	0.81	0.01	0.94	0.08	0.44	-0.02	0.83	0.04	0.71
Depot		-0.29	0.09	-0.10	0.56	-0.19	0.23	-0.24	0.16	-0.35	0.06	-0.33	0.06
Patient Age Correlation Values (Supplemental Fig. 1B)													
Healthy Controls		-0.16	0.26	-0.24	0.10	-0.17	0.24	-0.18	0.20	-0.14	0.33	-0.16	0.28
Clozapine		-0.01	0.94	-0.03	0.81	-0.12	0.27	-0.12	0.28	-0.05	0.63	0.03	0.80
Depot		-0.12	0.48	0.20	0.25	-0.20	0.23	0.44	0.01	0.44	0.01	0.40	0.02
BPRS Correlation Values (Supplemental Fig. 2A)													
Clozapine		-0.01	0.96	0.03	0.75	0.01	0.94	-0.02	0.87	0.02	0.84	-0.04	0.73
Depot		-0.03	0.10	-0.27	0.16	-0.16	0.36	0.00	1.00	-0.08	0.66	-0.13	0.44
SANS Correlation Values (Supplemental Fig. 2B)													
Clozapine		0.08	0.46	0.01	0.94	0.02	0.84	0.07	0.49	0.03	0.78	0.17	0.10
Depot		-0.24	0.17	-0.24	0.16	-0.19	0.27	-0.00	0.99	0.10	0.56	-0.15	0.40
BMI Correlation Values (Supplemental Fig. 3)													
Healthy Controls	Male	-0.34	0.23	0.26	0.37	-0.34	0.35	-0.46	0.19	-0.32	0.27	-0.25	0.38
	Female	-0.28	0.10	-0.19	0.28	-0.18	0.45	-0.16	0.36	-0.06	0.74	-0.19	0.26
Clozapine	Male	0.22	0.06	0.27	0.03	0.26	0.04	0.16	0.20	0.31	0.01	0.13	0.31
	Female	0.07	0.75	-0.04	0.86	-0.03	0.89	-0.02	0.93	-0.11	0.61	0.09	0.67
Depot	Male	-0.09	0.68	-0.24	0.20	0.08	0.72	0.23	0.28	0.01	0.97	-0.18	0.40
	Female	0.17	0.72	0.28	0.54	0.65	0.11	0.64	0.13	0.52	0.23	0.49	0.26

Table 2: Correlation values. Pearson’s correlations (r values) and p values (two tailed) are shown.

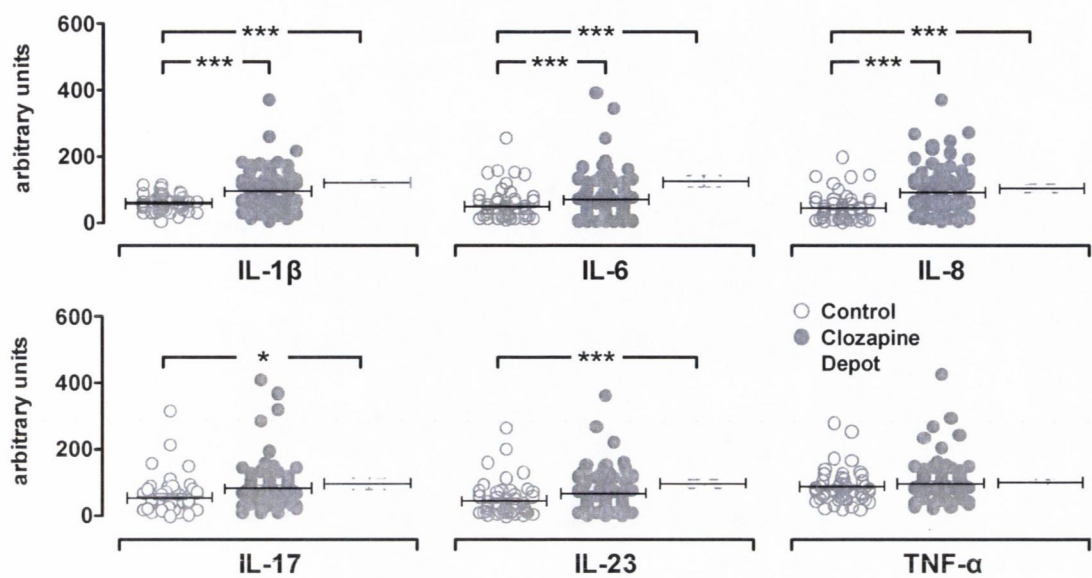


Figure 1: Levels of cytokines in patients diagnosed with schizophrenia. Serum samples were obtained from control subjects (n = 50), and patients treated with clozapine (n = 91) or depot medication (n = 36). Scatter plots show immunoreactive density levels of IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α analysed by dot blots (arbitrary units). Levels of IL-1 β (p<0.001), IL-6 (p<0.001) and IL-8 (p<0.001), but not IL-17, IL-23 and TNF- α , were raised in clozapine patients compared to controls. Levels of IL-1 β (p<0.001), IL-6 (p<0.001), IL-8 (p<0.001), IL-23 (p<0.001) and IL-17 (p<0.05), but not TNF- α were raised in depot patients compared to controls. Levels of IL-1 β (p<0.05) and IL-6 (p<0.05) in depot treated patients were raised compared to clozapine. Statistical analysis was performed using one-way ANOVA, Bonferroni post-hoc test.

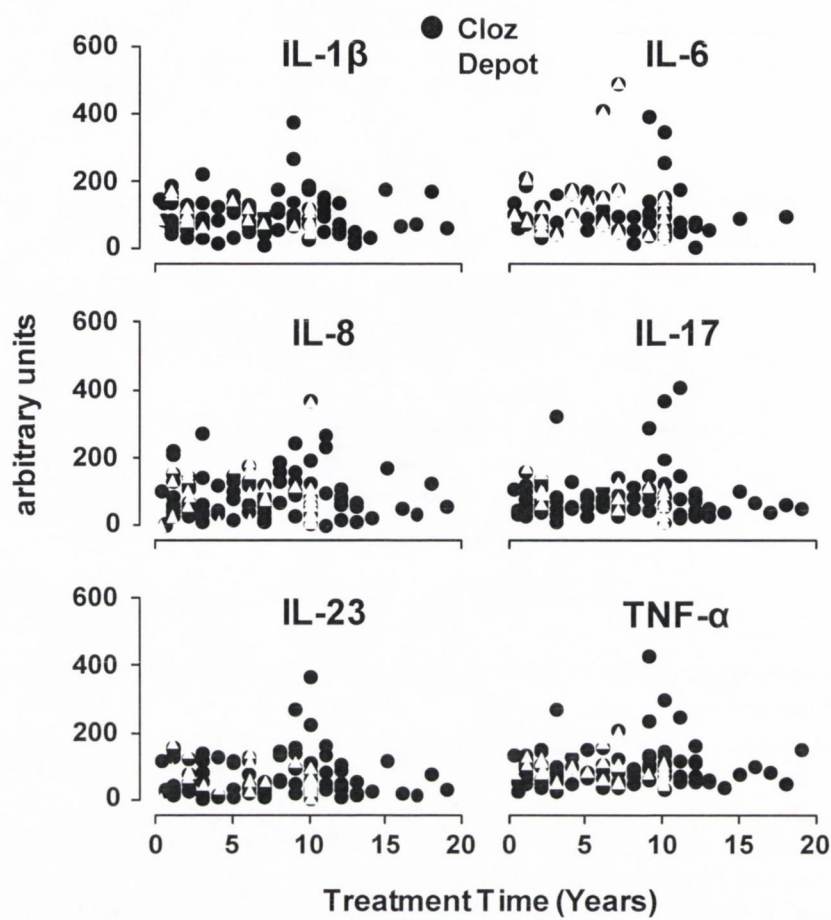


Figure 2: Correlation between cytokine levels and treatment time in patients with schizophrenia. Serum samples were obtained from control subjects (n = 50), and patients treated with clozapine (n = 91) or depot medication (n = 36). Correlation plots show treatment time plotted against the immunoreactive density levels of IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α analysed by dot blots (arbitrary units). No significant difference was observed between treatment times between any of the groups investigated. In addition, no correlation was observed between treatment time in any of the groups analysed (please see Table 2 for correlation values).

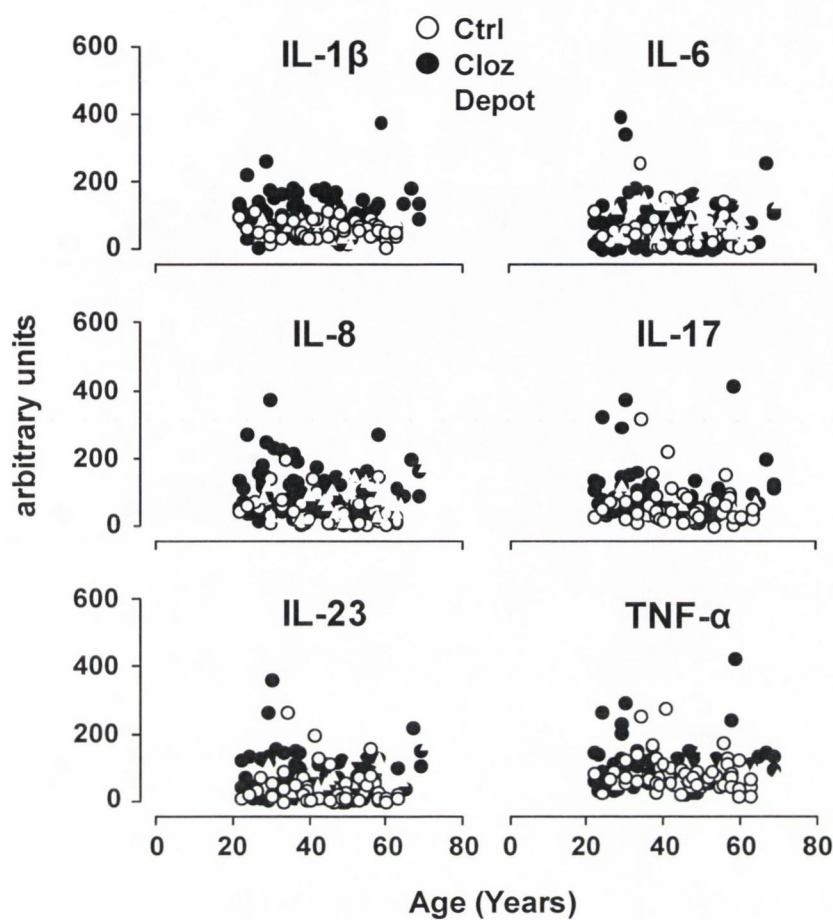


Figure 3: Correlation between cytokine levels and age in patients with schizophrenia. Serum samples were obtained from control subjects (n = 50), and patients treated with clozapine (n = 91) or depot medication (n = 36). Correlation plots show patient age plotted against the immunoreactive density levels of IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α analysed by dot blots (arbitrary units). No significant difference was observed between age in any of the groups investigated. In addition, no correlation was observed between treatment age in any of the groups analysed, with the exception of IL-17, IL-23 and TNF- α in the depot groups, which showed a positive correlation with patient age (please see Table 2 for correlation values).

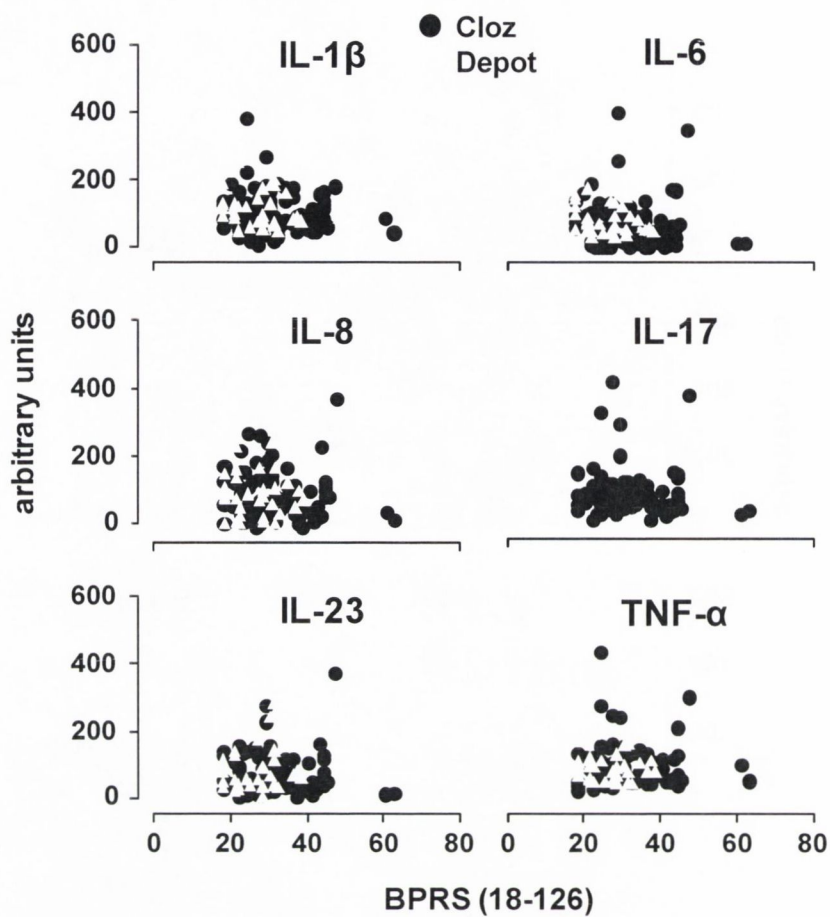


Figure 4: Correlation between cytokine levels and BPRS in patients with schizophrenia. Serum samples were obtained from control subjects (n = 50), and patients treated with clozapine (n = 91) or depot medication (n = 36). Correlation plots show BPRS (positive symptoms) score plotted against the immunoreactive density levels of IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α analysed by dot blots (arbitrary units). A significant increase was observed in BPRS score in the clozapine group compared to the depot group (p=0.026, Student’s unpaired t-test, two tailed). No correlation was observed between BPRS score in any of the groups analysed (please see Table 2 for correlation values).

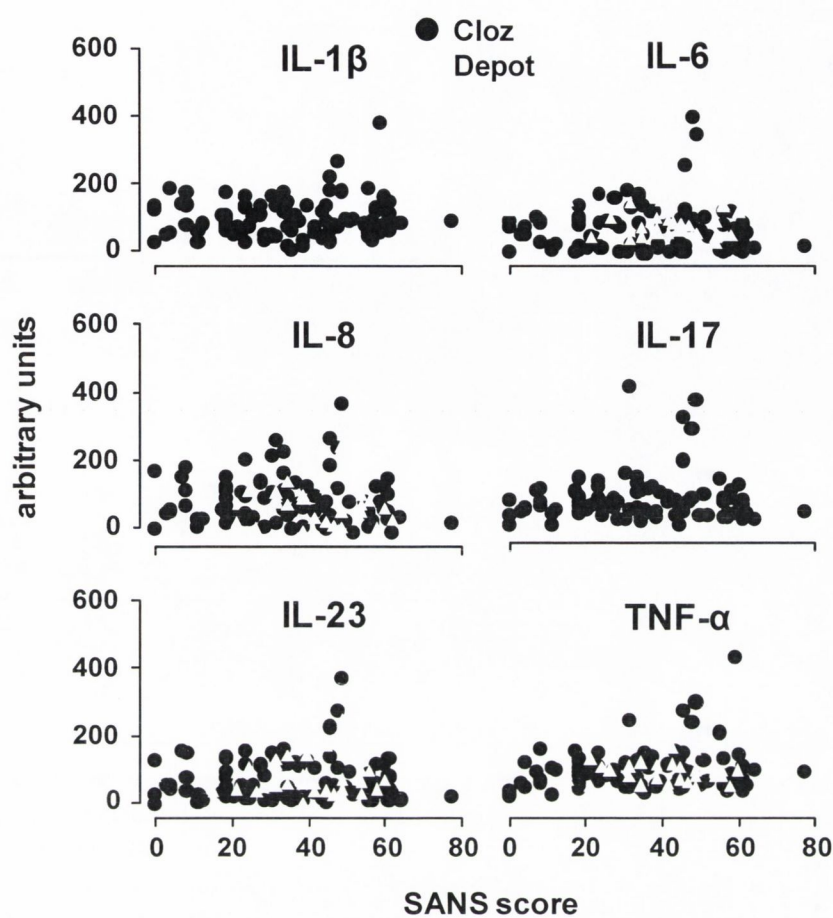


Figure 5: Correlation between cytokine levels and SANS in patients with schizophrenia. Serum samples were obtained from control subjects (n = 50), and patients treated with clozapine (n = 91) or depot medication (n = 36). Correlation plots show SANS (negative symptoms) score plotted against the immunoreactive density levels of IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α analysed by dot blots (arbitrary units). No difference in SANS score in the clozapine group compared to the depot group ($p=0.026$, Student’s unpaired t-test, two tailed). No correlation was observed between SANS score in any of the groups analysed (please see Table 2 for correlation values).

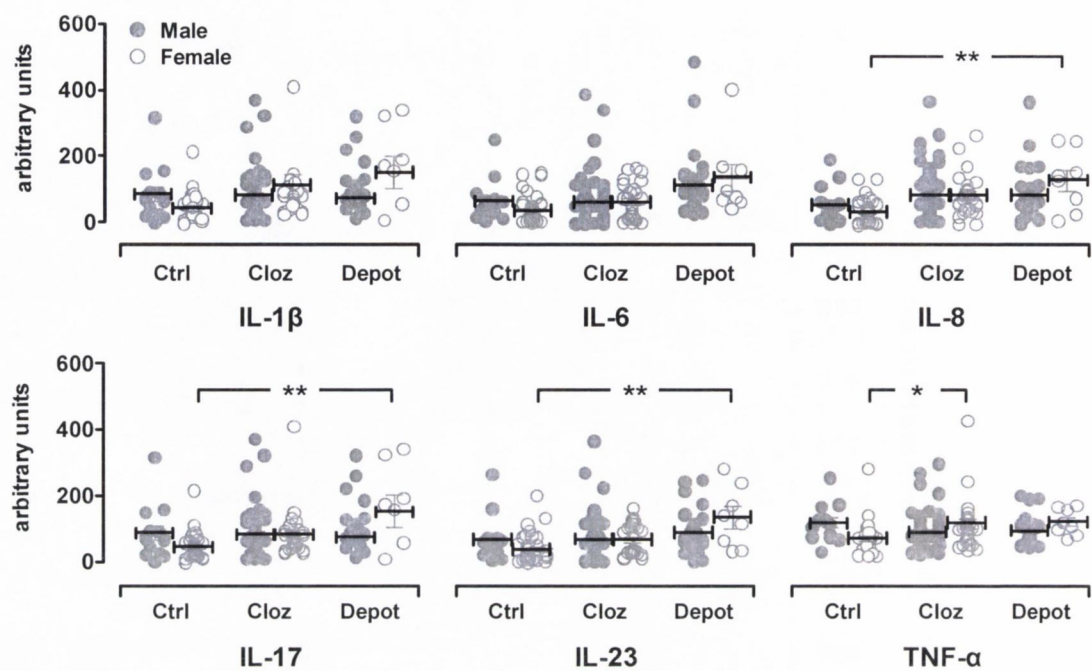


Figure 6: Levels of cytokines in male and female patients diagnosed with schizophrenia. Serum samples were obtained from control subjects (male, $n = 14$; female, $n = 36$), and patients treated with clozapine (male, $n = 65$; female, $n = 26$) or depot medication (male, $n = 28$; female, $n = 8$). Scatter plots show immunoreactive density levels of IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α analysed by dot blots (arbitrary units). The levels of cytokines were significantly raised in female clozapine patients (TNF- α , $p < 0.05$) and female depot patients (IL-8, $p < 0.01$; IL-17, $p < 0.01$ and IL-23, $p < 0.01$) compared to female controls (one-way ANOVA, Bonferroni post-hoc test).

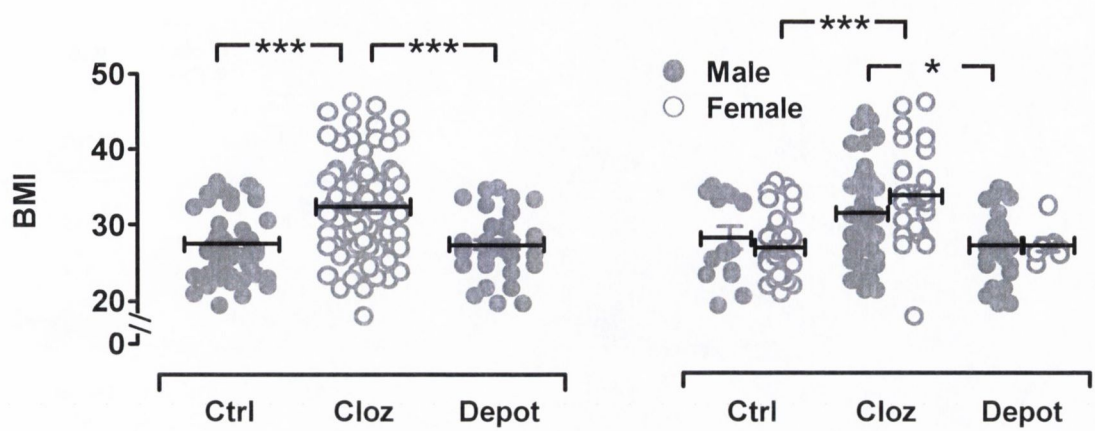


Figure 7: Levels of BMI in male and female patients diagnosed with schizophrenia. BMI values from control subjects (male, $n = 14$; female, $n = 36$), and patients treated with clozapine (male, $n = 65$; female, $n = 26$) or depot medication (male, $n = 28$; female, $n = 8$) are shown. Patients on clozapine had significantly raised levels of BMI compared to healthy controls and patients treated with depot medication ($p=0.001$; one-way ANOVA, Bonferroni post-hoc test). Both male and female clozapine groups showed a significant increase in BMI versus female control and male depot groups (clozapine male vs. female control $p=0.01$ and depot male $p=0.05$; clozapine female vs. female control $p=0.001$ and depot male $p=0.001$; one-way ANOVA, Bonferroni post-hoc test).

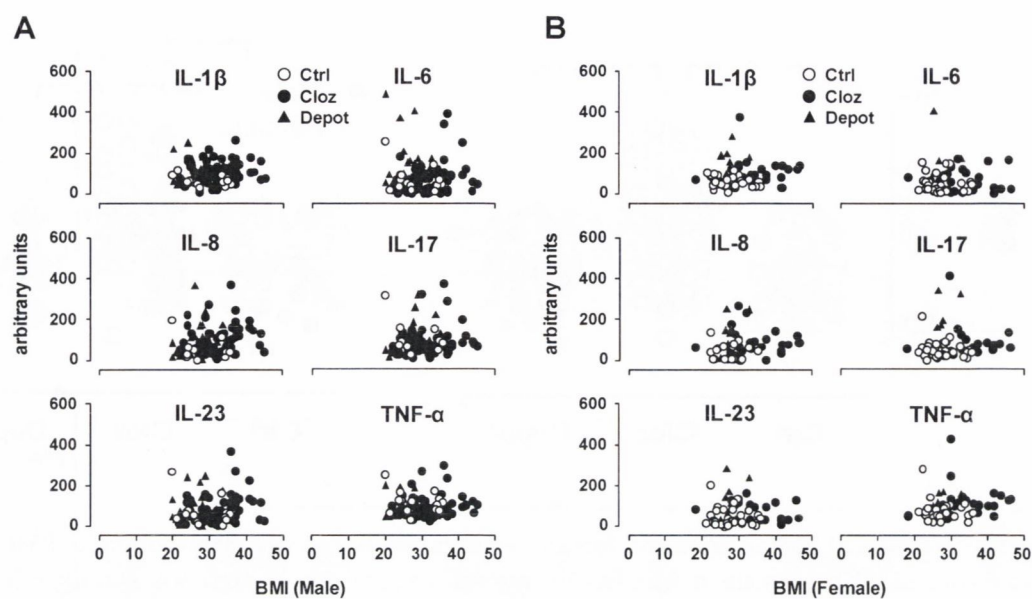


Figure 8: Correlation between cytokine levels and BMI in patients with schizophrenia. Serum samples were obtained from control subjects (male, n = 14; female, n = 36), and patients treated with clozapine (male, n = 65; female, n = 26) or depot medication (male, n = 28; female, n = 8). Correlation plots show BMI in (A) male groups or (B) female groups plotted against the immunoreactive density levels of IL-1 β , IL-6, IL-8, IL-17, IL-23 and TNF- α analysed by dot blots (arbitrary units). No significant correlation between levels of cytokines and BMI was observed for male or female healthy controls, patients on clozapine or patients treated with depot medication, with the exception of male patients on clozapine which showed a correlation between levels of the cytokines IL-6, IL-8 and IL-23 and BMI (see Table 2 for correlation values).

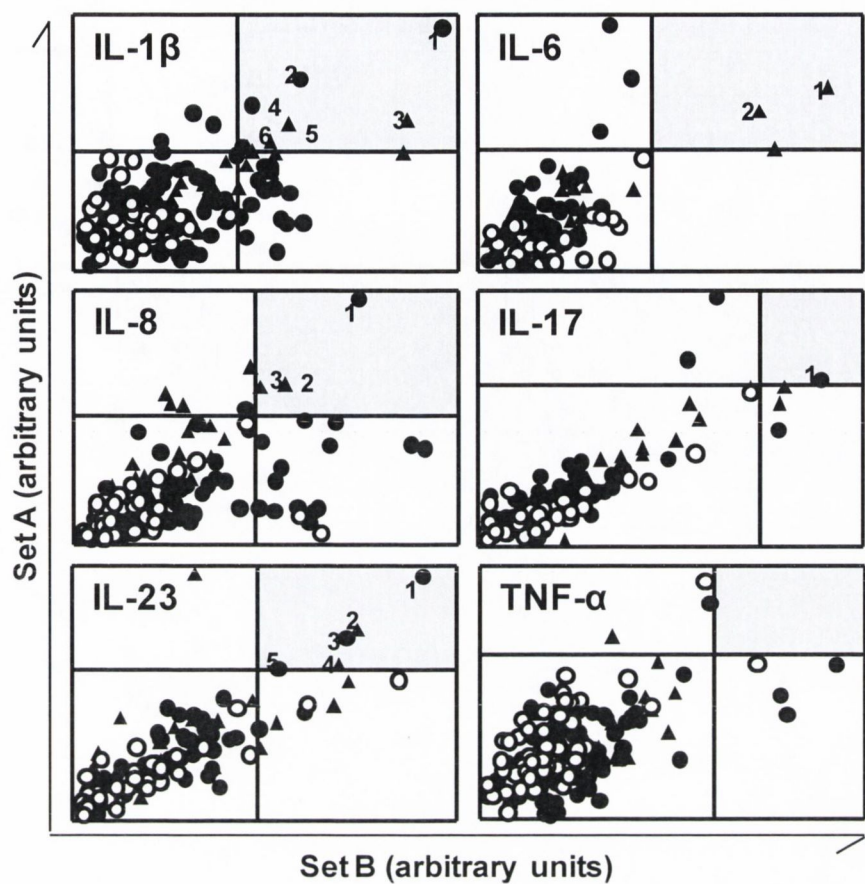


Figure 9: Cytokine signatures in patients with schizophrenia. Dot immunoblots were performed in two separate array sets (in duplicates) so that the data for individual cytokines could be subjected to 2-dimensional (2D) normalisation i.e. normalisation to remove both dot-to-dot and blot-to-blot variation, as we have reported previously (please see previous chapter; O’Connell et al 2013). A scatter plot for each cytokine was obtained by plotting the cytokine signals in the first array against that obtained in the second array. The samples lying at the extremities in this analysis displayed highest cytokine signals. The scatter plots were also divided into 4 quadrants, where x- and y-line cut-offs excluded all healthy control samples from the upper right quadrant.

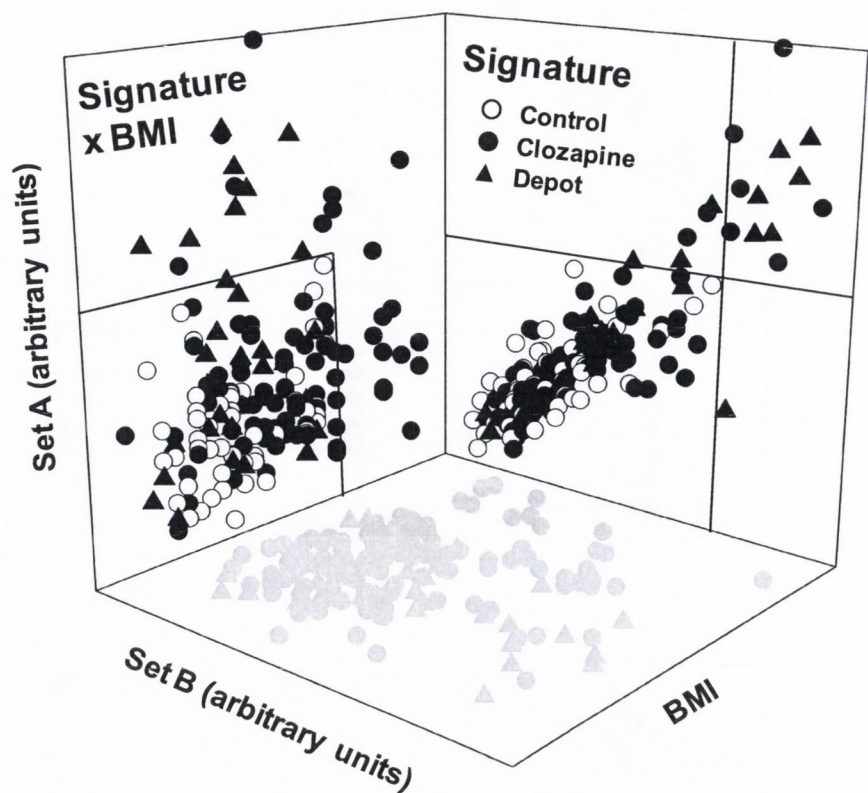


Figure 10: Cytokine signatures and BMI correlation in patients with schizophrenia. A 3D graph was generated by plotting the 2D cytokine signature data (signature face of graph) with BMI (signature x BMI face of graph). A greater number of samples were found separated from healthy controls using the cytokine signature compared to individual cytokines. The addition of BMI further improved the number of patients clearly separated from healthy controls.

3. Discussion

The current study examined the levels of cytokines in 91 patients with schizophrenia treated with clozapine and 36 patients treated with depot antipsychotic medication, as well as 50 healthy controls. Patients diagnosed with schizophrenia showed elevated levels of cytokines compared to control. Levels were also found to be modestly higher in those treated with depot medication compared to clozapine. Notably, there was no significant difference observed in treatment time or mean age between participating groups investigated. Furthermore, no noteworthy correlation of cytokine serum levels was found with treatment time or age of patients. While a significant increase in BPRS score was found in patients on clozapine compared to those on depot medication, no difference was noted in SANS score between the two groups; nor was there any correlation between BPRS or SANS score and levels of cytokines within the patient groups. We also found that levels of cytokines were increased in female patients with schizophrenia compared to control, although these raised levels of cytokines did not correlate with associated BMI levels. In this study, the six cytokines examined were summed and this 'cytokine signature' was also analysed. The level of 'cytokine signature' was raised in clozapine and depot patient groups compared to healthy controls. When stratifying for gender, the level of 'cytokine signature' was not altered between male controls, clozapine or depot treated groups. In contrast, female clozapine and female depot groups showed significantly raised level of 'cytokine signature' compared to female healthy controls. Taken together, this data showed higher levels of cytokines in patient groups compared to controls, where elevated levels were most prominent in female patients. Finally, analysis of 'cytokine signature' levels exhibited improved differentiation of individuals with schizophrenia from healthy controls, demonstrating the value of multiple cytokine profile analysis compared to that of individual cytokines.

Similar to our previous study, investigating the neurotrophic cytokine S100B (*Chapter 3*), these findings also show that the levels of both pro-inflammatory cytokines and BMI are

raised in female patients treated with clozapine. These results suggest a possible association between BMI and raised cytokine levels in this female patient group. However, in the current study, patients treated with depot medication had no significant differences in BMI compared to controls, while still displaying raised cytokine levels. Thus, the question remains as to why female patients with schizophrenia exhibit raised levels of pro-inflammatory cytokines? The observation that female patients are more predisposed to neurological diseases may provide some explanation for our findings. For example, it has been suggested that factors such as oestrogen may play a relevant role in regulating cytokine levels ([Czlonkowska et al., 2005](#)). Another question posed by this study is why patients treated with depot medication showed higher levels of cytokines compared to those treated with clozapine? One possible reason for this variance between clozapine and depot treatment may be due to differing immunomodulatory effects of specific antipsychotics on circulating systemic cytokines ([Røge et al., 2012](#)). Another reason for elevated levels of cytokines in depot treated patients, as opposed to clozapine, may be the potential unknown systemic low grade inflammatory effects of deep intramuscular injection. In the absence of a drug-naïve patient group and conclusive cellular studies investigating the effects of these drugs on cytokine levels in a range of cell types, these questions remain open.

A limitation of this study was the unequal distribution of genders among the three groups (controls, depot and clozapine treated patients). While ANOVA tests were performed, these assumed the sample size were equal and may therefore have been liberal. Another limitation of our study was our lack of inclusiveness of drug naïve and patient sibling groups, in helping decipher the role of genetics and impact of environment in our study. A caveat often observed in biomarker studies investigating levels of cytokines is the inability to separate healthy controls from patients with 100% fidelity. A number of previous studies have shown altered levels of cytokines associated with schizophrenia, however many of these studies have been contradictory in nature. One possible reason for this discrepancy may be due to technical limitations and lack of robustness in measuring single cytokines while investigating altered immune response.

4. Conclusions

The focus of previous research investigating cytokines in schizophrenia has often been with a view to identifying a clinical biomarker for this illness. By definition a biomarker needs to be reliable and reproducible across multiple laboratories and clinical settings and to be adequately sensitive and specific for that disease (ION consensus report, 2010). Previous studies regarding the cytokine profile in schizophrenia have been inconsistent in their findings and, as such, lack the attributes required of biomarkers in disease. Reasons for this discrepancy may include alterations in time of sample collection and lab technique and protocol. The subtle nature of cytokine changes and the effects of pathophysiological influences, other than that of schizophrenia, in the body may lead to masking of cytokine levels by a high noise to signal ratio, possibly contributing to variances between studies.

Given that schizophrenia appears to be a heterogeneous disorder it is important to consider that examining one cytokine, were it to be present, may not be appropriate to help categorise subtypes within this disorder. In our study, we aimed to account for these concerns by stratification of groups and by application of a 'cytokine signature', compared to the use of individual cytokines. We therefore hypothesized that the adoption of such a 'cytokine signature' model when examining altered immune response in disease may result in less contradictory findings between studies. We therefore suggest more studies directed toward answering such questions rather than focused solely on identifying individual cytokines as biomarkers for schizophrenia, which given the complexity of this illness, will unlikely yield auspicious results.

We note, however, that a 'cytokine signature' approach is also open to several questions such as (i) what are the minimal number of cytokines required to make a 'cytokine signature' (ii) which cytokines should be included in a 'cytokine signature' analysis and (iii) will a 'cytokine signature' approach be successful in reliably stratifying schizophrenia patients from healthy controls? To further investigate if this 'cytokine

signature' approach can help stratify patient groups from control group we choose to investigate this method in a well known autoimmune disease, namely, multiple sclerosis. The application of single cytokines versus a 'cytokine signature' in differentiating healthy controls from patient groups with multiple sclerosis is the focus of the last results chapter (please see Chapter 5).

Chapter 5.
The Role of Cytokines in Multiple Sclerosis.

Aims

- to analyse demographic data of patient cohort studied
- to investigate if drug naive patients with multiple sclerosis (MS) display similar cytokine levels compared to controls
- to demonstrate if patients with MS treated with interferons show reduced levels of cytokines
- to demonstrate if patients with MS treated with natalizumab show significant decreased levels of cytokines
- to use 'cytokine signatures' in determining immune dysfunction in MS

Abstract

Background: Multiple sclerosis (MS) is an autoimmune illness characterised by demyelination and subsequent axonal neurodegeneration. Here, the aim was to develop a method that would separate individual MS patients from controls using cytokine profiling and to demonstrate if 'cytokine signature' patterns can separate MS patients treated with interferon or natalizumab, or drug naïve patients from controls.

Methods: Cytokines serum levels of patients with MS treated with interferons (n=11), natalizumab (n=14) or drug naïve (n=12) were measured and compared to controls (n=18). Six cytokines (TNF α , IL1 β , S100B, IL-6, IL-8 and IL-23) were examined using dot immunoblotting. In addition, 'cytokine signatures' (i.e. summed value of all six cytokines) were analysed.

Results: Compared to controls, drug naïve and interferon treated groups showed reduced levels of cytokines, with the natalizumab group showing significant reduction in cytokine levels. The 'cytokine signatures' showed the following rank order: control > drug naïve = interferon > natalizumab; where natalizumab treated patients were separated from controls by almost 100% fidelity.

Conclusions: MS patients treated with natalizumab had reduced levels of pro-inflammatory cytokines compared to drug naïve and interferon treated patients. The use of 'cytokine signatures' is a potential method to separate individual MS patients from controls.

1. Introduction

Multiple sclerosis, while it does not meet the Witebsky criteria for autoimmune pathogenesis, is described as a chronic inflammatory possible (or putative) autoimmune disease. This disorder involves autoreactive T cells entering the central nervous system, destruction of oligodendrocytes, axonal loss and eventual neurodegeneration (Weiner, 2004; Sospedra and Martin, 2005). These cellular events manifest in a myriad of clinical syndromes including optic neuritis, cerebellar ataxia, dysarthria and myelitis or partial transverse myelitis. At an incidence of 0.1% in Europe and North America, this illness is more common in women, and has a typical age of onset between 20-40 years. While the exact aetiology remains unclear, environmental and genetic factors have been proposed (Noseworthy, 2000; Compston and Coles, 2002; Kerschensteiner et al., 2004; Martino, 2004; Frohman et al., 2005; McQualter, 2007). MS is a heterogeneous illness, the most common form being relapsing-remitting disease (RRMS) (Lassmann et al., 2001; Franklin, 2002). These RRMS patients, as well as those with secondary-progressive (SPMS) and primary-progressive (PPMS) forms of MS display incomplete remyelination, heightened axonal loss and neurodegeneration. The majority of current modalities of treatment are centred towards relapse prevention in the RRMS stage, with only a few agents employed in SPMS and none found to be efficacious in primary progressive MS (PPMS). Even with significant therapeutic advances in recent times it remains difficult to accurately determine disease activity or progression apart from clinical assessment and imaging parameters. A disease-specific biomarker which correlates with disease activity would aid in making increasingly complex therapeutic decisions.

Previous studies have shown a number of cytokines to be altered in MS (Hamann et al., 2008; Ubogu et al., 2006). Th2-related anti-inflammatory cytokines including interleukin IL-4, IL-5 and IL-10 are associated with symptomatic improvement in MS patients (Ramos-Cejudo et al., 2012), while Th1 pro-inflammatory cytokines such as interferon-gamma (INF- γ) and tumor necrosis factor-alpha (TNF- α) are associated with worsening of the disease (Sharief and Hentges, 1991; Vartanian et al., 1995; Murphy and Reiner,

2002; Segal, 2003; Miller et al., 2004; Imitola et al., 2005). Such reports clearly outline altered immune response in a grouped population of patients with MS but have yet to develop methods to distinguish single individuals with MS from controls with high reliability. Thus the use of cytokines as a *bona fide* method to identify individual patients with MS from controls has been limited.

Here, to examine changes in immune function in patients diagnosed with multiple sclerosis, the levels of a set of cytokines were investigated, namely TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23. The relationships between levels of these six cytokines and treatment were examined in MS patients treated separately with injectible interferons, natalizumab, in drug naive patients and controls. Moreover, the utility of generating and an employing a 'cytokine signature' over individual cytokine analyses to further distinguish patient groups was studied.

2. Results

2.1. Demographic data of patient cohort studied

In order to determine changes in cytokine levels in patients diagnosed with MS, a total number of 37 subjects diagnosed with MS, treated with interferons (Rebif n=5, Betaferon n=6), natalizumab (n=14) or drug naive (n=12) and 18 control subjects were recruited (**Table 1**). The EDSS for patients with MS treated with interferons (2.06 ± 1.47), natalizumab (2.27 ± 1.54) or drug naive (1.64 ± 1.32) were not statistically different (one-way ANOVA, Bonferroni post-hoc test). Moreover, the age of patients with MS treated with interferons (40.4 ± 10.1), natalizumab (40.9 ± 8.9) or drug naive (41.5 ± 12.1) was not significantly different (one-way ANOVA, Bonferroni post-hoc test). The control group was notably younger with an average age of 29.1 ± 6.4 years old and was statistically different to the drug naive ($p < 0.01$), interferon ($p < 0.05$) and natalizumab ($p < 0.01$) MS patient groups (one-way ANOVA, Bonferroni post-hoc test). While the male:female ratio was 25-29% for the patient group, this ratio was 44% for the control group. We thus firstly investigated if the levels of cytokines (TNF α , IL1 β , S100B, IL-6, IL-8 and IL-23) in serum samples obtained from female (n=10) and male (n=8) control subjects differed. No significant difference was found between female and male control subject groups for any of the individual cytokines measured (**Figure 1**) or when the levels for all 6 cytokines were examined as a whole (**Figure 2**) (Student's unpaired t-test (two-tailed)).

2.2. Drug Naive MS patients display similar cytokine levels compared to controls

We next examined if the levels of cytokines in drug naive MS patients differed from controls. When using our previously reported 2D analysis method ([Chatterjee et al 2009](#)) we found 30% or more of the drug naive patients in the lower quadrant compared to controls for 2 out of the 6 cytokines: IL-6 (28% vs. 75%) and IL-8 (18% vs. 50%) (**Figure 3**). When taking all 6 cytokines as a whole, only 9 control data points were clearly separated from drug naive patients (**Figure 4A**). In agreement with the 2D

analysis, a significant decrease in drug naive patients compared to controls was found in the average values (expressed as % of controls) for 2 of the 6 cytokines: IL-6 ($57.7 \pm 9.0\%$, $p < 0.001$) and IL-8 ($67.7 \pm 6.4\%$, $p < 0.01$), but not TNF- α ($84.0 \pm 9.7\%$), IL-1 β ($83.0 \pm 14.9\%$), S100B ($88.8 \pm 11.8\%$) or IL-23 ($77.7 \pm 9.8\%$) (one-way ANOVA, Bonferroni post-hoc test) (**Figure 4B**). Overall, the data suggest a reduction in cytokine amounts in drug naive patients compared to controls.

2.3. MS patients treated with interferons show reduced levels of cytokines

To examine the effects of interferons on cytokine levels in MS patients, patients were treated with Rebif (n=5) or Betaferon (n=6) and were analysed collectively. The 2D analysis of each cytokine showed 30% or more interferon treated patients in the lower quadrant compared to controls for 3 of the 6 cytokines: IL-6 (28% vs. 82%), IL-8 (18% vs. 73%) and IL-23 (47% vs. 91%) (**Figure 5**), more than compared to drug naive patients (**Figure 3**). When taking all 6 cytokines as a whole, 19 control data points were completely separated from patients treated with interferons (**Figure 6A**), again more than that found for the drug naive patients (**Figure 4A**). A significant decrease in interferon treated patients compared to controls was found in the average values (expressed as % of controls) for 2 of the 6 cytokines: IL-6 ($54.0 \pm 8.1\%$, $p < 0.001$) and IL-8 ($59.7 \pm 5.2\%$, $p < 0.001$), but not TNF- α ($93.8 \pm 10.4\%$), IL-1 β ($75.3 \pm 15.3\%$), S100B ($83.6 \pm 9.8\%$) or IL-23 ($79.9 \pm 8.0\%$) (one-way ANOVA, Bonferroni post-hoc test) (**Figure 6B**). Given that the age between controls and patients with MS were significantly different (with controls being younger) (**Table 1**), we considered comparison between the levels of cytokines in drug naive patients and interferon treated patients would be better justified than comparison with controls. Notably, this analysis showed no significant difference in any of the 6 cytokines examined between drug naive patients and interferon treated patients (one-way ANOVA, Bonferroni post-hoc test). Collectively, the data suggested a reduction in cytokine amounts in interferon treated patients compared to controls, but not compared to drug naive patients.

2.4. MS patients treated with natalizumab show significant decreased levels of cytokines

The effect of natalizumab on cytokine levels in MS patients was also demonstrated. The 2D analysis of each individual cytokine showed 30% or more natalizumab treated patients in the lower quadrant compared to controls for 5 of the 6 cytokines: IL-1 β (56% vs. 86%), S100B (41% vs. 79%), IL-6 (28% vs. 100%), IL-8 (18% vs. 85%) and IL-23 (47% vs. 100%) (**Figure 7**), more than compared to the drug naive (**Figure 3**) and interferon treated patients (**Figure 5**). When taking all 6 cytokines as a whole, 22 control data points were completely separated from patients treated with natalizumab (**Figure 8A**), again more than that found for drug naive (**Figure 4A**) and interferon treated patients (**Figure 6A**). A significant decrease in natalizumab treated patients compared to controls was found in the average values (expressed as % of controls) for 4 out of the 6 cytokines: S100B ($55.7 \pm 8.2\%$, $p < 0.01$), IL-6 ($27.1 \pm 3.9\%$, $p < 0.001$), IL-8 ($33.8 \pm 3.5\%$, $p < 0.001$) and IL-23 ($43.9 \pm 5.5\%$, $p < 0.001$), but not TNF- α ($103.6 \pm 13.4\%$) or IL-1 β ($56.4 \pm 10.7\%$) (one-way ANOVA, Bonferroni post-hoc test) (**Figure 8B**). We also found a significant decrease in levels of IL-8 (but not in any of the other 5 cytokines examined) in natalizumab treated patients compared to drug naive patients ($p < 0.01$) and interferon treated patients ($p < 0.05$) (one-way ANOVA, Bonferroni post-hoc test). In summary, natalizumab treated patients appear to have lower levels of cytokines compared to interferon treated and drug naive patients and also compared to controls.

2.5. The use of 'cytokine signatures' in determining immune dysfunction in MS

Population studies examining the levels of cytokines have revealed a great deal of information regarding the role of the immune system in disease. However, the correlation of cytokine/chemokine levels and illness state as a predictor of disease in individual patients has been more difficult limiting their use as *bona fide* biomarkers. To determine if disease state in single individuals is better predicted using a set of cytokines rather than single cytokines, the overlap in 'cytokine signatures' (i.e. sum of 6 cytokines signals for each patient) between controls and patients with MS was

investigated using 2-dimensional (2D) normalisation methods as we have reported previously (Chatterjee et al., 2009; O'Connell et al., 2013). A scatter plot using a 'cytokine signature' was generated (**Figure 9**). In this case, less scatter along the x/y-axis was found compared to any of the individual cytokines analysed. Moreover, the number of controls found in the upper right quadrant separated from MS patients as follows: drug naive (1 control), interferon group (5 controls) and natalizumab (11 controls) (**Figure 9A**). These data suggest benefits in the use of 'cytokine signature' analysis, rather than single cytokines, to separate patients with MS from control individuals. Compared to the control group, a significant decrease in the average values of cytokine signature was found in natalizumab ($39.4 \pm 3.7\%$, $p < 0.001$) and interferon treated ($65.5 \pm 4.0\%$, $p < 0.01$), as well as drug naive patients ($69.1 \pm 6.3\%$, $p < 0.01$) (one-way ANOVA, Bonferroni post-hoc test) (**Figure 9B**). We also found a significant decrease of cytokine signature in natalizumab treated patients compared to drug naive patients ($p < 0.01$) and interferon treated patients ($p < 0.05$) (one-way ANOVA, Bonferroni post-hoc test).

Treatment	Subjects (Male:Female)	Age	EDSS
Healthy Control	18 (8:10)	29.1 ± 6.4	n/a
Drug Naive	12 (3:9)	41.5 ± 12.1	1.64 ± 1.32
Interferons	11 (3:8)	40.4 ± 10.1	2.06 ± 1.47
Natalizumab	14 (4:10)	40.9 ± 8.9	2.27 ± 1.54

Table 1: Demographic Data. Demographics of patients treated with interferons or natalizumab, as well as drug naive patients and control subjects. Expanded Disability Status Scale (EDSS).

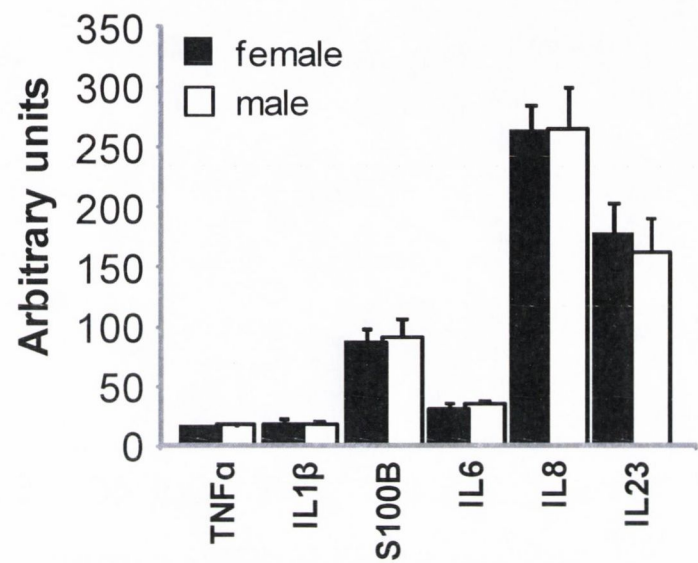


Figure 1: Levels of cytokines in male and female healthy control subjects. Bar graph shows levels of TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23 in serum samples obtained from female (n=10) and male (n=8) control subjects as measured by dot blot analysis, where the density of each dot (arbitrary units) was measured. Data is represented as average $\pm$ SEM.

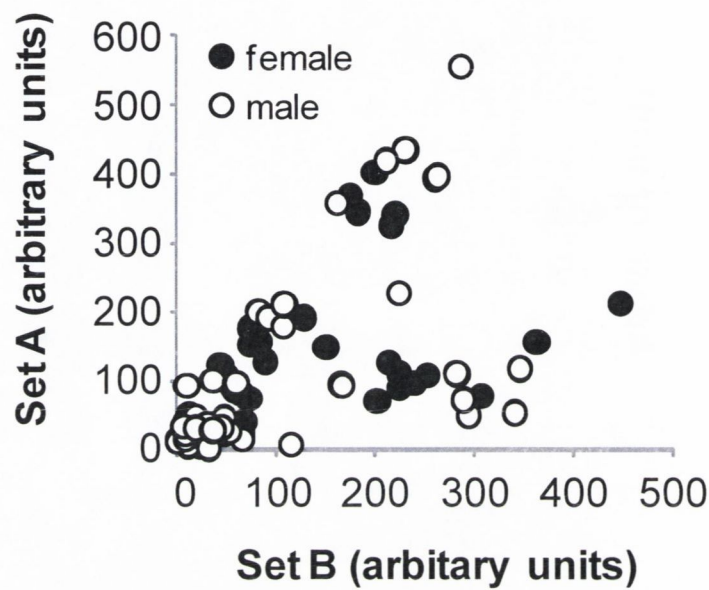


Figure 2: Overlap of cytokine levels in male and female healthy control subjects. Scatter plot shows data from all 6 proteins (TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23). The density of each dot (arbitrary units) from each serum sample in the first experiment (x-axis) is plotted against that obtained in the second experiment (y-axis). No clear separation was noted between female and male control subjects.

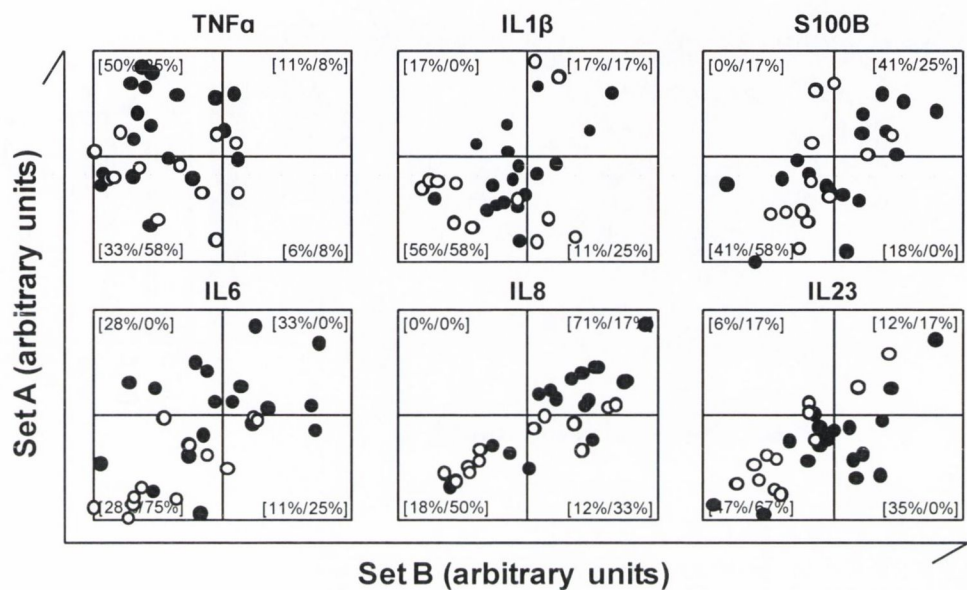


Figure 3: Drug Naive MS patients show similar cytokine profile to control subjects. Scatter plots show data from dot blots performed on serum samples obtained from control subjects (n=17-18) and drug naive MS patients (n=12). Dot blot were performed using antibodies for TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23. The density of each dot (arbitrary units) from each serum sample in the first experiment (x-axis) is plotted against that obtained in the second experiment (y-axis). Data points at the top right corner represent high cytokine signals while those at the bottom left corner represent low cytokine signals.

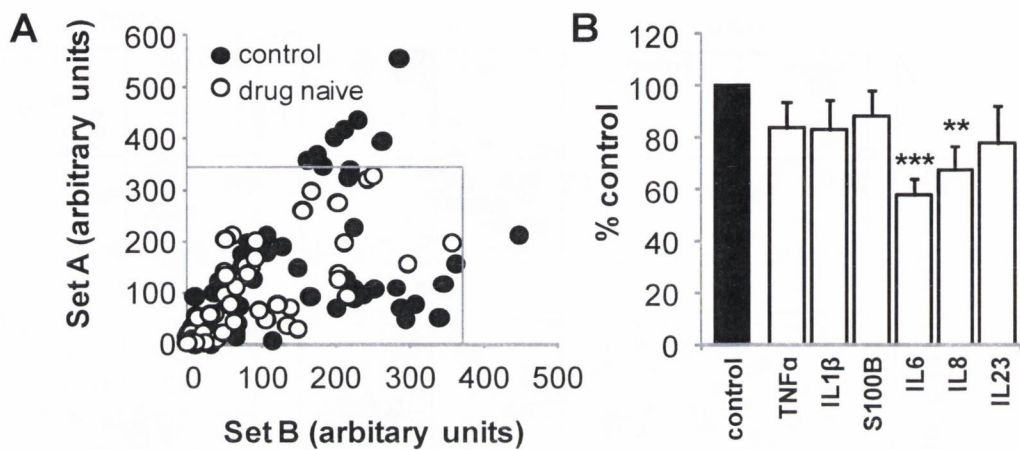


Figure 4: Overlap of cytokine levels in healthy control subjects and drug naive MS patients. (A) Scatter plot representing the data from all the 6 proteins is shown. No clear separation was noted between control and drug naive MS patients. **(B)** Bar graph shows levels of TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23 in serum samples obtained from drug naive MS patients (n=12), where data is shown as average $\pm$ SEM and is represented as % of control subjects (n=17-18). Statistical analysis was performed using one-way ANOVA, Bonferroni post-hoc test (**, $p < 0.01$; ***, $p < 0.001$, drug naive MS patients vs controls).

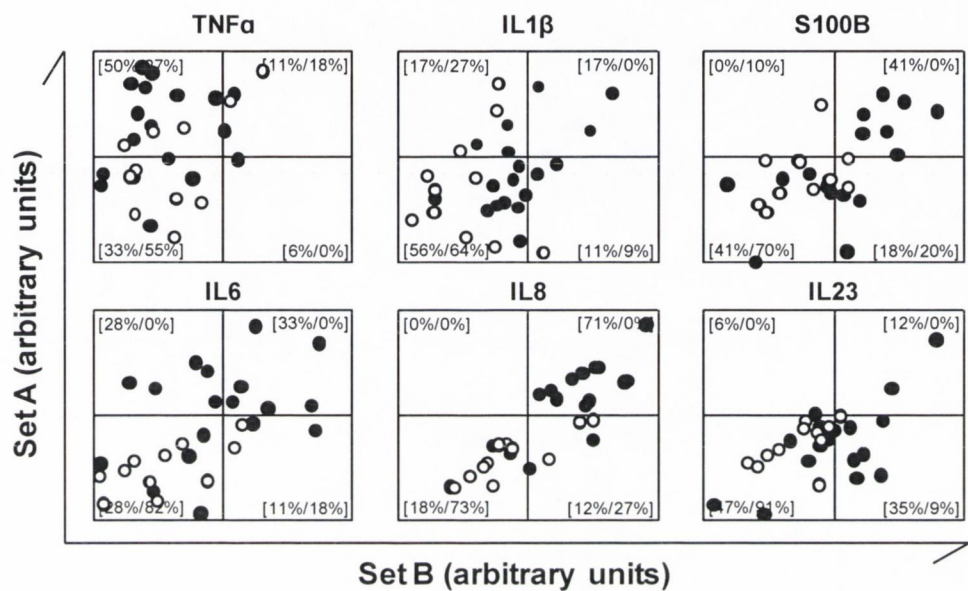


Figure 5: MS patients treated with interferons partially reduced levels of cytokines compared to control subjects. Scatter plots show data from dot blots performed on serum samples obtained from control subjects (n=17-18) and MS patients treated with interferons (n=10-11). Dot blot were performed using antibodies for TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23. The density of each dot (arbitrary units) from each serum sample in the first experiment (x-axis) is plotted against that obtained in the second experiment (y-axis). Data points at the top right corner represent high cytokine signals while those at the bottom left corner represent low cytokine signals.

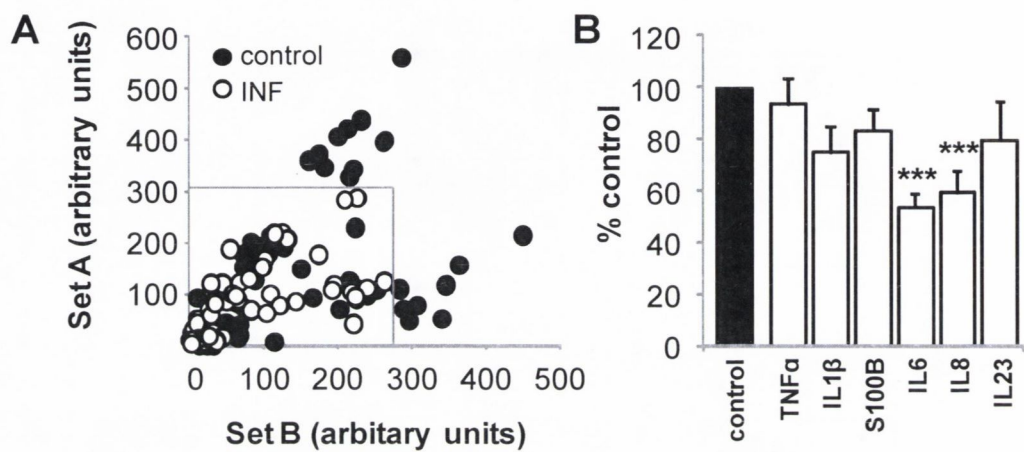


Figure 6: Overlap of cytokine levels in healthy control subjects and interferon treated MS patients. (A) Scatter plot representing the data from all the 6 proteins is shown. Cytokines signals in serum obtained from MS patients treated with interferons were primarily found in the lower quadrant compared to control patients. (B) Bar graph shows levels of TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23 in serum samples obtained from drug naive MS patients (n=10-11), where data is shown as average $\pm$ SEM and is represented as % of control subjects (n=17-18). Statistical analysis was performed using one-way ANOVA, Bonferroni post-hoc test (***, $p < 0.001$, interferon treated MS patients vs control).

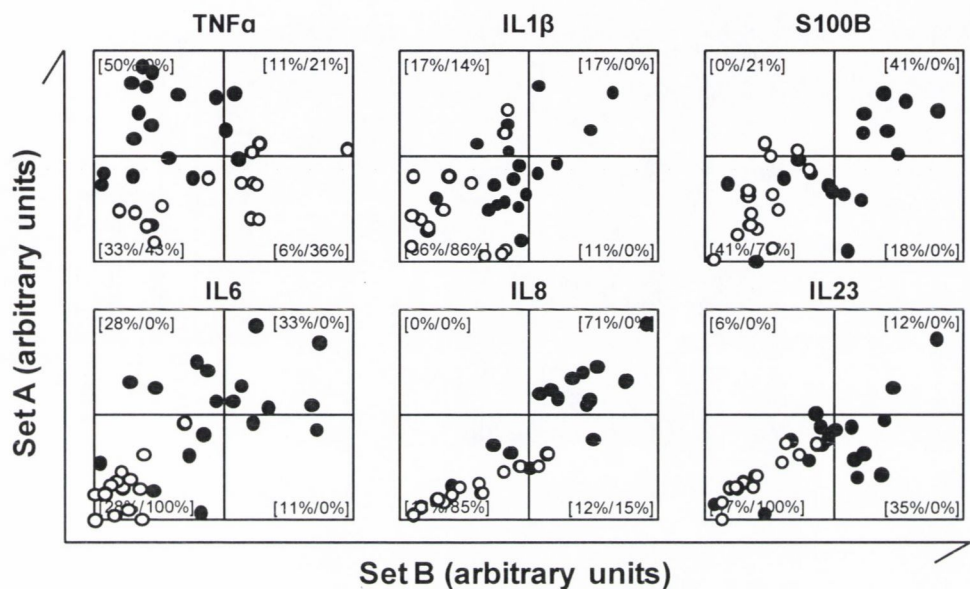


Figure 7: MS patients treated with natalizumab show reduced levels of cytokines compared to control subjects. Scatter plots show data from dot blots performed on serum samples obtained from control subjects (n=17-18) and MS patients treated with interferons (n=14). Dot blot were performed using antibodies for TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23. The density of each dot (arbitrary units) from each serum sample in the first experiment (x-axis) is plotted against that obtained in the second experiment (y-axis). Data points at the top right corner represent high cytokine signals while those at the bottom left corner represent low cytokine signals.

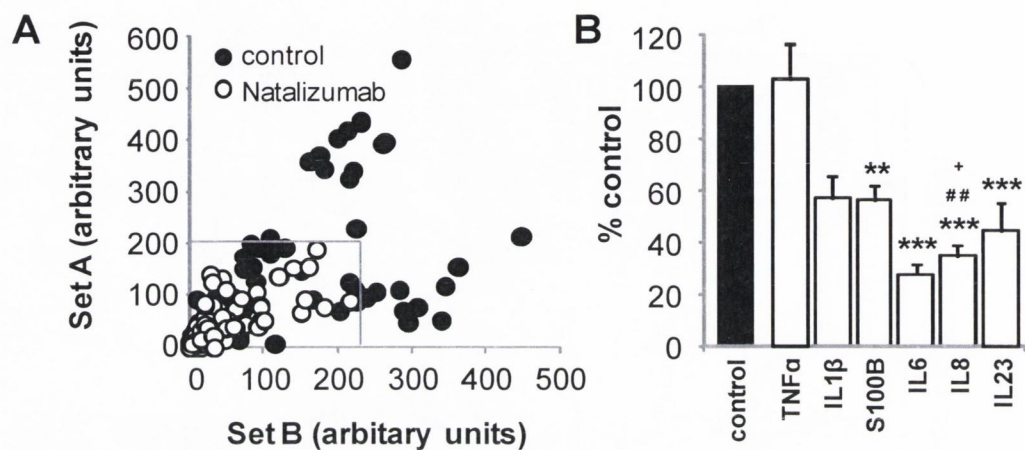


Figure 8: Overlap of cytokine levels in healthy control subjects and natalizumab treated MS patients. (A) Scatter plot representing the data from all the 6 proteins is shown. Cytokines signals in serum obtained from MS patients treated with natalizumab were found in the lower quadrant compared to control patients. **(B)** Bar graph shows levels of TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23 in serum samples obtained from drug naive MS patients (n=14), where data is shown as average $\pm$ SEM and is represented as % of control subjects (n=17-18). Statistical analysis was performed using one-way ANOVA, Bonferroni post-hoc test (**p < 0.01; ***, p < 0.001, natalizumab treated MS patients vs control; #, p < 0.01, natalizumab treated MS patients vs drug naive; +, p < 0.05, natalizumab treated MS patients vs interferon treated MS patients).

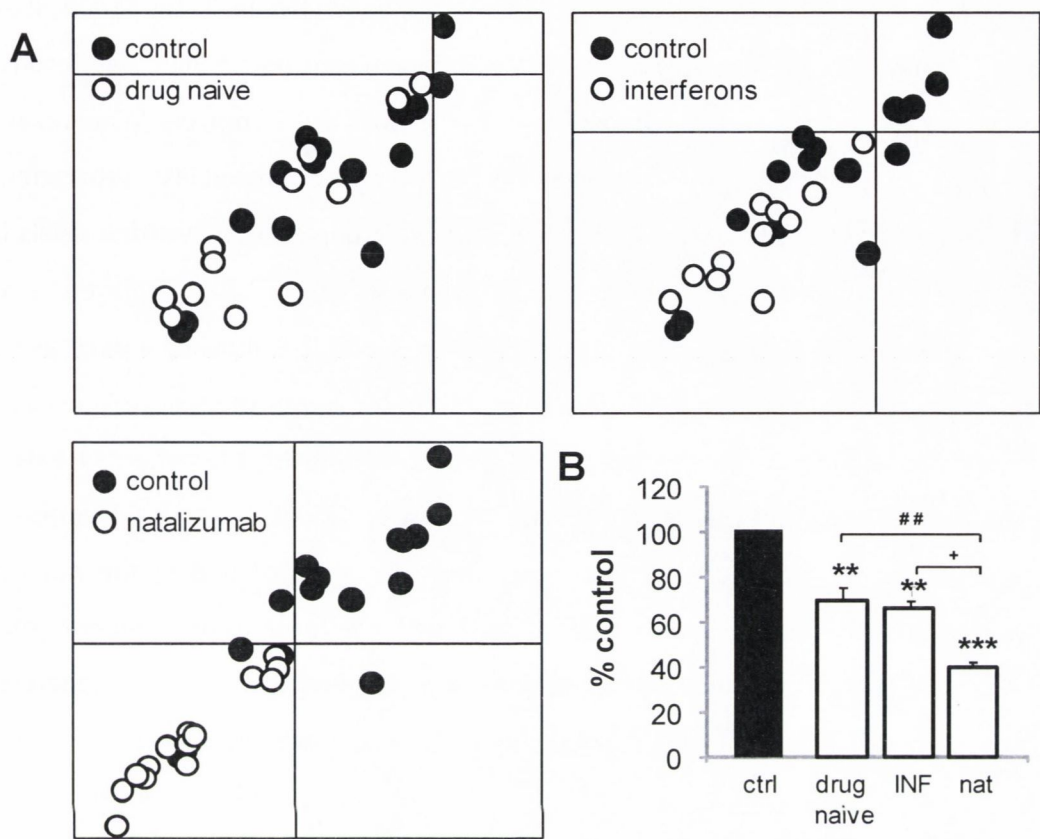


Figure 9: Cytokine signatures improve fidelity in separating MS patients from controls. (A) Dot blot were performed using antibodies for TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23. The summed density for each cytokine of each dot (arbitrary units) from each serum sample in the first experiment (x-axis) is plotted against that obtained in the second experiment (y-axis). Data points at the top right corner represent high 'cytokine signature' levels while those at the bottom left corner represent low 'cytokine signature' levels. Scatter plot representing 'cytokine signature' (i.e. summed density levels of 6 cytokines) is shown. The 'cytokines signatures' in serum obtained from drug naive MS patients (n=12), MS patients treated with interferons (n=14), and MS patients treated with natalizumab (n=14) are shown compared to control subjects (n=17-18). **(B)** Bar graph shows 'cytokine signatures' in serum samples obtained from drug naive MS patients (n=14), MS patients treated with interferons (n=14), and MS patients treated with natalizumab (n=14) where data is shown as average $\pm$ SEM and is represented as % of control subjects (n=17-18). Statistical analysis was performed using one-way ANOVA, Bonferroni post-hoc test (**, $p < 0.01$; ***, $p < 0.001$, MS patients vs control; # #, $p < 0.01$, natalizumab treated MS patients vs drug naive; +, $p < 0.05$, natalizumab treated MS patients vs interferon treated MS patients).

3. Discussion

The current study examined the levels of six cytokines (TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23) in 37 patients with MS treated with interferons (Rebif, n=5; Betaferon, n=6), natalizumab (n=14) or were drug naive (n=12), as well as 18 controls. When examining levels of single cytokines, the drug naive MS patient group showed little separation from the control group, with only IL-6 and IL-8 showing a decrease in cytokine levels in this patient group. The interferon treated MS patient group also displayed a similar separation profile from the control group, with IL-6 and IL-8 showing a decrease in this patient group. No significant difference was found for any of the six cytokines between drug naive and interferon treated patient groups. In contrast, MS patients treated with natalizumab showed significant decrease in S100B, IL-6, IL-8 and IL-23 compared to controls. We also found a significant decrease in levels of IL-8 in the natalizumab treated patient group compared to drug naive and interferon treated patient groups. In this study, the six cytokines examined were summed and this 'cytokine signature' was also analysed. The level of 'cytokine signature' was lower in drug naive, interferon treated and natalizumab treated patient groups compared to controls. The data demonstrated that natalizumab treated patients had lower levels of cytokines compared to interferon treated and drug naive patients and also compared to controls. Analysis of 'cytokine signature' showed the following rank order for the groups: control > drug naïve = interferon > natalizumab. Importantly, analysis of 'cytokine signature' allowed separation of individual natalizumab treated patients from controls by almost 100% fidelity, demonstrating the value of analysing multiple cytokine signature patterns compared to that of single cytokines.

In this study we investigated the levels of 6 cytokines, namely TNF- α , IL-1 β , S100B, IL-6, IL-8 and IL-23, in MS patients and controls. These cytokines were chosen for a number of reasons. In particular, previous studies have shown that both TNF- α and IL-1 β are increased in MS (Hauser et al., 1990; Spuler et al., 1996; Dujmovic et al., 2009; Reale et al., 2012). The astrocyte marker S100B has also been shown to be increased in MS and a

number of other brain related illnesses including schizophrenia (O'Connell et al., 2013), brain trauma and ischemia in addition to other neurodegenerative, inflammatory and psychiatric diseases, such as Alzheimer's disease, Down's syndrome, and Tourette's syndrome (Rothermundt et al., 2003; Sen and Belli, 2007). Elevated levels of IL-6 in the serum and CSF have also been shown in patients suffering from viral and bacterial meningitis, MS, Alzheimer's disease and systemic lupus erythematosus (Gruol and Nelson, 1997). In addition levels of IL-8 are raised in MS patients and decreased in MS patients on interferon (Lund et al., 2004), and natalizumab treatment (Mellergard et al., 2010). The role of IL-23 in MS has been less well studied, although reports have shown an increase in CSF and serum IL-23 in MS patients compared to non-inflammatory neurological disease (Wen et al., 2012; Chen et al., 2012; Tang et al., 2012). Collectively these publications supported the use of these six cytokines in generating a 'cytokine signature' profile for MS patients and comparison with controls.

A limitation of this study was, however, that we did not include anti-inflammatory cytokines such as IL-10, TGF- β to develop pro/anti-inflammatory ratios. The inclusion of gene expression into such analysis would also likely provide a more robust diagnostic measure. That being said, the inclusion of additional cytokines and gene expression studies would likely need to be weighed against additional costs and process time, which in a hospital setting, may be challenging for use of potential diagnostic kits. Another caveat to this study was that the control group was statistically younger by approximately 10 years than the drug naive, interferon treated and natalizumab treated MS patient groups. While previous studies have suggested increased levels of cytokines in MS patients compared to controls, we found no change in four of the six cytokines (TNF- α , IL-1 β , S100B and IL-23) examined or a decrease in two of the cytokines analysed (IL-6 and IL-8), when comparing the drug naive MS patient group with the control group. We suggest that the age difference between controls and drug naive MS patient groups likely explains this anomalous finding. We circumvented this concern by comparing interferon and natalizumab treated patient groups with drug naive MS patients, which were aged-matched, as well as comparing with the younger control group. When doing

so, we found no significant difference between the drug naive and interferon treated patient groups in any of the six cytokines examined. Importantly, we did find a significant decrease in levels of IL-8 (but not in any of the other 5 cytokines examined) in natalizumab treated patient group compared to drug naive and interferon treated patient groups. These current finding are in agreement with previous studies showing levels of IL-8 is decreased in MS patients treated with interferon and natalizumab compared to drug naive patients ([Lund et al., 2004](#); [Mellergard et al., 2010](#)). In our study, the reasons why the natalizumab treated patient group showed a lower 'cytokine signature' compared to the interferon treated patient group is presently unclear. It may potentially be due differences in the mechanism-of-action between these two drugs and certainly warrants further study.

4. Conclusions

In conclusion, we observed an enhanced separation between groups when the 'cytokine signature' pattern was used, compared to the use of single cytokines. We note that this approach still has several open questions such as (i) what are the minimal number of cytokines required to make a 'cytokine signature?', (ii) which cytokines should be included in a 'cytokine signature' analysis?, and (iii) will a 'cytokine signature' approach be successful in reliably stratifying MS patients from controls? (iv) will a cytokine signature approach be useful in monitoring response to individual therapies?

The usefulness of a diagnostic test would also need to be tested for its ability to separate MS patients from 'MS mimics' including Neuromyelitis Optica (NMO), Acute Disseminated Encephalomyelitis (ADEM), Systemic lupus erythematosus (SLE), Sjogren's syndrome, inherited diseases of CNS myelin and anti-phospholipid antibody syndrome or migraine. Nevertheless, our findings support the use of 'cytokine signature' patterns rather than the use of individual cytokines when investigating this illness. We suggest that follow-up studies using cytokine collections may aid in the development of reliable diagnostic kits for MS.

Chapter 6.
Discussion

1. Opening Remarks

1.1. Overview of schizophrenia

A Pubmed search of the word 'Schizophrenia' (conducted on 25th Aug 2013) retrieved a total of 104,697 publications. The first schizophrenia publication cited on PubMed is dated more than nine decades ago, in 1921. Assuming the average cost of such a publication is about 5,000 euros, this, rather underestimated, value would still exceed a sum total of 500 million euros (see **Figure 1**). However, despite this concerted research effort, the underlying aetiology of schizophrenia still remains unknown. Perhaps, unsurprisingly, this limited progress has made pharmaceutical companies averse to spending further resources to create novel therapies.

The pathophysiology of schizophrenia is likely to be neurodevelopmental in origin with a combination of factors, such as genetic influence and environment insults to the brain, determining who develops full psychotic disorder. Schizophrenia remains a lifelong illness affecting about one in every hundred people. Current treatments which can help allivate the 'positive' symptoms, such as delusions and hallucinations, are not always successful in all cases of the illness, in addition to having a range of significant side effects. There are currently no definitive clinical treatments available that can stop the progression of 'negative symptoms' and cognitive decline that accompany this illness.

1.2 Current pharmacological treatments used in schizophrenia

It has long been postulated that neurotransmitters such as dopamine and, in more latter times, serotonin and glutamate play a role in the pathophysiology of the symptoms we observe in schizophrenia. All of the current antipsychotic treatments licenced for schizophrenia act on at least one of these neurotransmitter receptors. However, it remains unclear as to why some antipsychotic medications, such as clozapine, are superior to others, based of their receptor profile. Furthermore, the burdensome side effect profile of antipsychotic medications makes them undesirable for patients. In addition, by nature of the illness, the belief and insight for the

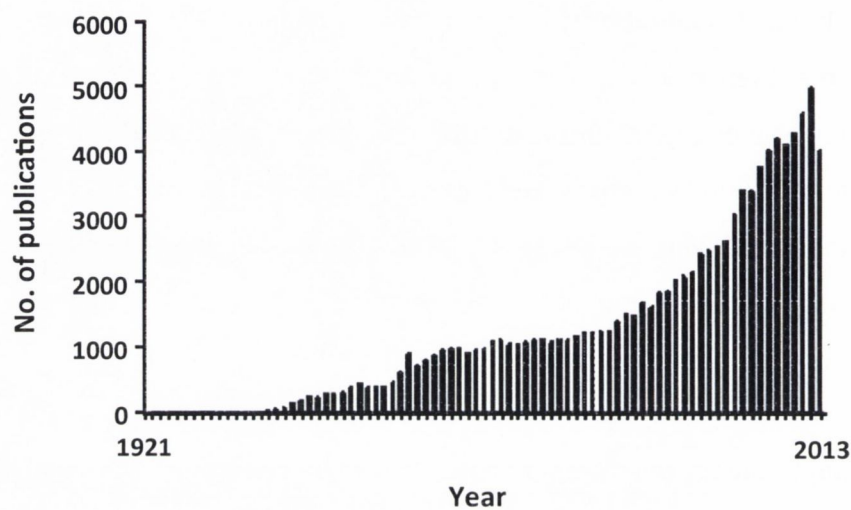


Figure 1: PubMed articles on schizophrenia. The graph shows the pattern of publications on schizophrenia, as found in the PubMed database. To date, there are 104,697 publications on schizophrenia in PubMed.

requirement of pharmacological, or other treatments for this illness, is a challenge for this patient group. Taken together, schizophrenia remains a perplexing disorder to treat and our lack of full understanding as to why people fall ill with schizophrenia has limited our progression to developing more effective pharmacological treatments.

1.3. Schizophrenia as an endophenotype disorder

It has been suggested that schizophrenia presents in a heterogenous fashion. This may help explain why a number of studies into this illness have failed to replicate findings, for example, despite a known high heritability there has been a high proportion of non-replication of genetic analysis data (Sanders et al., 2008). In addition, imaging studies have also shown heterogeneity in brain variation and white matter abnormalities in patients with schizophrenia (White et al., 2012; Nenadic et al., 2012). As such, in order to understand emerging studies of this disorder we may need to approach it's aetiology from an endophenotypic viewpoint. An endophenotype may be neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive or neuropsychological in nature (Gottesman et al., 2003). They are a quantitative inheritable trait related deficit closely linked to heritable risk factors and as such are generally accepted to be caused by genetic polymorphisms (Braff et al., 2007). Looking forward, if viewed as a heterogenous disorder, each of the 'subgroups' of schizophrenia may potentially be characterised endophenotypically. Current evidence supports these subgroups, which have specific neurobiological and genetic architecture, within the spectrum of schizophrenia. This potential segregation of patients into subgroups within the umbrella of schizophrenia may have profound benefits on future drug treatment choices. These efforts may help stratify patients, with the prospect of individual tailored pharmacological treatment based upon their subgroup within this illness.

1.4. Inflammation and schizophrenia

Evidence suggests that inflammation plays a role in schizophrenia (Meyer, 2013; Iritani, 2013). Anti-inflammatory medications have been shown to help alleviate psychotic symptoms in schizophrenia and animal studies have shown that prenatal and perinatal

rise in pro-inflammatory cytokines causes schizophrenia-like symptoms in the offspring (Müller et al., 2010; Akhondzadeh, et al., 2007). Previous studies examining the presence of cytokines in the plasma, serum or CSF of patients with schizophrenia have yielded contradictory data, however, in general there appears to be an increase in pro-inflammatory cytokines in schizophrenia compared to healthy controls (Potvin et al., 2008). Inconsistencies and failure to replicate studies of schizophrenia may be understandable in the context of ascertainment bias, population stratification and cohort variance due to for example gender, age, treatment, illness onset and smoking or other lifestyle differences (Braff et al., 2007).

In agreement, in our study, serum levels of the protein S100B, which is known to play a role in neuroinflammation, were raised in female patients with schizophrenia compared to healthy controls. Serum levels of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, IL-8, IL-17 and IL-23 were also increased in schizophrenia compared to healthy controls and, furthermore, the use of a 'cytokine signature' was used to examine if a signature was more definitive at segregating patients from controls. This method of data analysis was superior to individual cytokine levels at separating patients from controls, however this only applied to a subgroup of patients with schizophrenia and there were no correlating factors for this subgroup, such as illness severity, patient demographic or metabolic status. In addition, to further investigate this method of 'cytokine signature' data analysis we examined these cytokines in serum of patients with multiple sclerosis, a known autoimmune disorder. Patients with multiple sclerosis were more clearly delineated from healthy controls using the 'cytokine signature' data analysis method, compared to schizophrenia.

1.5. Metabolic syndrome, inflammation and schizophrenia

It is recognised that schizophrenia is often accompanied by physical illness (Leonard, 2012) and is commonly associated with the metabolic syndrome (Holt et al., 2005). Inflammation appears to play a role in both schizophrenia and the metabolic syndrome. Whether the metabolic syndrome seen in this patient group is due to unhealthy lifestyle

choices associated with schizophrenia or due to genetic influences and antipsychotic treatment remains uncertain. Evidence does suggest that metabolic syndrome seen in schizophrenia may be a result of an interaction between heritable factors and adverse environmental factors, including antipsychotic treatments (O'Rahilly, 2009). Furthermore, it is hypothesized that inflammation in schizophrenia initiates changes in metabolic factors such as glucose and lipid metabolism, contributing to insulin resistance. Additionally, evidence has suggested that antipsychotic medication may attenuate this inflammation, although how this mechanistically occurs has yet to be established. However, physical illness in this disease group remains and the underlying cause of this low grade inflammation observed in schizophrenia remains speculative. Future studies into the use of anti-inflammatory medications, for symptom relief and coinciding observation of physical health symptoms, are thus still required.

2. Closing Remarks on the results

2.1. The role of S100B in schizophrenia (Results 1)

Previous studies have reported altered levels of S100B in patients with schizophrenia (Qi et al., 2009). S100B is a protein released by a number of cell types in the body, within the brain it is primarily released by astrocyte cells. Moreover, it is known to play a role as a marker of structural damage within the brain. In addition to investigating the levels of S100B in patients with schizophrenia, this thesis aimed to examine the influence of metabolic factors, such as glucose and lipid abnormalities, as well as demographic and medication influences on the serum levels of S100B. Data showed serum levels of S100B were increased in female patients with schizophrenia compared to male patients and healthy controls. Furthermore, these increased serum concentrations of S100B demonstrated a positive correlation with BMI for these patients. This study supports previously findings suggesting that visceral fat and altered adipocyte function may contribute to raised levels of S100B in patients with schizophrenia (Steiner et al., 2010a; Barreira et al., 2012).

2.2. The role of cytokines in schizophrenia (Results 2)

While it is putative that inflammation plays a role in schizophrenia, conflicting studies and lack of complete understanding of the causal pathophysiology have led to a lack of clarity in this area. In this study, levels of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, IL-8, IL-17 and IL-23 were modestly raised in patients with schizophrenia compared to controls. Furthermore, although analysis of 'cytokine signature' levels exhibited greater differentiation between patients with schizophrenia and healthy controls, compared to that of individual cytokines, this 'cytokine signature' analysis also requires further improvements before it can be considered clinically useful. In addition, female treatment groups showed significantly raised 'cytokine signature' levels compared to healthy controls, however, these did not correlate with BMI. Reasons for this gender imbalance remain unclear, although it has been postulated that females are more predisposed to neurological illnesses and that oestrogen plays a role in regulating cytokine levels (Czlonkowska et al., 2005). Mechanistic and cellular experiments examining the effects of antipsychotic medications on neuronal and glial cells, such as astrocytes, and indeed further investigation regarding the role of oestrogen on these cell types may be of interest.

2.3. The role of cytokines in multiple sclerosis (Results 3)

In order to determine the value of cytokines and, moreover, 'cytokine signature' as biomarkers in diseases with immune dysfunction a further disease group of patients with MS was examined. In this study, the levels of IL-6, IL-8, IL-23 and S100B in patients with MS treated with natalizumab were significantly lowered compared to controls. Natalizumab treated patients also had a decreased 'cytokine signature' compared to that of patients who were drug naïve or treated with interferon and also compared to healthy controls. These results are in keeping with previous data showing individual pro-inflammatory cytokines are increased in MS (Wen et al., 2012; Hauser et al., 1990) and that natalizumab treatment decreases levels of IL-8 in this patient group (Møllergaard et al., 2010). The better separation of MS patients from control groups (compared to schizophrenia patients separated from their control groups) is likely suggestive of MS

being strongly driven by altered immune response compared to schizophrenia. This comparison suggests that alterations in immune response in schizophrenia are likely to be subtle, possibly also providing a reason for the numerous disparities found in the literature that investigate immune function in this illness.

3. Last words and Future Perspectives

In closing, the studies carried out in this thesis have demonstrated that patients with schizophrenia have, in general, increased serum levels of pro-inflammatory cytokines and cytokine signatures compared to healthy controls. Notably, however the relatively poor segregation between individual patients and healthy controls limits the use of this method as a clinical diagnostic tool. In contrast, further investigation of the serum levels of pro-inflammatory cytokines and cytokine signatures in MS demonstrated a clearer separation between disease and control groups, and supported the use of cytokine signature analysis in inflammatory based illnesses. This work concludes that the immune system plays a role in schizophrenia, however this is likely less clearly defined than in other inflammatory disorders.

When considering further research in the area of schizophrenia, it appears no longer tenable to consider this illness as only a brain-related disorder. Given that the body of research conducted to date has primarily rested on investigating neuronal dysfunction in schizophrenia and that this research has provided little advancement in the development of novel therapies, it seems prudent to look at additional mechanisms for the underlying cause of schizophrenia. Even at the risk of investigating incorrect hypothesis, or ruling out alternative mechanisms, research beyond neuronal function appears to be warranted. In this regard, a multi-system approach to this illness is likely required, involving investigation of communications at a systems level, inclusive of immune, metabolic and the central nervous systems. At the cellular level, inclusion of lymphocyte, astrocyte and adipocyte (dys)function in addition to neuronal function is also still needed (*please see Figure 2*). These concerted research efforts may help us as

yet discover the genesis of schizophrenia, aid in the development of new therapies and help in the better clinical management of patients with this illness.

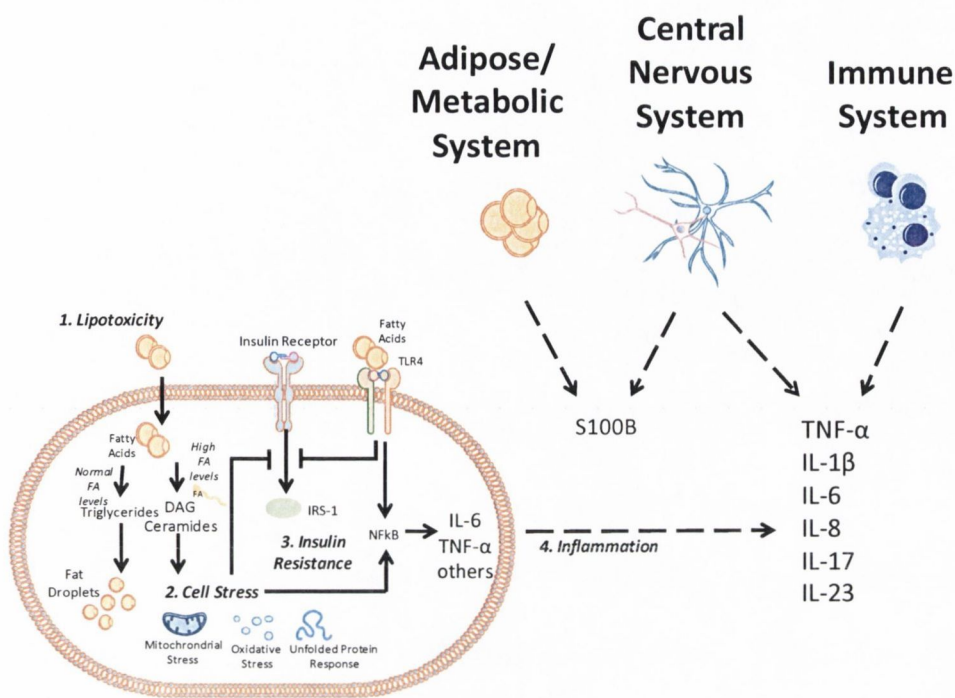


Figure 2: The alternative ‘tripartate’ synapse. Cross-communication between the central nervous system, immune system and metabolic system is shown. A dysfunction in this communication may play a role in development and maintenance of ill health in schizophrenia.

Chapter 7
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Chapter 8. Appendix I
Phenotype of Subjects

The purpose of this section was to give a more detailed description of the subjects phenotype recruited during this study.

1. Phenotype of Participant Subjects

1.1. Participants in schizophrenia study

All patients attending clozapine and depot clinics within an urban mental health setting (St. Vincent's Hospital, Fairview, Dublin 3, Ireland) were invited to participate in this study. This is an urban mental health service which provides publicly funded healthcare for patients with mental illness whom live in the surrounding area. Inclusion criteria were a DSM-IV-TR ([Diagnostic and Statistical Manual of Mental Disorders, 4th edition, Revised, 2000](#)) diagnosis of schizophrenia, age >18 years, known compliance with prescribed antipsychotic medication and those able to give informed consent to participate in the study. Exclusion criteria included (i) other psychiatric and neurological diagnoses, (ii) a co-morbid diagnosis of substance abuse disorder and (iii) those with an IQ < 70. Once invited to participate in the study, patients voluntarily opted in or out of the study. Patients attending clozapine and depot clinics were invited to participate as their compliance status was more certain than those patients taking oral antipsychotic medications. Informed written consent was obtained from all subjects. All participants were educated to at least secondary school level in Ireland or equivalent and psychometric testing was not performed. Once recruited all patients had their blood samples collected between the hours of 8-9am, so as to decrease intersample cortisol effects. Menstrual cycle phase of those female patients was not collected during the study. The Demographic data (male:female ratio, age range, treatment duration, daily dose, BPRS, and SANS), biochemical data (fasting glucose, triglycerides, HDL, LDL, total cholesterol), and cardiovascular disease risk factor and lifestyle data (smoking status, diabetes mellitus status, body mass index, hypertension treatment, systolic blood pressure, and Framingham score) were collected for each patient participating in the study.

Healthy controls were recruited from the same urban area as those patients, with matched demographics. They were primarily recruited from domestic and nursing staff working within St. Vincent's Hospital, Fairview, Dublin 3. Advertisement for healthy control recruitment took place within the hospital and surrounding community. This ensured that patient and control subjects were recruited from a similar community setting. Participants also benefitted from a 'health check' of their cardiovascular risk status, which was relayed to them following biochemical data and BMI results. Informed, written consent was gained from all participants. Healthy controls recruited did not have a significant physical disability or disease, or a personal or family history of psychiatric illness. The Demographic data (male:female ratio, age range), biochemical data (fasting glucose, triglycerides, HDL, LDL, total cholesterol), and cardiovascular disease risk factor and lifestyle data (smoking status, diabetes mellitus status, body mass index, hypertension treatment, systolic blood pressure, and Framingham score) were collected. Healthy controls had their blood samples collected between the hours of 8-9am, so as to decrease intersample cortisol effects. Menstrual cycle phase of those female controls was not collected during the study. All participants were educated regarding healthy diet and exercise in reducing their cardiovascular risk. Further details of patients with schizophrenia and healthy control participant phenotype for this study are presented in results *Chapter 5*.

1.2. Subjects in multiple sclerosis study

In order to further investigate immune function in schizophrenia, patients with multiple sclerosis, a disorder with known immune pathology, were chosen as a comparative group. In this study, patients with MS treated with interferon or natalizumab were recruited from outpatient clinics, while the drug naive MS group were recruited from an inpatient setting, all within Cork University Hospital (CUH). Controls were recruited from medical staff, as well as healthy volunteers following advertisement within CUH. All participants were caucasian and resident in south/south east Ireland. Informed, written consent was gained from all participants. Once recruited all patients and controls had their blood samples collected between the hours of 8-9am, so as to decrease

intersample cortisol effects. Menstrual cycle phase of those female patients and controls was not collected during the study. These patients were treated with either interferon beta 1a or 1b (Rebif or Betaferon), natalizumab or were drug naive. The patient group treated with interferons were as follows: Rebif and Betaferon. The patient group treated with natalizumab received from between 4-60 infusions. All confirmed MS patients had a relapsing-remitting form of MS as defined by the revised McDonald criteria. Disease severity was scored at time of collection using the Kurtzke's Expanded Disability Status Scale (EDSS). Further details of multiple sclerosis and healthy control participant phenotype for this study are presented in results *Chapter 5*.

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Investigating the Role of Inflammation in Schizophrenia

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